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**Studies on virulence-critical proteins of enteropathogenic
Escherichia coli (EPEC)**

Azzeldin Zaid Hamed Madkour

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Medical School

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) virulence depends on a Type-3 Secretion System (T3SS) that transfers many 'effector' proteins into human gastrointestinal cells. The components for the effector-delivery apparatus (T3SS and translocator proteins), virulence-critical surface protein (Intimin) and seven effectors (EspG, EspF, Map, EspH, EspZ, Tir, EspB; latter also a translocator) are encoded on the Locus of Enterocyte Effacement (LEE) pathogenicity island. The EspA translocator protein extends the T3SS and is tipped with EspB and EspD which insert into the plasma membrane enabling effector delivery into the host cytoplasm. Two LEE effectors, Tir and EspZ, have virulence-critical functions with both inserted into the plasma membrane and linked, for Tir, as a receptor for EPEC (via Intimin) and, for EspZ, to prevent a cytotoxic response. It is controversial how Tir becomes inserted into membranes and how EspZ prevent cytotoxicity. Previous work predicted that Tir insertion depends on LEE effector activities. Here, we demonstrate LEE sufficiency for Tir insertion and rule out roles for Intimin and classical LEE (EspG, Map, EspF, EspH, EspZ) effectors. Surprisingly, our data implicated roles for the EspA and EspD translocators in stable Tir-intimin interactions and revealed a new EspZ protective mechanism i.e. prevention of cytotoxicity triggered as a consequence of Tir-Intimin interaction. Furthermore, we provide bioinformatic and experimental support that an unusual *Edwardsiella tarda* LEE-like region encodes a functional effector-delivery system. The swapping of protein homologues between *E. tarda* and EPEC has also provided an opportunity to gain insights on the structure/function of ~20 T3SS/translocon components and virulence-critical Tir, Intimin and EspZ proteins.

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List of Abbreviations

A/E lesion: Attaching and effacing lesion

Ab: Antibody

ARP2/3: Actin related protein 2/3

BFP: Bundle forming pilus

BSA: Bovine serum albumin

Carb: Carbenicillin

Cdc42: Cell division cycle 42

Cmp: Chloramphenicol

CR: *Citobacter rodentium*

C-terminal: Carboxyl-terminal

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's minimal Eagle's medium

E2348/69: EPEC strain E2348/69

EAF: EPEC adherence factor

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

EHEC: Enterohemorrhagic *Escherichia coli*

EIEC: Enteroinvasive *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

Erk: Extracellular signal-regulated kinase

Esc: EPEC secretion

Esp: EPEC secreted/signalling protein

FL: Full-length

FCS: Foetal calf serum

GEF: Guanine exchange factor

IE: Integrative element

IKK: IκB kinase kinase

ITIM: Immune receptor tyrosine based inhibition motifs

JAM: Junction adhesion molecule

Km: Kanamycin

LB: Luria broth

LDH: Lactate dehydrogenase

LEE: Locus of enterocyte effacement

Ler: LEE encoded regulator

LPS: Lipopolysaccharide

Map: Mitochondrial associated protein

MOI: Multiplicity of infection

Nal: Nalidixic acid

NF- κ B: Nuclear factor-kappa B

NHE: Sodium hydrogen (Na⁺/H⁺) exchanger

Nle: Non-LEE encoded

N-WASP: Neural Wiskott-Aldrich syndrome protein

OD₆₀₀: Optical density 600 nm

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

Per: Plasmid encoded regulator

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PMSF: Phenylmethane sulfonyl fluoride

PPR: Polyproline region

RT: Room temperature

RTK: Receptor tyrosine kinase

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Ser: Serine

SGLT1: Sodium glucose transporter 1

SHIP2: SH2 domain containing inositol 5-phosphatase

SH2: Src homology 2

SNX9: Sorting nexin 9

Src tyrosine kinase: Sarcoma tyrosine kinase

SOC: Super optimal broth with catabolite repression

T2SS: Type II secretion system

T3SS: Type III secretion system

TVSS: Type V secretion system

Tet: Tetracycline

Tir: Translocated intimin receptor

Thr: Threonine

TLR: Toll-like receptor

TNF α : Tumour necrosis factor alpha

TNFR: TNF receptor

TRADD: Tumour necrosis factor receptor type1-associated Death domain protein

TRAF: TNF receptor associated factor

Tyr: Tyrosine

UPEC: Uropathogenic *Escherichia coli*

WT: Wild type

1.1 Pathogenic Bacteria

1.1.1 *Escherichia coli* and enteropathogenic *E. coli* (EPEC)

Escherichia coli (*E. coli*) is an aerobic gram-negative bacterium within the family of *enterobacteriaceae* and the genus *Escherichia* (Kaper *et al.*, 2004). It is a commensal organism of the small intestine of humans and animals (Nicolas-Chanoine *et al.*, 2014) with some strains causing diarrhoea in humans, whereas others can spread throughout the body where they cause urinary tract infection or meningitis (Weintraub, 2007). Diarrhoeagenic *E. coli* are divided into six main pathotypes: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffuse adherent *E. coli* (DAEC), enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). During infection all pathogenic *E. coli* strains remain extracellular except EIEC, which is an intracellular pathogen that can invade and replicate within epithelial cells and macrophages, while other *E. coli* strains, which might be internalized at low levels, cannot replicate intracellularly (Gruenheid & Finlay, 2003).

EPEC is one of the first *E. coli* pathotypes recognised to be responsible for millions of paediatric diarrhoeal cases each year (Clements *et al.*, 2012, Levine *et al.*, 1978). It targets the absorptive epithelia (enterocytes) of the human small intestine where alteration leads to severe watery diarrhoea. Although EPEC outbreaks of neonatal diarrhoea in developed countries are now rare, it is one of the most important pathogens infecting infants and causing nonspecific gastroenteritis in the developing world (Dutta *et al.*, 2013, Levine & Edelman, 1984). EPEC with other pathogenic strains including enterohaemorrhagic *E. coli* (EHEC), rabbit specific enteropathogenic *E. coli* (REPEC-1) and the mouse specific *Citrobacter rodentium* are members of the attaching and effacing (A/E) family of pathogens. These members share the ability to induce pathophenotypic attaching and effacing (A/E) lesions following adherence to the apical surface of the intestinal epithelial layer (Wong *et al.*, 2011). The distinguishing features of these lesions are i) localised loss of brush border microvilli, ii) intimate bacterial adherence to the enterocyte apical plasma membrane, and iii) production of actin-rich pedestal-like structures beneath the adherent bacteria (Figure 1) (Mundy *et al.*, 2005, Robins-Browne *et al.*, 1994, Nataro & Kaper, 1998).

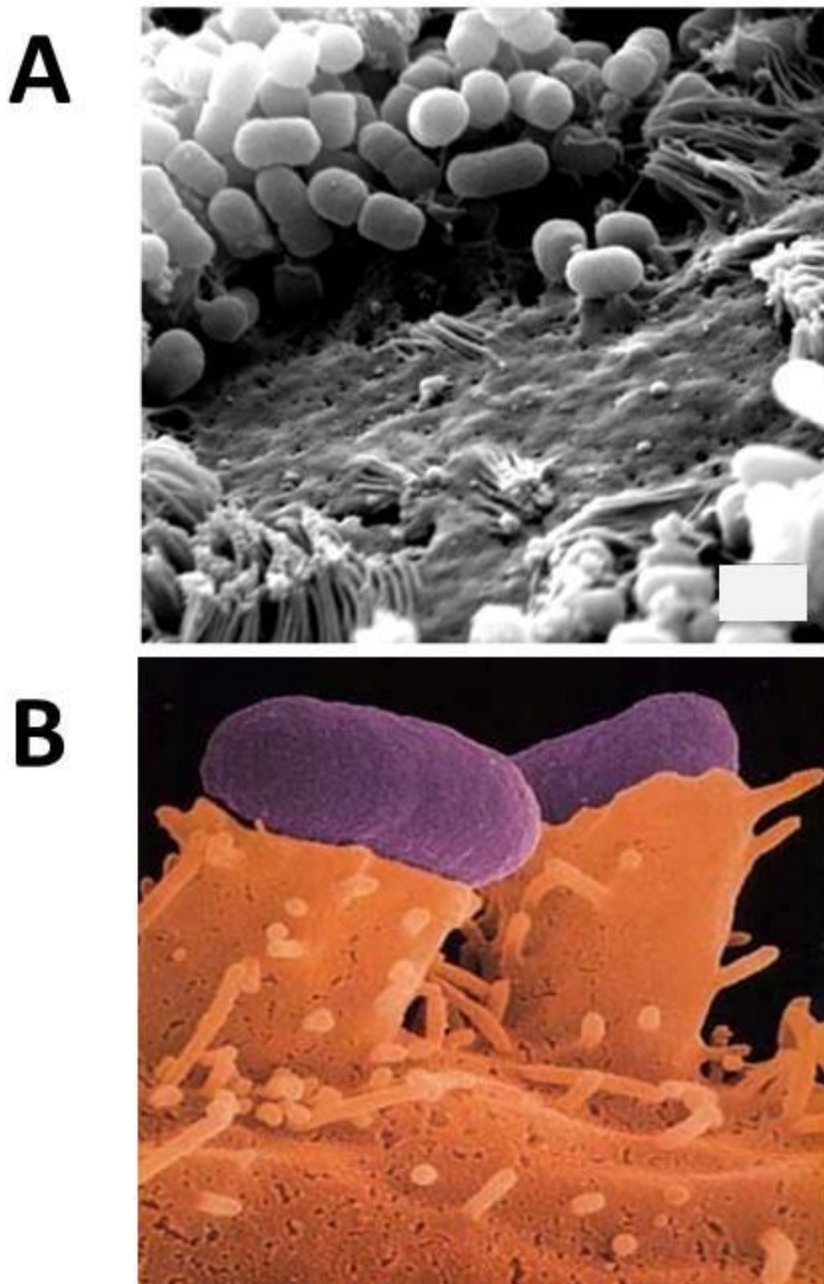


Figure 1: EPEC induced attaching and effacing lesions with actin rich pedestals. A) The extensive effacement of microvilli on the apical surface of epithelial cells (Caco-2 cell model) infected with wild type EPEC. Taken from (Dean *et al.*, 2006). B) EPEC induces pedestal-like structures in HeLa cell model. These pedestal structures protrude from the apical surface, cupping individual bacteria. The image was artificially coloured to distinguish bacteria (purple) from HeLa cell (brown). Taken from (Rosenshine *et al.*, 1996).

Chapter 1 Introduction

1.1.2 Interactions of EPEC with epithelial cells

Infection with EPEC occurs through ingestion or drinking of contaminated food or water. Upon entering the gut, EPEC adhere to epithelial cells via bundle-forming pili (BFP) (Giron *et al.*, 1991). The bundle-forming pili (BFP) is encoded by the EPEC adherence factor (EAF) plasmid (Jarvis *et al.*, 1995). EPEC BFP is a member of the type IV pilus and promotes EPEC aggregation to form microcolonies and bind enterocytes in a manner named localised adherence (LA) (Giron *et al.*, 1991). The disease process depends on the ability of the EPEC to penetrate the intestinal mucus layer, attach to enterocytes and transduce signal cascades that control important cellular functions, including actin and cytoskeletal dynamics (Donnenberg & Kaper, 1992). The gastrointestinal epithelial cells are also covered by a glycocalyx (glycoprotein-polysaccharide) layer, which covers the apical surface of mucosal epithelial cells (McGuckin *et al.*, 2011).

EPEC subverts different mechanisms to colonise intestinal epithelium and penetrate the mucus barrier to access the epithelial surface (Clements *et al.*, 2012). EPEC possesses a type II secretion system (T2SS) that secretes a surface-associated lipoprotein, SsIE (Baldi *et al.*, 2012, Valeri *et al.*, 2015). SsIE is a mucin-binding protein involved in the degradation of mucin substrates and facilitates EPEC penetration of the mucus layer to access the host cells (Valeri *et al.*, 2015). EPEC also secretes a serine protease auto-transporter, EspC, which is secreted by type V secretion system (TVSS) to the extracellular milieu, causing epithelial damage (Stein *et al.*, 1996, Navarro-Garcia *et al.*, 2004). EPEC then utilizes a type III secretion system (T3SS) to translocate proteins into the host cell's cytoplasm. The T3SS with its substrates are encoded on the Locus of Enterocyte Effacement (LEE) pathogenicity island, which encodes over 40 gene products (Elliott *et al.*, 1998).

1.2 Locus of Enterocyte Effacement (LEE)

The locus of enterocyte effacement (LEE) is a 35 kb pathogenicity island present in all attaching and effacing pathogenic strains (Mundy *et al.*, 2005, Robins-Browne *et al.*, 1994, Nataro & Kaper, 1998). In EPEC (E2348/69), the LEE region consists of 41 ORFs organized in seven operons from LEE1 to LEE7 (Figure 2) (Gaytán *et al.*, 2016). EPEC LEE expression is regulated by a non-LEE transcriptional regulator (PerC) encoded by the *perABC* operon on the EAF plasmid (Gomez-Duarte & Kaper, 1995).

Chapter 1 Introduction

The LEE genes, based on their functionality, are classified into a number of groups, including the components of a T3SS apparatus (Sep, for Secretion EPEC protein and Esc, for EPEC secretion components), secreted translocators and effector proteins (Esp, for EPEC secretion protein), chaperones (Ces), regulatory factors (Ler, GrIA, GrIR, and CesL), an adhesin (Eae), Intimin (Lin *et al.*, 2014). The LEE operons (LEE2 to LEE7) are activated by gene located on the LEE1 operon. This gene encodes the master regulator Ler (LEE encoded regulator) (Mellies *et al.*, 1999). In addition to Ler, LEE also has genes that encode a negative (GrIR) and a positive (GrIA) regulator of the Ler expression (Iyoda *et al.*, 2006). CesL can also modulate LEE expression by interacting with Ler (Younis *et al.*, 2010). Once EPEC LEE is activated and proteins are expressed, this leads to the production of an effector-delivery apparatus composed of a T3SS and translocators.

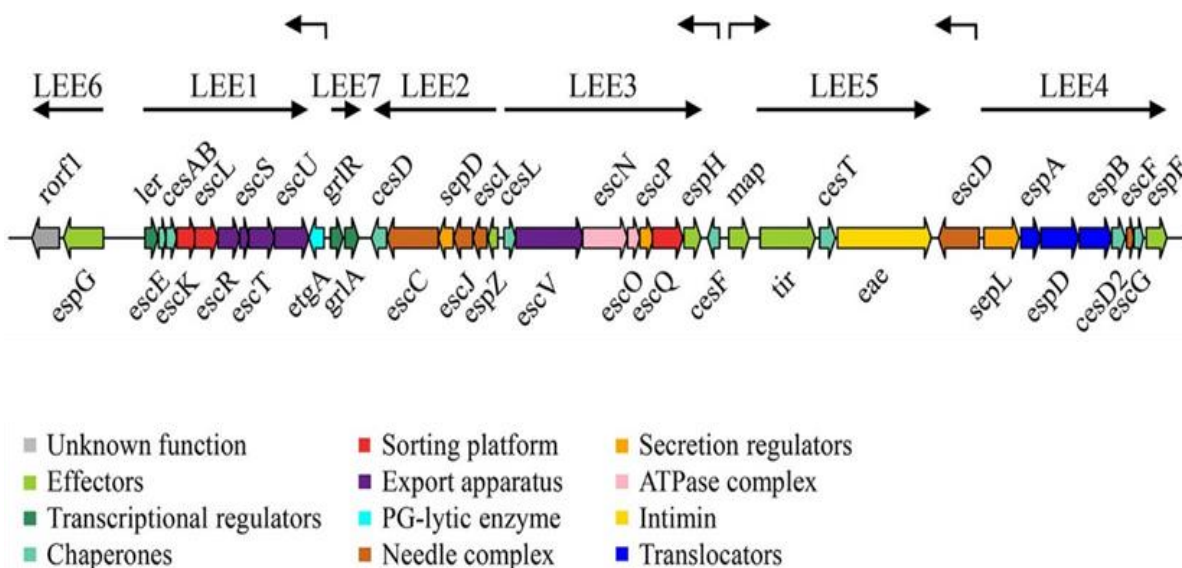


Figure 2: Genetic organisation of EPEC LEE Pathogenicity Island. EPEC LEE 41 genes are organised into seven polycistronic operons (LEE1 to LEE7). Taken from (Gaytán *et al.*, 2016).

1.3 The EPEC type III secretion system (T3SS)

The T3SS apparatus is a macromolecular complex that spans both bacterial membranes with a short needle-like projection to enable direct delivery of 'effector' proteins across the plasma membrane of host cells (Hueck, 1998, Burkinshaw & Strynadka, 2014). The T3SS is composed of ~20 proteins that form a basal body,

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extracellular appendages (needle, filament and the translocation pore), and cytoplasmic components (Figure 3). The basal body of the T3SS system, which spans both the inner and outer bacterial membrane, consists of ring structures associated with the inner (EscJ, EscD), outer (EscC) bacterial membranes and a periplasmic inner rod (EscI) (Figure 3) (Romo-Castillo *et al.*, 2014). EscJ, EscD and EscC proteins are critical for T3SS assembly and are required for the secretion of the Esp proteins (Ogino *et al.*, 2006). EscI, a rod/needle protein, interacts with the outer membrane secretin EscC and it is essential for type III secretion (Sal-Man *et al.*, 2012b).

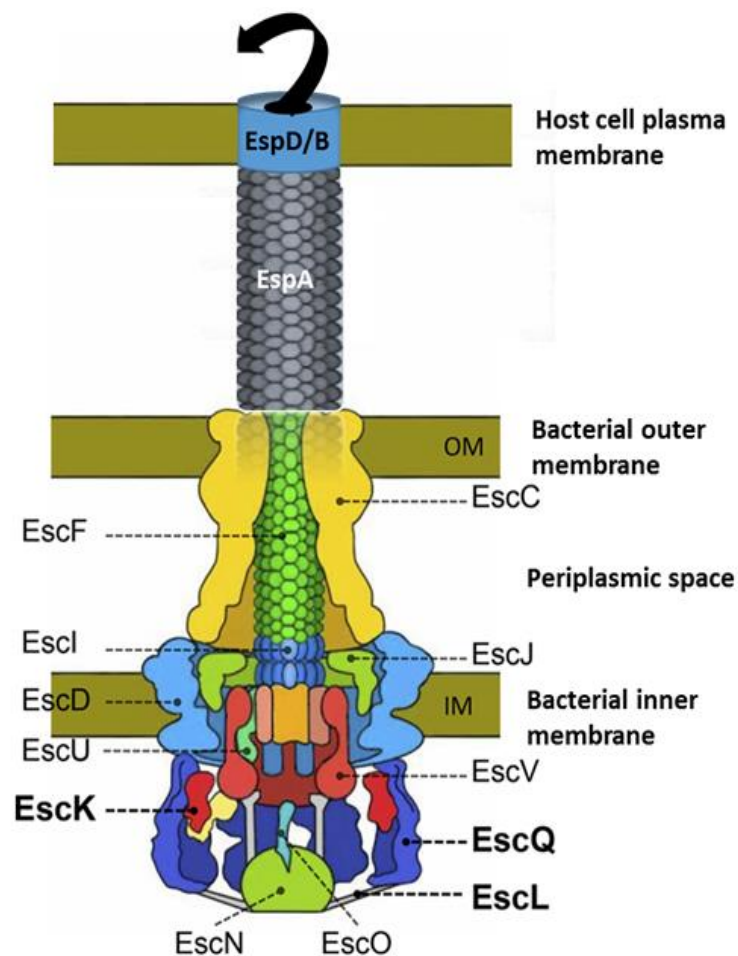


Figure 3: Schematic illustration of the EPEC effector delivery system. The effector delivery system is composed of a T3SS and the translocators. T3SS is encoded by genes located on the locus for enterocyte effacement (LEE), and composed of ~20 proteins that form the basal body, extracellular appendages, and cytoplasmic components. T3SS spans the inner and outer bacterial membranes to deliver EPEC effector proteins into the host cells. The basal body of the TTSS (EscC and EscV, and the EscJ) spans the periplasm forming a cylindrical structure. EscF constitutes a short needle structure that is extended by a long filament formed by EspA subunits. EspA filament to form a hollow tube and at the end of EspA the translocator proteins EspB and EspD are placed to form the pore in the host cell plasma membrane. T3SS is energized by the cytoplasmic ATPase EscN that provide the energy to the system by hydrolysing ATP into ADP. SepD and SepL regulate the secreting of the effector proteins. Adapted from (Soto *et al.*, 2017).

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The basal structure of the T3SS export apparatus consists of five essential transmembrane proteins (EscR, EscS, EscT, EscU, and EscV) (Figure 3). These proteins form the integral inner membrane proteins (Gauthier *et al.*, 2003). EscF is a needle protein that is required for T3SS secretion and EspA filament assembly (Wilson *et al.*, 2001). EspA (Kenny *et al.*, 1996), EspB and EspD (Donnenberg *et al.*, 1993) function to form the translocon portions of the T3SS (Figure 3). EspA is a filamentous protein that forms a translocation tube linking the EscF needle to the host cell's membrane (Knutton *et al.*, 1998). These structures act as a channel to translocate proteins from the bacteria into the host cells. EspB and EspD are located at the end of the EspA filament (Figure 3) where they insert and form a pore into the host cell membrane (Frankel *et al.*, 1998). In EPEC, EscN is T3SS ATPase, which is essential for the functioning of the T3SS translocator and a potential source of energy for T3SS proteins secretion (Zarivach *et al.*, 2007). EscN forms a complex with two other essential T3SS proteins EscL/Orf5 (putative ATPase-negative regulator) and EscQ/SepQ (a predicted component of cytoplasmic C ring) (Biemans-Oldehinkel *et al.*, 2011). Orf16/EscP, a T3SS-secreted substrate, acts as a molecular measuring device that regulates the needle length and the secretion of the inner rod component EscI (Monjarás Feria *et al.*, 2012). In addition, the secretion of both translocators and effectors are controlled by SepL (gatekeeper). SepL functions, together with SepD, to regulate the secretion hierarchy between translocators and effectors (Deng *et al.*, 2015). The stability and secretion of some LEE encoded T3SS proteins are mediated by LEE encoded chaperones. The LEE have genes encoding the translocator chaperones CesA, CesD and CesD2 (Creasey *et al.*, 2003b) and the needle protein EscF chaperone EscE and EscG (Sal-Man *et al.*, 2013). The T3SS must traverse both bacterial membranes and the peptidoglycan layer. This requires a dedicated peptidoglycan lytic enzyme, EtgA, to locally degrade peptidoglycan. EtgA also interacts with the T3SS inner rod component, EscI, and this interaction enhances PG-lytic activity of EtgA (Burkinshaw *et al.*, 2015).

1.4 EPEC LEE and non-LEE effector proteins

Enteropathogenic *Escherichia coli* (EPEC) prototypic strain E2348/69 encodes at least 21 putative effector proteins (Dean & Kenny, 2009, Iguchi *et al.*, 2009). Seven effector proteins are LEE encoded (Tir, EspZ, EspF, Map, EspH, EspG, and the EspB

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translocator protein), with other effector proteins encoded outside the LEE region, in prophages located elsewhere on the chromosome, and termed non-LEE encoded (Nle) effector proteins (Iguchi *et al.*, 2009).

1.5 EPEC LEE effector proteins

1.5.1 Translocated intimin receptor (Tir)

Tir is the first effector protein translocated into host cells via the T3SS where act as a receptor for outer bacterial membrane protein, Intimin (Kenny *et al.*, 1997b, Mills *et al.*, 2008). Tir, a 550-amino-acid-protein, has a predicted molecular mass of 56.8kD but has an SDS/PAGE gel mobility of 78kD (Kenny *et al.*, 1997b). Upon translocation to the host cells, Tir adopts a hairpin-loop conformation with two transmembrane domains (residues 234–259 and 353–382) (TMDs), resulting in a central extracellular domain (residues 260–352) containing the Intimin-binding domain (IBD) that serves as a binding site for Intimin with both N- (residues 1–233) and C-terminal (residues 383–550) domains exposed to the cytoplasm (Figure 4) (Kenny *et al.*, 1997b, de Grado *et al.*, 1999, Kenny, 1999).

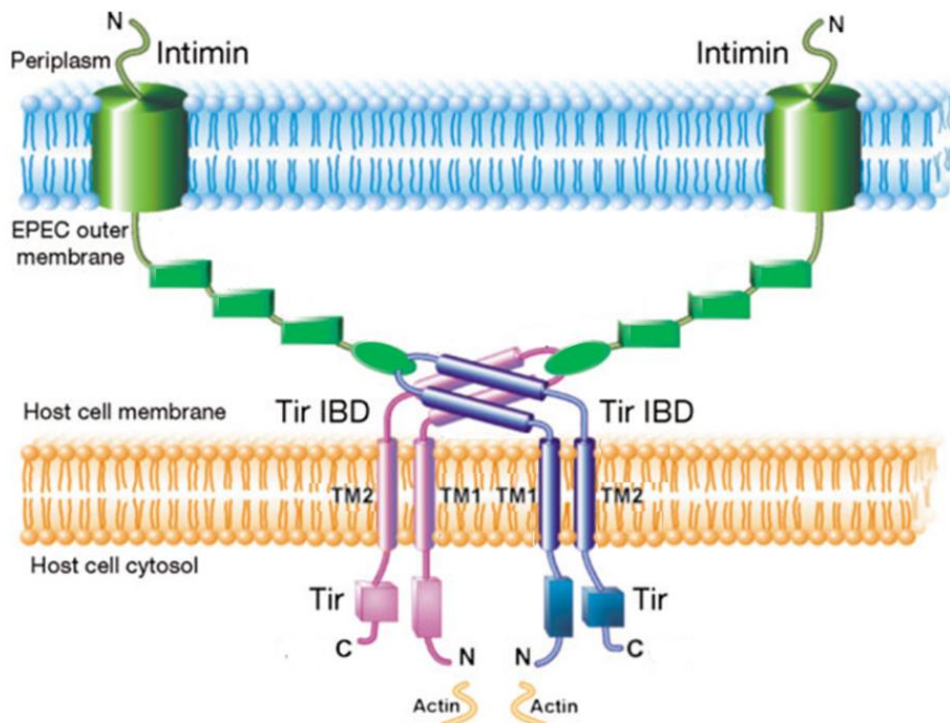


Figure 4: Interactions between the EPEC Intimin and Tir proteins. Intimin is localised in the bacterial outer membrane where the C-terminal fragment can bind Tir. Tir is inserted in the host plasma membrane with an extracellular intimin binding domain (IBD), that mediate intimin-Tir interaction, and the two N- and C-terminal regions are localised in the host cytoplasm. Adapted from (Luo *et al.*, 2000).

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1.5.1.1 Tir features and domains

Tir facilitates different functions according to its domains. The N-terminal 233 amino acid of Tir interacts with numerous host proteins such as α -actinin, talin, and vinculin (Freeman *et al.*, 2000). Recruitment of these proteins to the N-terminal of Tir might stabilise pedestal formation, influences pedestal length and thus binding of A/E pathogens to the host cytoskeleton (Campellone *et al.*, 2006). The N-terminal region is also required for efficient delivery, with 26 amino acids containing a T3SS signal sequence and the first 100 amino-terminal residues of Tir function as a CesT-binding sequence that aids sufficient Tir delivery into the host cells (Crawford & Kaper, 2002). In addition, the N-terminal also possesses a polyproline region (PPR) that is essential for Tir's subversive activities - recruits host tyrosine kinases to modify Tir residues and pedestal formation (Bommarius *et al.*, 2007). By contrast, the carboxy terminus of Tir is targeted by host kinase-mediated phosphorylation events at defined serine and tyrosine residues including S434, S463, Y474, Y454, Y511 and Y483 (Figure 5) (Phillips *et al.*, 2004); while the extracellular domain interacts with Intimin, which mediates intimate bacterial attachment, via binding Tir, to the host cells and production of actin-rich pedestals (Kenny, 1999).

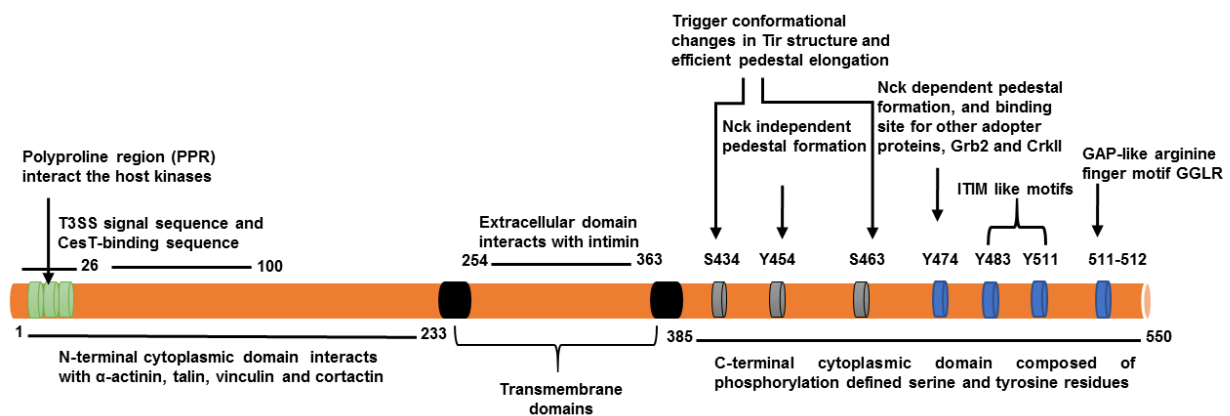


Figure 5: Schematic of EPEC translocated Intimin receptor Tir protein. Tir is 550aa protein with three domains. The N-terminal domain (1-233aa) by which Tir bind to the host proteins such as α -actinin, talin, vinculin and cortactin. The N-terminal also possess a CesT binding sequence and Polyproline motif. The C-terminal domains (385-550aa) comprises residues linked to EPEC Tir's subversive activities (S434, S463, Y454, Y474, arginine finger motif (518-521aa) and ITIM like motifs (Y483 and Y511), while the area between them (254-363aa) is critical for Tir interaction with intimin. Adapted from (Dean & Kenny, 2009).

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1.5.1.2 Tir phosphorylation and pedestal formation

Following its delivery into the host cells, Tir acts as a substrate for host kinases, including protein kinase A (PKA), which phosphorylate Tir on two serine residues S434 and S463 (Warawa & Kenny, 2001). This phosphorylation is linked to a conformational change in the Tir structure, which may promote insertion of Tir into the host plasma membrane, consequently causing an increase in Tir molecular mass by 5kD to the partially modified form (T'), which can be detected in the cytoplasm, followed by the second modification of a 2kD increase to the fully modified form (T''), which can be detected in the membrane on SDS-PA gels (Kenny & Warawa, 2001). Once Tir-serine residues (S434 and S463) are phosphorylated, Tir undergoes a tyrosine phosphorylation event on tyrosine residues Y474, which, while not correlating with any apparent increase in molecular mass, is critical for pedestal formation (Kenny, 1999). This phosphorylation is mediated by host tyrosine kinases (Fyn of the Src family member and Tec/Abl-family kinases) with sufficient role for Abl-family tyrosine kinases Abl1 and Abl2 (Figure 6) (Phillips *et al.*, 2004, Swimm *et al.*, 2004). Tyrosine phosphorylation of Tir on Y474 residue is essential for generating a binding site for the SH2 domain of the adapter protein Nck and other adapter proteins such as Grb2 and CrkII (Lai *et al.*, 2013). This interaction is sufficient to trigger localised actin assembly by binding to the proline motifs of N-WASP (Wiskott Aldrich syndrome protein family members) and stimulating N-WASP to interact with actin-related protein (Arp2/3) and stimulate actin nucleation beneath attached bacteria (Figure 6) (Lai *et al.*, 2013, Campellone *et al.*, 2002, Rohatgi *et al.*, 2001, Gruenheid *et al.*, 2001).

However, in addition to Y474 (Kenny, 1999), Tir is also phosphorylated on residue Y454, although at lower efficiency, and induces pedestal formation in a Nck-independent manner (Frankel & Phillips, 2008). Phosphorylation of Tir on 454 residue recruits phosphatidylinositol 3-kinase (PI3K) and prompts the transient accumulation of PI(3,4,5)P(3) below adherent EPEC (Sason *et al.*, 2009). Phosphoinositides (PIs) are present in the the plasma membrane to modulate the activity of proteins involved in EPEC infection. They are required for EPEC adherence to the host cell surface and for the construction of the actin pedestal, and may influence host cell death during EPEC infection (Sason *et al.*, 2009). Interestingly, during in vitro organ cultures (IVOC) EPEC infection, neither EPEC Tir tyrosine phosphorylation nor Tir:Nck signalling complexes are necessary for A/E lesion formation and N-WASP recruitment (Schuller *et al.*, 2007).

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Tir also, within its C-terminal, encompasses two other tyrosine residues Y483 and Y511, which share sequence similarity with cellular immune-receptor tyrosine-based inhibition motifs (ITIM). Tir is phosphorylated within the ITIM motif, and recruits protein tyrosine phosphatases (SHPs) and subsequently regulates pedestal formation and inhibits immune responses (Yan *et al.*, 2013, Smith *et al.*, 2010). In addition, Tir also possesses a polyproline region (PPR) in its N-terminal that interact with SH3 domains of the kinases. This interaction is essential for pedestal formation. Phosphorylation of Tir on Y474 residue by tyrosine kinases leads to the additional recruitment of kinases by both PPR–SH3 and Y474–SH2 interactions; consequently, other Tir will be phosphorylated, as well as formation of actin pedestals (Bommarius *et al.*, 2007).

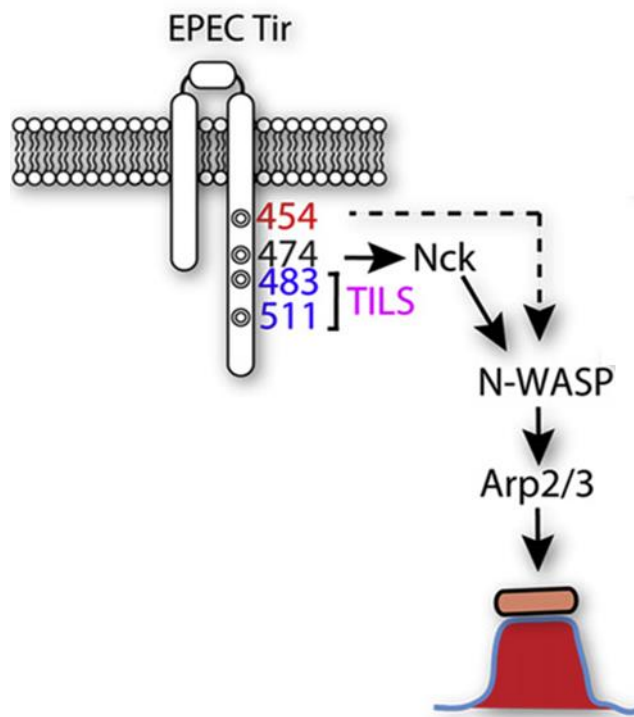


Figure 6: Actin accumulation mechanism in EPEC. In EPEC, actin accumulation and pedestal formation can be either Nck dependent (tyrosine phosphorylation of Tir-Y474) or independent (tyrosine phosphorylation of Tir-Y454). Tyrosine phosphorylation of Tir (Y474 or Y454) within its C-terminal by host kinases lead or not to recruit the adaptor protein Nck that activates N-WASP, which initiates actin polymerisation mediated by the Arp2/3 complex. Taken from (Campellone *et al.*, 2006).

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1.5.1.3 Tir–Intimin dependent and independent function

Initially, Tir was believed to function only in an intimin-dependent manner, acting as a receptor for Intimin. Following its delivery into the host plasma membrane (Tir–intimin interaction), Tir triggers downstream signalling events leading to the formation of actin-rich pedestals beneath the adherent bacteria in a Tir tyrosine phosphorylation dependent manner (Kenny *et al.*, 1997b). Tir–Intimin interaction also aids bacterial sinking into the brush border, causing microvilli effacement and inactivation of sodium glucose co-transporter SGLT-1, which leads to the severe watery diarrhoea during EPEC infection (Kenny *et al.*, 1997b, Dean *et al.*, 2006).

However, recent studies have illustrated the Tir independent functions of Intimin. Tir inhibits nuclear factor kappa B (NF- κ B) activation, leading to a decreased expression of antimicrobial and inflammatory molecules (Ruchaud-Sparagano *et al.*, 2011). Moreover, during EPEC infection, EspG/EspG2 promotes calpain activity whose function is to induce host cell detachment and cleavage of the host proteins. This function is regulated by Tir (Dean *et al.*, 2010a). While Tir downregulates filopodia formation triggered by Mitochondrial associated protein (Map), both effector proteins stimulate invasion by synergistic mechanisms (Lai *et al.*, 2013, Jepson *et al.*, 2003). Down-regulating filopodia formation requires phosphorylation of a Tir tyrosine (Y474) residue and sequestering N-WASP from the Cdc42-GTP pathway by Nck (Tomasevic *et al.*, 2007). Simultaneously, Tir also possesses a putative arginine finger motif, found in GTPase-activating proteins (GAPs), in its C-terminal domain; disrupting this motif impairs the ability of Tir to downregulate filopodia (Kenny *et al.*, 2002b).

1.5.2 EspZ

EPEC secreted/signalling protein Z (EspZ) is a small (9kDa) protein consisting of 98 amino acids and detected at high levels at an early post-infection time point comparable to the transmembrane intimin receptor (Tir) (Mills *et al.*, 2008). EspZ is translocated by T3SS and predicted to be integrated in a hairpin-loop topology into the host cell plasma membrane (Figure 7) with two transmembrane domains and an extracellular loop responsible for EspZ activities (Kanack *et al.*, 2005). EspZ localises to the pedestal-like structures induced by EPEC and to the mitochondria (Smith *et al.*, 2010, Kanack *et al.*, 2005) and functions as a pro-survival effector to prevent rapid

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death of cells during EPEC infection. The N-terminal consists of a translocation signal (20 amino acids) (Kanack *et al.*, 2005), while an extracellular loop 10-amino-acid (from 65 to 74) is critical for EspZ specific activities (Berger *et al.*, 2012). Comparison of EspZ sequences from 12 different A/E pathogen strains revealed that EspZ is a hypervariable protein, particularly among the extracellular loop 10-amino-acid (Kanack *et al.*, 2005).

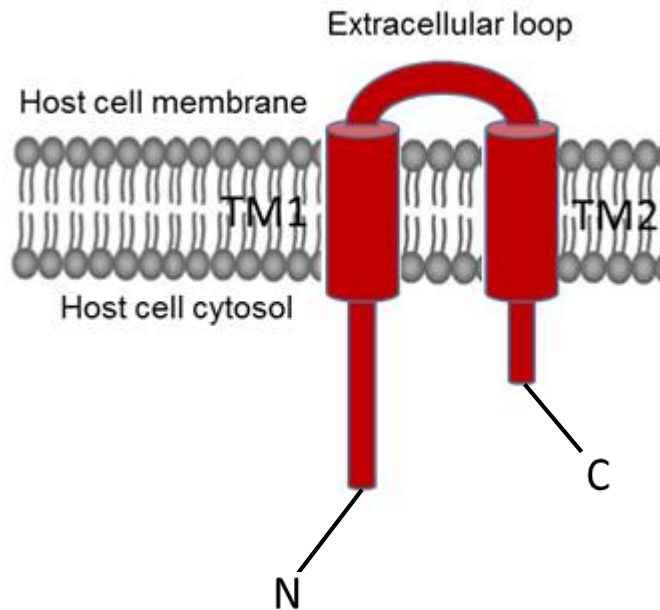


Figure 7: The topology of EPEC EspZ. EspZ integrates into the host cells membrane in the hairpin like structure with two transmembrane domains. The N and C termini of EspZ face the cytosolic leaflet of the plasma membrane and an extracellular domain plays an important role in the EspZ activity. Adapted from (Berger *et al.*, 2012).

1.5.3 EspF

EPEC secreted/signalling protein F (EspF) is a 206-amino-acid-protein with several distinct functional domains (Figure 8) (Zhao *et al.*, 2013). The N-terminal region (residues 1 to 20) is sufficient for EspF secretion and translocation into host cells. EspF N-terminal also possess a mitochondria targeting sequence (MTS; residues 13 to 17) by which EspF targets mitochondria, causing mitochondrial dysfunction and cell death (Nagai *et al.*, 2005). In addition, EspF also targets the nucleolus by the nucleolar targeting sequence (residues 21 to 74), leading to the disruption of nucleolar factors (Dean *et al.*, 2010b). By contrast, the C-terminal region of EspF (residue 74 to 206) (Figure 8) comprises the 3 proline-rich repeat (PRR) domains of 47 amino acids (Alto *et al.*, 2007) by which EspF interacts with several host proteins including N-WASP and

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sorting nexin 9 (SNX9) to induce actin polymerization and membrane remodelling in the host cells (Alto *et al.*, 2007). EspF plays an important role during EPEC infection, with several cellular processes linked to diarrhoea-related symptoms ascribed to the EspF. EspF downregulates sodium hydrogen exchanger 3 (NHE) activity, by which it contributes to diarrhoea (Hodges *et al.*, 2008). Other cellular processes are also attributed to EspF, including prevention of bacterial uptake into macrophages by inhibiting PI-3 kinase, disruption of tight junctions (TJ), disruption of water reabsorption, microvilli effacement and elongation around the bacteria (Quitard *et al.*, 2006, Elliott *et al.*, 2002, Dean *et al.*, 2006).

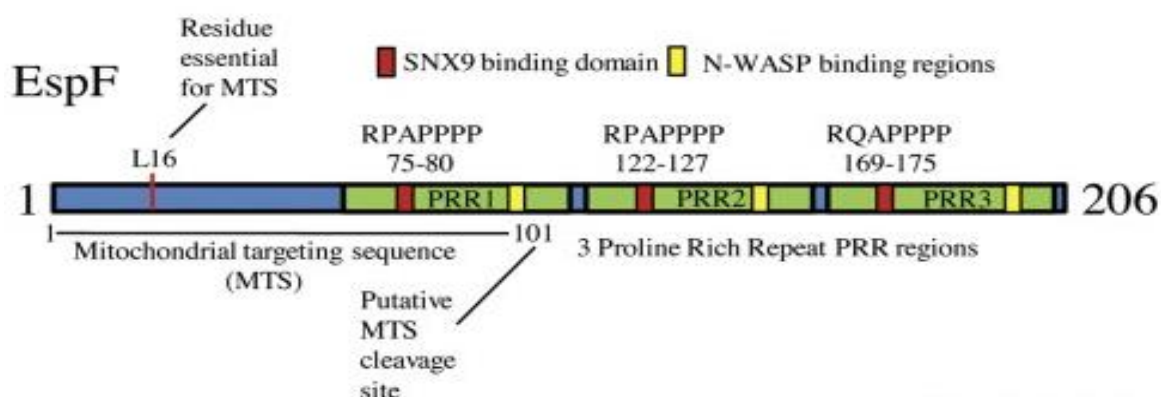


Figure 8: Schematic of EPEC translocated effector protein (EspF). EspF is 206 amino acid protein with an N-terminal encodes a mitochondrial-targeting sequence and possess three proline-rich repeat (PRR) domains of 47 amino acids within its C-terminal, that facilitate EspF interaction with several host proteins including N-WASP and sorting nexin 9 (SNX9). Taken from (Dean & Kenny, 2009).

1.5.4 Map

Mitochondrial associated protein (Map) is a 203 amino acid protein with an N-terminal (44 residues) that encodes a mitochondrial-targeting sequence (Figure 9) by which Map targets a host mitochondria, leading to mitochondrial dysfunction (Kenny & Jepson, 2000, Papatheodorou *et al.*, 2006). Map is a guanine nucleotide exchange factor (GEF) that possess WxxxE motif (74-78) at its N-terminal. This motif activates host Rho GTPase Cdc42 (cell division cycle 42) at the cell membrane, leading to the localised formation of filopodia during the early stages of EPEC infection (Wong *et al.*, 2012a). Filopodia structures are short-lived (retraction is Tir dependent) and have only

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been identified on cultured human cell lines, thus, the requirement of filopodia formation during infection is unknown (Kenny *et al.*, 2002, Berger *et al.*, 2009). In contrast, Map, at the carboxy-terminal, has a classical PDZ1-binding motif (TRL) (Figure 9) that interacts with ezrin/radixin/moesin (ERM)-binding phosphoprotein 50 (EBP50), also known as scaffold protein sodium/hydrogen exchanger regulatory factor-1 (NHERF1), and this leads to stabilisation of filopodia. Moreover, Map also possesses a mitochondrial toxicity region at its C-terminal (residues 101–152) (Papatheodorou *et al.*, 2006). Map also contributes to the maintenance of EPEC colonisation (Nguyen *et al.*, 2015) and disrupts intestinal tight junctions, leading to the onset of diarrhoea (Singh & Aijaz, 2015).

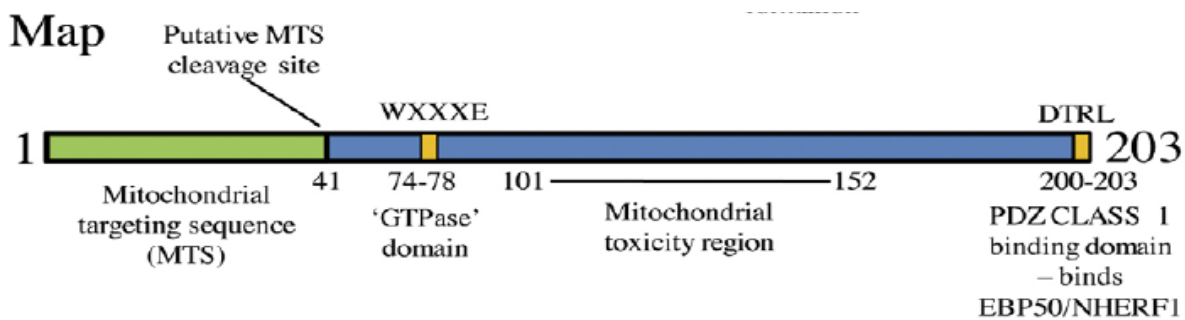


Figure 9: Schematic of mitochondrial associated protein (Map). Map is 203 amino acid protein with an N-terminal encodes a mitochondrial-targeting sequence and possess WxxxE motif (74-78) that activate host The Rho GTPase Cdc42. While its C-terminal possess has a classical PDZ1-binding motif to facilitate its interaction with ezrin and a regulatory factor-1 (NHERF1). Taken from (Dean & Kenny, 2009).

1.5.5 EspH

EPEC secreted/signalling protein H (EspH) is a small protein of 20kDa that is delivered by the T3SS to localise at the host cell membrane (Tu *et al.*, 2003). EspH functions as a RhoGEF inhibitor by binding to the DH-PH domain in RhoGEFs, leading to inactivation of mammalian RhoGEFs, and subsequently inducing changes in cell morphology, triggering cell detachment and inducing cytotoxicity (Wong *et al.*, 2012a, Dong *et al.*, 2010). However, bacteria replaced a mammalian RhoGEFs with a bacterial mimic mammalian RhoGEFs (Map), and translocating bacterial RhoGEFs (Map) to the host cells leads to neutralisation of EspH associated phenotypes, which induces cell adhesion and survival (Wong *et al.*, 2012a). EspH also plays a critical role in EPEC

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resisting macrophage phagocytosis by its ability to bind and inhibit DH-PH domain-containing Rho GEFs (Dong *et al.*, 2010). EspH plays a critical role for efficient pedestal formation and pedestal elongation. It promotes N-WASP recruitment and Arp2/3 to the bacterial attachment site in Tir tyrosine residues Y474 and Y454 independent manner (Wong *et al.*, 2012a). Whereas deletion of EspH leads to a short pedestal and improved filopodia formation, over-expression of EspH leads to pedestal elongation (Tu *et al.*, 2003).

1.5.6 EspG

EPEC secreted/signalling protein G (EspG), previously named *rorf2*, is a 398-amino-acid protein that is secreted and translocated via T3SS into the host cells (Elliott *et al.*, 2001). It is encoded in the LEE region while its homologous Orf3 (EspG2) is encoded on the EspC pathogenicity islet. EspG shows 21% identity of amino acids with the *Shigella flexneri* effector VirA, which has been shown to trigger the destabilization of host microtubule (Elliott *et al.*, 2001). EspG interacts with tubulins and stimulates microtubule destabilization, consequently leading to activation of microtubule-bound GEF-H1 (RhoA-specific guanine nucleotide exchange factor), resulting in the formation of actin stress fibres (Matsuzawa *et al.*, 2004). Recently, it was reported to act as a scaffolding protein and a regulator of GTPase signalling (Selyunin *et al.*, 2011). EspG interacts with master regulators of membrane trafficking (ARF and RAB1), leading to inactivation of ARF GTPase and disruption of endoplasmic reticulum to Golgi trafficking (Selyunin *et al.*, 2014).

1.6 Intimin

An outer bacterial membrane protein, Intimin is expressed on the EPEC cell surface, but not delivered by bacterial T3SS, to form a major adhesin of enteropathogenic *Escherichia coli* (EPEC). It is a 94kDa protein (939 amino acids) encoded by the *eae* gene located in the LEE pathogenicity island (Frankel *et al.*, 1998b). Intimin possesses two functional regions, with the N-terminal region (residues 1–550) containing a signal peptide region that is responsible for its secretion to the bacterial surface and mediating its binding to the peptidoglycan (Leo *et al.*, 2015, McWilliams & Torres, 2014). In contrast, Intimin uses the 280 amino acids of the C-terminal domain (Int280 α) to mediate an intimate interaction with translocated intimin receptor (Tir) (Figure 10)

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(Fairman *et al.*, 2012). Int280 α comprises four globular domains (D1, D2, D3 and D4) extending from the bacterium with two immunoglobulin-like domains and a C-type lectin-like domain responsible for cell-surface carbohydrate recognition and Tir-independent cell binding (Kelly *et al.*, 1999, Hartland *et al.*, 1999). Additionally, Intimin–Tir interaction is required for intimate interaction to the infected host cells and pedestal formation in a Tir tyrosine phosphorylation dependent manner, as well as for triggering tyrosine phosphorylation of phospholipase C-gamma1 (PLC-gamma1) (Kenny & Finlay, 1997). Moreover, Intimin also has additional host cell receptors, including β_1 integrin and nucleolin (Frankel *et al.*, 1996, Sinclair *et al.*, 2006). Interaction of Intimin with these receptors (β_1 integrin and nucleolin) may help Intimin to control the barrier-disrupting activities of Map and EspF independently of Tir (Dean & Kenny, 2004).

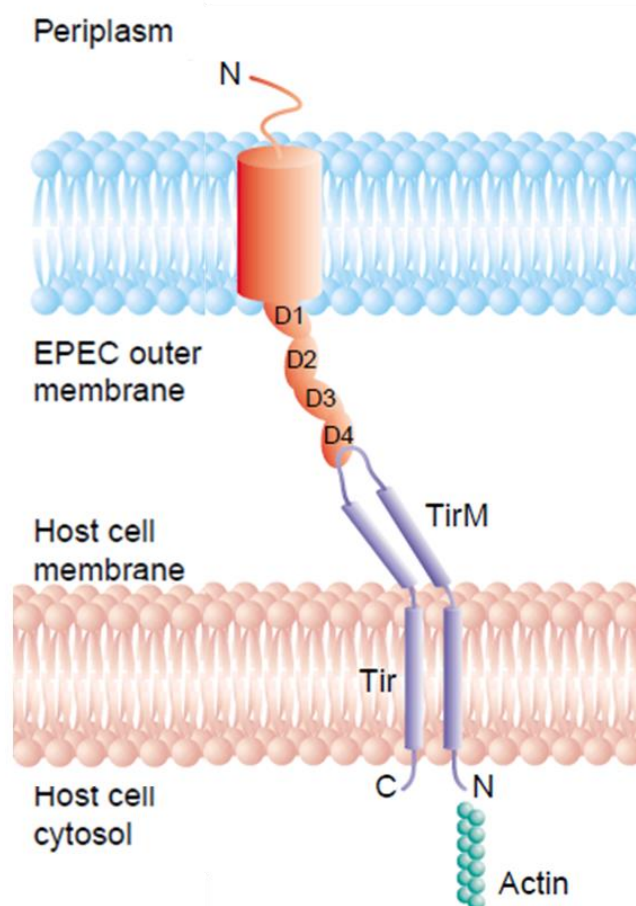


Figure 10: The model of Intimin topology and binding to Tir. The Intimin extracellular carboxy-terminal domain (Int280 α) comprises three immunoglobulin domains (D1, D2 and D3) with a terminal C-type lectin cell-binding domain (D4). Intimin binds the extracellular Intimin binding domain of Tir (IBA). Taken from (Frankel *et al.*, 2001).

1.7 Non-LEE encoded effectors

Non-LEE effector proteins (NLe) are encoded by genes located outside the LEE region, in prophages and integrative elements, and clustered in six pathogenicity islands (PP2, PP4, PP6, IE2, IE5 and IE6) (Figure 11) (Creuzburg & Schmidt, 2007). In EPEC (strain E2348/69) around 24 non-LEE putative effectors were identified and translocated by the LEE encoded T3SS (Dean & Kenny, 2009, Iguchi *et al.*, 2009, Deng *et al.*, 2004b). The non-LEE effector proteins function to inhibit phagocytosis and activate the innate immune response, and some effectors play a role in colonisation and virulence (Wong *et al.*, 2011). During infection, EPEC translocates the non-LEE protein, NleB, into the host cells to activate the transcriptional regulator NF-kappaB (NF- κ B). In contrast, NleC, NleH1/NleH2, NleE and NleA inhibit the activation of NF- κ B (Kim *et al.*, 2007, Yen *et al.*, 2015). While EspJ is reported to inhibit phagocytosis, NleB and LifA promote bacterial colonisation of the host cells and NleD promotes barrier disruption and loss of barrier function (Pearson *et al.*, 2013, Marches *et al.*, 2008, Long *et al.*, 2014). Recently, EspL has been proven to inhibit cell death and inflammation (Pearson *et al.*, 2017).

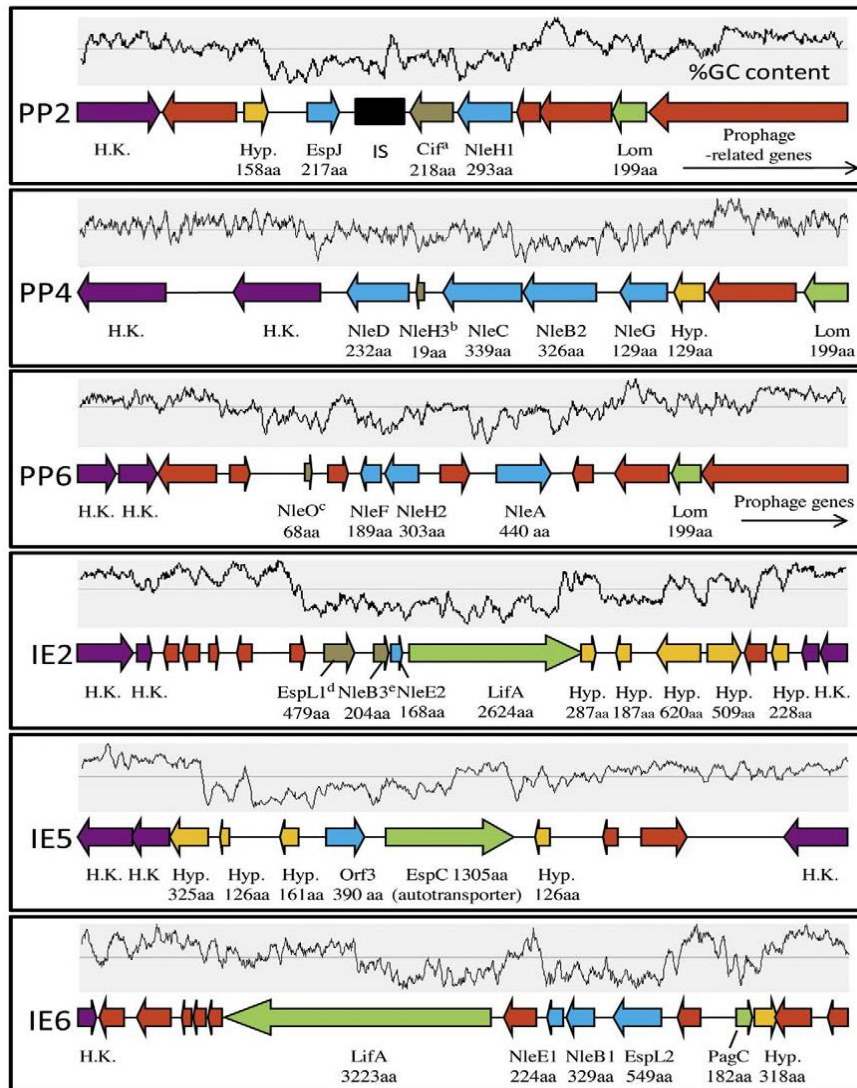


Figure 11: The EPEC non-LEE effectors encoded in the prophages and integrative elements. 24 non-LEE predicted effector genes were identified and clustered in six pathogenicity islands (PP2, PP4, PP6, IE2, IE5 and IE6). Arrows refer to the genes and strand direction, while different colours refer to housekeeping genes (purple), effector genes (blue), virulence genes (green), hypothetical genes (orange), prophage-like transposase genes (red) and pseudogenes (gray). Taken from (Dean & Kenny, 2009)

1.8 EPEC chaperones

Most T3SS substrates rely on chaperones to aid their stability and/or efficient secretion and translocation (Thomas *et al.*, 2005). The type III chaperones are usually small (15-20kDa), acidic and remain within the bacterial cytoplasm even after trafficking a paired protein into an infected host cell (Ramu, 2013). They are grouped into class I (bind effectors), class II (bind translocators) or class III (bind needle-forming) proteins. Class I is subdivided, on the basis of the number of effectors that the chaperone binds, into

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class IA (binds one effector) and class IB (binds multiple effectors). EPEC LEE encodes eight different chaperones, CesF, CesL, CesT, CesAB, CesD, CesD2, EscE, and EscG/CesA2. EPEC has a class IA (CesF for EspF) and class IB (CesT for >10 effector proteins) with several class II chaperones (CesB/D and CesAB that both aid EspB secretion) and class III chaperones (EscE, and EscG) (Ramu *et al.*, 2013, Wainwright & Kaper, 1998, Creasey *et al.*, 2003b, Sal-Man *et al.*, 2013).

CesT, with 156 amino acids, is a multi-cargo class IB chaperone that interacts with more than 10 LEE and non-LEE effectors including Tir, Map, EspH, and EspZ (Thomas *et al.*, 2005). The N-terminal of CesT is implicated in chaperone dimerization, whereas the C-terminal region of CesT is important for CesT dependent effector translocation into host cells (Ramu *et al.*, 2013). CesF is a class IA chaperone that interacts with EspF to aid EspF stability and translocation into the host cells (Elliott *et al.*, 2002). Crucially, CesL, (previously named Orf12/ multiple point controller “Mpc”) a 117-amino-acid EPEC LEE encoded protein, is classified as a class IA chaperone for an aberrant effector SepL (Younis *et al.*, 2010). CesL also interacts with Ler (regulator of LEE gene expression), with over-expression of CesL leads to modulation of Ler activation activity (Tsai *et al.*, 2006).

EPEC class II chaperones are composed of three chaperones, CesD, CesD2 and CesAB. CesD, a 17.5-kDa protein, functions as a chaperone for the EPEC translocators, EspD and EspB. CesD interacts with EspD, but not with EspB, and is required for sufficient EspD secretion (Wainwright & Kaper, 1998). CesD2 is another EspD chaperone that also interacts with EspD and aids its stability and secretion (Neves *et al.*, 2003). By contrast, EspA has two chaperones CesAB and CesA2 (formerly Orf29, renamed EscG in EPEC) that prevent premature oligomerization of EspA and assist in its stabilisation in the cytoplasm (Creasey *et al.*, 2003b, Su *et al.*, 2008). Finally, EscE and EscG/CesA2, an EPEC class III chaperone, function as chaperones for the needle protein, EscF. EscE and EscG chaperones interact with EscF and prevent premature polymerization of the needle (Sal-Man *et al.*, 2013).

1.9 EPEC proteins secretion and regulation

The EPEC LEE and non-LEE encoded proteins are secreted and translocated into host cells, with Tir being the first effector to be translocated, followed by EspZ, NleA, NleH1, EspF, EspH, NleH2, EspJ, Map, EspG, NleD, NleF, NleB1, NleE1, NleB2, NleC, NleG, NleE2, EspG2, and EspL2 (Mills *et al.*, 2013). The EPEC proteins secretion is induced in response to many environmental conditions, with 37 °C an optimal temperature, and other factors such as pH, osmolarity, calcium, iron and salt concentrations also critical (Kenny *et al.*, 1997a). The EPEC proteins are also hierarchically secreted via different mechanisms, which is important for effectors' function stages (Mills *et al.*, 2013, Deng *et al.*, 2017).

1.9.1 Hierarchical control of EPEC proteins secretion

The hierarchical secretion of EPEC proteins is regulated by two T3SS specificity-switching mechanisms that are known as molecular switches (Deane *et al.*, 2010). Molecular switch one, from the needle to the translocators, starts when the needle reaches its proper length, which is regulated by EscP and EscU (Monjaras Feria *et al.*, 2012). Once the needle has reached the final length, EscP interacts with the C-terminal domain of EscU, leading to a conformational change that regulates the switching event from needle to the translocators (Monjaras Feria *et al.*, 2012). Recently, EscP has been shown to interact with the gatekeeper protein SepL to generate EscP-SepL complex (Shaulov *et al.*, 2017). Once bacterial contact with the host cells is made and calcium concentration was dropped, EscP-SepL complex is dissociated, triggering the secretion of effector proteins (Shaulov *et al.*, 2017).

The second molecular switch from the translocators to the effectors is regulated by a family of proteins known as gatekeepers (SepL and SepD), which prevent effector secretion before host cell contact. SepL interacts with SepD, and deletion of either SepL or SepD results in decreased secretion of the translocators and enhanced secretion of effectors (Deng *et al.*, 2015). SepL is thought to interact with EscV, resulting in blocking of the access of effectors to the secretion apparatus (Lee *et al.*, 2014). However, this interaction might be disrupted by low calcium concentration, leading to an increase in translocators' secretion (Lee *et al.*, 2014). In addition, the SepL-SepD complex also interacts with a SepL chaperone (CesL) and regulates the secretion hierarchy in response to pH changes (Yu *et al.*, 2010). Once pores are formed in the host cell membrane, pH is increased and transmitted to the T3SS base,

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resulting in disrupted SepL/SepD/CesL interaction and consequently allowing secretion of effectors (Yu *et al.*, 2010). Furthermore, SepL binds to the effector protein Tir and delays its secretion while the translocators are secreted (Wang *et al.*, 2008). Recently, SepD and SepL have been shown to regulate secretion hierarchy between translocators and effectors by recognising EspB (translocator) export signals (Deng *et al.*, 2015).

1.9.2 Substrate–chaperone binding regulates T3SS secretion

Each effector protein has a chaperone recognition site (50–100 amino acids) and a secretion signal sequence located in the N-terminal (first 20 amino acids), and together these are necessary and sufficient for targeting effector proteins to the T3SS sorting platform and may also contribute to secretion hierarchy (Deng *et al.*, 2015). CesT targets effectors to the T3SS by interacting with a component of the T3SS sorting platform, ATPase EscN, and is implicated in coordinating hierarchical effector secretion (Thomas *et al.*, 2007, Gauthier & Finlay, 2003). CesAB, the EPEC EspA chaperone, interacts with the EscN ATPase following CesAB-EspA binding. Interestingly, prevention of the interaction between EscN and the CesAB-EspA complex resulted in severe secretion (Chen *et al.*, 2013).

1.10 Bacterial protein pore-forming and insertion

Pathogenic bacteria have evolved proteins that can undergo transitions from soluble to membrane-inserted forms (Pedelacq *et al.*, 1999). Pore-forming proteins (PFPs) are produced in a soluble monomeric form that can assemble into oligomeric complexes with the capacity to insert into membranes (Iacovache *et al.*, 2010). The PFT families are either multi domain or multi subunit proteins and called AB toxins, where the B subunit is responsible for binding to the host cells and forming transmembrane pores to translocate the A subunit into the cytoplasm to the target organelle (Reig & van der Goot, 2006). PFTs form transmembrane pores via insertion of a generated transmembrane β -barrel (β -PFTs) or by inserting a bundle of hydrophobic or amphipathic α -helices of PFTs into the membrane (Peraro & van der Goot, 2016, Parker & Feil, 2005). PFTs bind to specific receptors (sugars, lipids or proteins) on the target cell, leading to an increase in the local concentration and oligomerization of PFTs. Thus, exposure of hydrophobic surfaces leads to membrane insertion. In

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addition, charged bacterial membranes and acidic residues with low pH promote the membrane insertion by making some PFTs more hydrophobic, unfolding or inducing a conformational change (Peraro & van der Goot, 2016, Parker & Feil, 2005).

The pore-forming colicins, a class of antibiotics produced by various strains of *E. coli* (Lakey & Slatin, 2001), consist of a hydrophobic helical hairpin that initiates insertion into the lipid bilayer in the colicin umbrella-like model (Figure 12), in which this helical hairpin pair leads insertion of the toxin into target membranes followed by spontaneous insertion of the entire hairpin (Parker *et al.*). In contrast, the diphtheria pore-forming protein insertion mechanism is dependent on a conformational change triggered by a low pH and accompanied by increasing hydrophobicity of the toxin and the ability to form ion channels, reviewed in (Peraro & van der Goot, 2016)

In addition, EPEC translocator protein EspD, *Salmonella* SipB and IpaB from *Shigella* (Hume *et al.*, 2003, Dasanayake *et al.*, 2011) share approximately the same features and insertion mechanism. These proteins contain the amphipathic domains spanning residues that are critical for binding to membrane lipid bilayers in pH dependent manner. Low pH leads to a conformational change correlated with increasing the α -helix content of this protein and promoting insertion into the hydrophobic core of the lipid bilayer. In addition, phosphatidylserine is also critical for the incorporation of the *E. coli* bacterial receptor protein Tir (Race *et al.*, 2006) and a *Pseudomonas aruginosa*, PopD (Faudry *et al.*, 2006) into the host cell membrane. However, while SipB, a virulence factor from *Salmonella*, inserts into the membrane and penetrates through the outer leaflet of membranes to translocate a 50-residue hydrophilic domain across the bilayer, Tir interacts and penetrates through the inner leaflet of the plasma membrane (Race *et al.*, 2006).

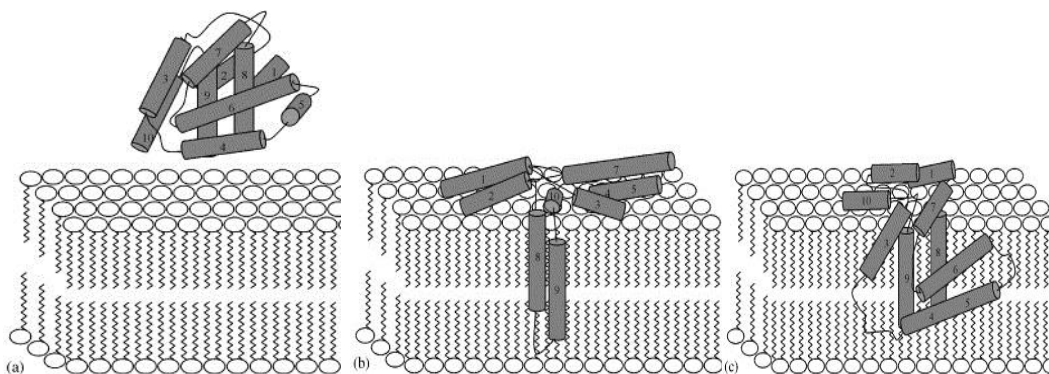


Figure 12: The pore-forming mechanism. (a) The protein form a helical hairpin pair. (b) Followed by umbrella conformation with spontaneous insertion. (c) Formation of the channel. Taken from (Parker & Feil, 2005).

1.11 Manipulation of host cell functions by EPEC effector proteins

1.11.1 EPEC effector proteins induce watery diarrhoea

EPEC infection is correlated with severe watery diarrhoea, particularly in under-developed countries (Dutta *et al.*, 2013, Levine & Edelman, 1984). Although the mechanism by which EPEC causes watery diarrhoea is unclear, EPEC disrupts enterocyte cellular and barrier functions with alteration in ion transport (Figure 13).

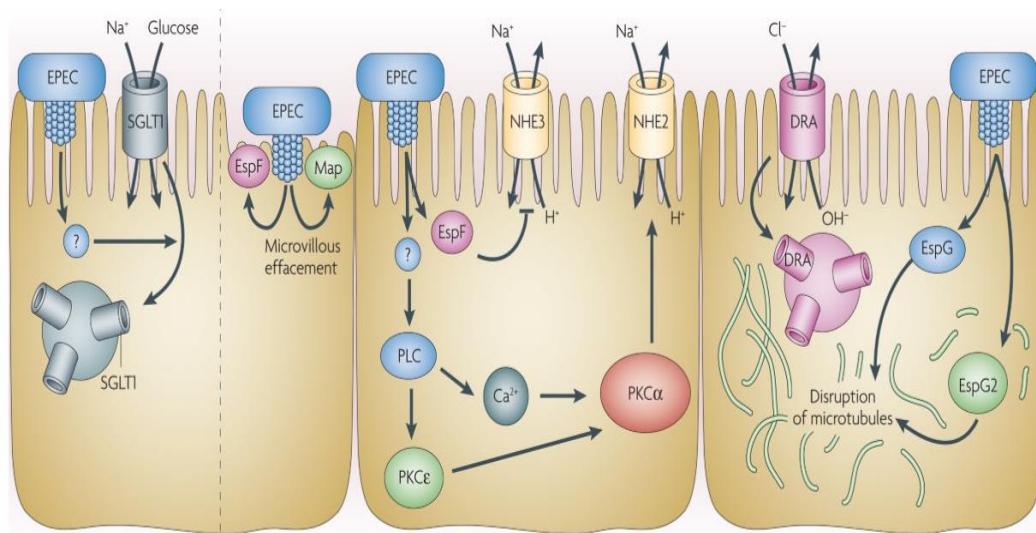


Figure 13: EPEC alters ion transport and induce watery diarrhoea. EPEC infection induced diarrhoea via different mechanisms and all depend on T3SS effectors. EPEC induce rapid Na⁺/glucose (SGLT1) inactivation in pathway dependent on a loss of microvilli, a process that is dependent on Map, EspF, Tir effector proteins and Intimin. EPEC infection also inhibits Na⁺/H⁺ exchangers (NHEs), a process dependent on EspF effector protein. In addition, EPEC alters the Na⁺ uptake and Cl⁻ secretion by injecting effector proteins EspG and EspG2. These effectors disrupt microtubules, preventing protein trafficking, thus reducing DRA expression at the membrane. Taken from (Viswanathan *et al.*, 2009).

EPEC induced diarrhoea is dependent on the effacement of absorptive microvilli and reduction in the absorptive capacity of enterocytes (Hodges & Gill, 2010). This requires bacterial adherence to the brush border membrane with cooperative actions of three LEE encoded effectors (Map, EspF & Tir) and the outer membrane protein Intimin (Dean *et al.*, 2006). EPEC has been shown to rapidly inactivate sodium-D-glucose transporter (SGLT-1), which is responsible for fluid uptake from the normal small intestine, in a T3SS dependent manner with a critical role for Map, EspF, Tir effectors, and Intimin (Figure 13) (Dean *et al.*, 2006, Meinild *et al.*, 1998). Since water moves to high salt concentration areas, changes in ion absorption or secretion leads to diarrhoea (Hodges & Gill, 2010). EPEC infection alters the activity of intestinal absorption via Na

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(+)/H (+) exchanger (NHE), resulting in the inhibition of the uptake of sodium ions (Na⁺) from the gut lumen with an alteration in the Cl⁻ secretion (Hodges *et al.*, 2008). Crucially, EPEC EspF effector protein is shown to be responsible for induced inhibition of NHE activity (Figure 13) (Hodges *et al.*, 2008). Parallel to EPEC mediated inhibition of NHE3, EPEC also mediates a decrease in the expression of major apical anion exchanger DRA (SLC26A3), resulting in inhibition of the Cl(-)/OH(-) exchange activity (Gill *et al.*, 2007). This EPEC effect is attributed to the T3SS dependent effector proteins EspG and EspG2 (Figure 13) (Gill *et al.*, 2007).

Tight junctions are formed between adjacent enterocytes to hinder the movement of membrane components (Hartsock & Nelson, 2008). However, disruption of the tight junction influences diarrhoea through unregulated movement of ions, fluids and antigens between cells (Abreu, 2010). The ability of EPEC to disrupt the tight junction barrier is dependent on the T3SS and the translocated effector proteins EspF, Map and NleA (Thanabalasuriar *et al.*, 2010, Shifflett *et al.*, 2005). While EspF is responsible for redistributing the transmembrane protein, occludin, NleA inhibits the host cell protein trafficking (Thanabalasuriar *et al.*, 2010, Shifflett *et al.*, 2005).

1.11.2 EPEC effectors regulate host cell survival

Programmed cell death occurs either as a normal mechanism to maintain cell populations in tissues or as a defence mechanism during infection (Fink & Cookson, 2005). The programmed cell death results from stimulation of specific signalling pathways, and can be divided into lytic (necrosis and pyroptosis) and non-lytic (Apoptosis) cell death (Jorgensen *et al.*, 2017).

The apoptotic, non-lytic cell death, is induced via extrinsic or intrinsic pathways (Rudel *et al.*, 2010), and characterised by cell shrinkage, membrane blebbing or nuclear condensation (Figure 14) (Lamkanfi & Dixit, 2010). Apoptosis can be balanced by effector(s) with anti-apoptotic activity that neutralise the effects of other, pro-apoptotic effectors, and promote cell survival, as reviewed in (Santos & Finlay, 2015). Indeed, EPEC infection is not correlated with a late apoptotic phenotype, suggesting it has the ability to antagonize host apoptosis during infection (Crane *et al.*, 2001). The ability of EPEC to interfere with host apoptotic pathways depends on the T3SS apparatus to deliver effector proteins into host cells. Some of these effector proteins promote cell

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death, while others inhibit cell death by suppressing either an extrinsic or an intrinsic apoptotic pathway (Rudel *et al.*, 2010). EPEC, within its surface structure's "bundle-forming pili", induces the extrinsic pathway of apoptosis by stimulating the transmembrane death receptors such as tumor necrosis factor (TNF), Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) (Abul-Milh *et al.*, 2001, Peter & Krammer, 2003, Strasser *et al.*, 2000). However, the non-LEE effector proteins NleD, NleB1 and NleB2 suppress extrinsic apoptosis (Baruch *et al.*, 2011, Pearson *et al.*, 2013).

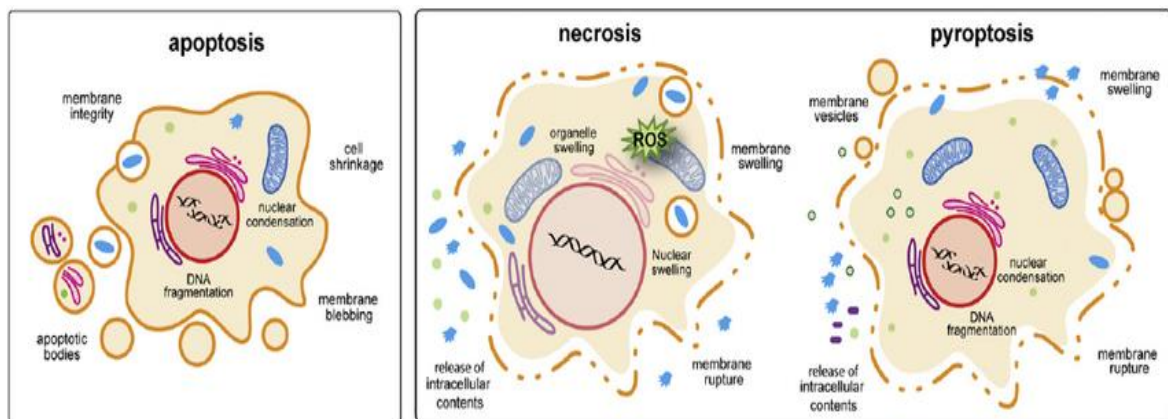


Figure 14: Programmed Cell Death Modes. Programmed cell death can be either apoptosis, necrosis or pyroptosis. While apoptosis is characterised by the retention of plasma membrane integrity. In contrast, both necrosis and pyroptosis are characterised by the release of cytoplasmic content into the extracellular space. Apoptosis and pyroptosis are characteristic by nuclear condensation and DNA fragmentation, whereas the nuclei of necrotic cells swell along with other organelles. Taken from (Lamkanfi & Dixit, 2010).

In contrast, the intrinsic apoptotic pathway is triggered by intracellular stresses, leading to the permeabilization of the outer mitochondrial membrane (MOMP). The MOMP, which is formed by Bcl-2 family members Bak or Bax, leads to the release of cytochrome *c*, activation of caspase-9, caspase-3 and subsequently cell death (Rudel *et al.*, 2010). EPEC also trigger intrinsic apoptotic pathways via the EspF, Map and Cif effector proteins. EspF and Map effector proteins have a mitochondrial-targeting sequence through which both target a host mitochondrion and induce mitochondrial lysis, leading to cytochrome *c* release, caspase activation and intrinsic apoptosis (Nagai *et al.*, 2005, Kenny & Jepson, 2000). While the cycle inhibitory factor (Cif) does not target the mitochondria, it induces cell cycle arrest and subsequently a delayed form of apoptosis in infected cells (Samba-Louaka *et al.*, 2009). In addition, intrinsic

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apoptosis is also induced via a RhoGEF inhibitor, EspH (Wong *et al.*, 2012b). Over-expression of EspH activates the caspase-3 in the infected epithelial cells, which can be inhibited by bacterial RhoGEF mimics EspT and EspM2 (Wong *et al.*, 2012b).

The intrinsic pathway can be prevented via the anti-apoptotic activity of non-LEE effector proteins NleH1 and NleH2, which inhibit pro-caspase-3 cleavage at the bacterial attachment site (Hemrajani *et al.*, 2010). Comparably, while the non-LEE effector protein, NleF, prevents both intrinsic and extrinsic apoptotic pathways by inhibiting caspases-4, -8 and -9 (Blasche *et al.*, 2013), EspZ, an EPEC LEE effector protein, inhibits intrinsic apoptosis, promotes host survival and prevents rapid death of cells during EPEC infection (Shames *et al.*, 2010).

Pyroptosis is a lytic cell death mode that is characterized by cytoplasmic swelling, plasma membrane permeabilization and release of the intracellular components into the extracellular space (Figure 14) (Fink & Cookson, 2005). Pyroptosis is initiated by caspase 1 or caspase 11, which are activated by inflammasomes (Lamkanfi & Dixit, 2009, Martinon *et al.*, 2002). The inflammasome consists of cytosolic sensors such as the nucleotide-binding oligomerization domain-like receptors, known as NOD-like receptors (NLRs), which detect cytosolic contamination by the T3SS needle, bacterial flagellin or toxin (Lamkanfi & Dixit, 2009). Once caspase 1 is activated, it cleaves pro-interleukin-1 β and IL-18 into their mature forms (Lamkanfi & Dixit, 2009) and also cleaves gasdermin D (Shi *et al.*, 2015), a member of the enigmatic gasdermin protein family. The cleavage of gasdermin D releases the N-terminal domain which moves to plasma membrane and induces the formation of a membrane pore and pyroptosis (Qiu *et al.*, 2017).

Necrosis is another mode of cell death that results in a similar cellular morphology to pyroptosis (Figure 14), including cell lysis which leads to the loss of intracellular contents into the extracellular space (Vanden Berghe *et al.*, 2010). Bacterial infection leads to the stimulation of the serine/threonine kinase receptor interacting protein (RIP1 & RIP3) activities, which are required for tumor necrosis factor (TNF)-induced necroptosis. RIP1 kinase binds to death receptors such as TNF receptor 1 (TNFR1), Fas, and tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAILR1) (Schutze *et al.*, 2008). It is also recruited to the Fas-associated death domain protein (FADD), resulting in the activation of calpain, an increase in reactive oxygen species

(ROS) production and lysosomal membrane permeabilization that thus induces necrosis (Hitomi *et al.*, 2008, Declercq *et al.*, 2009).

1.11.3 EPEC effectors manipulate inflammatory signalling pathways

Intestinal epithelial cells possess pattern recognition receptors (PRRs), including toll-like receptors (TLRs), that detect of bacterial stimuli on apical and basolateral surfaces of epithelial cells (Girardin *et al.*, 2002, Peterson & Artis, 2014). This lead to activate inflammatory signalling pathways such as nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways, leading to regulation of the production of cytokines such as IL8, tumour necrosis factor- α (TNF- α) and mediation of host defence (Figure 15) (Peterson & Artis, 2014, Neurath *et al.*, 1998).

EPEC is able to manipulate host innate immune defences, prior to the disruption of barrier function, by the action of several T3SS-dependent secreted effector proteins. Tir is the first T3SS dependent effector translocated into host cells during EPEC infection and is implicated in the suppression of NF- κ B activation (Ruchaud-Sparagano *et al.*, 2011, Mills *et al.*, 2008). Tir interacts with TNF receptor-associated factor (TRAF) and inhibits NF- κ B activation (Ruchaud-Sparagano *et al.*, 2011). Tir possesses ITIMs like motifs (Y483 and Y511) at its C-terminal region by which Tir recruits the regulatory protein tyrosine phosphatase SHP-2, enhancing their inhibitory associations with TRAF6, resulting in suppression of host cytokine production and prevention of downstream NF- κ B activation (Figure 15) (Yan *et al.*, 2013). In addition, while the non-LEE effector proteins NleE and NleB prevent activation of I κ B kinase (IKK β) and consequently the degradation of the NF- κ B inhibitor, I κ B (Nadler *et al.*, 2010), NleH1 and NleH2 inhibit the nuclear translocation of NF- κ B gene transcription via binding to ribosomal protein S3 (RPS3) of NF- κ B complexes, thus preventing transcription of pro-inflammatory genes (Figure 15) (Gao *et al.*, 2009). NleC, a zinc metalloprotease, is localised to the cytoplasm beneath infecting bacteria and to the nucleus of infected cells and possesses zinc metalloprotease activity (Yen *et al.*, 2010). The NleC cleaves the p65 subunit (NF- κ B heterodimer subunit), resulting in inhibition of translocation of NF- κ B to the nucleus and disruption to NF- κ B signalling (Figure 15) (Yen *et al.*, 2010). In contrast, NleD, a zinc metalloprotease, cleaves the MAPK signalling pathway components, thus preventing activation of the transcriptional activator of pro-inflammatory cytokine production factor (Baruch *et al.*, 2011).

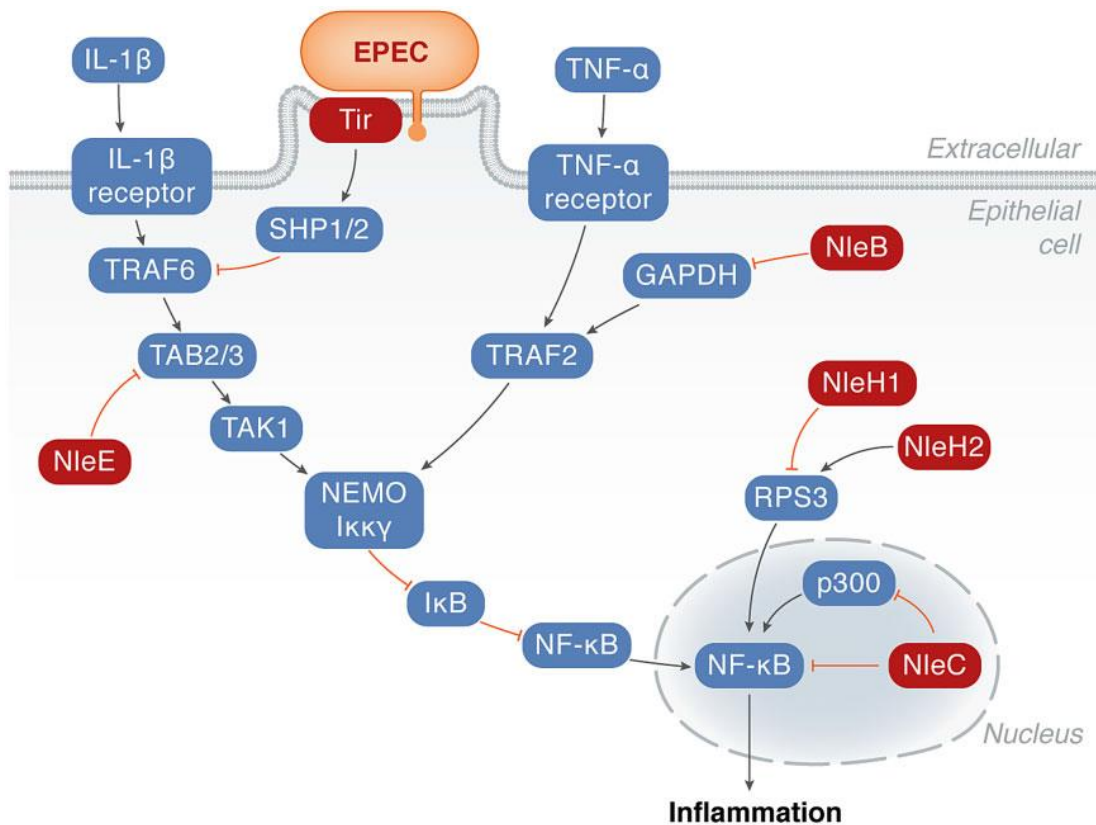


Figure 15: EPEC effectors inhibit NF- κ B activation in the intestinal epithelial cells. EPEC effector Tir promotes SHP1/2 interaction with TRAF6, resulting in inhibition of NF- κ B induced inflammation. While NleE methylates TAB2/3, inhibiting TAK1 activation, NleB glycosylates GAPDH, preventing its activation of TRAF2. NleH1 binds to ribosomal protein S3 (RPS3), inhibiting the nuclear translocation of the NF- κ B complex, while NleH2 promotes RPS3 nuclear translocation. NleC inhibits the signalling pathways through the cleavage of host proteins p65 subunit of NF- κ B. NleC also cleaves p300, an acetyltransferase that promotes p65 activity. (EPEC effectors are highlighted in red). Taken from (Santos & Finlay, 2015, Cozzone, 2005).

1.12 Project aims

Enteropathogenic *E. coli* (EPEC) deliver 24 known T3SS dependent effector proteins into host cells (Dean & Kenny, 2009, Iguchi *et al.*, 2009, Deng *et al.*, 2004b). Seven of them are encoded in the LEE region with one, Tir, acting as a receptor for the pathogen, and another, EspZ, functioning as an anti-cytotoxic factor (Kenny *et al.*, 1997b, Shames *et al.*, 2010). The mechanisms by which Tir is inserted into the host cells membrane and EspZ protects against cell death are slowly being unravelled. Thus, the project aims are to examine the possible role for LEE encoded factor(s) in the modification of Tir to the T⁺ form; its insertion into the membranes of host cells; and to investigate the mechanism by which EPEC EspZ protects against EPEC triggered cell death. Moreover, this project also will focus on the *Edwardsiella tarda* (*E.tarda*) type III secretion system as a useful model to investigate the functionality of T3SS components.

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.1 Mammalian cell culture

The human cervical epithelial cancer cell line (HeLa cells; American Type Culture Collection ATCC® CCL-2™) was retrieved from liquid nitrogen stocks for growth in Dulbecco's minimal Eagles medium (DMEM, high glucose; Sigma Cat #D5796) supplemented with 10% foetal bovine serum (FBS, Lonza Bio Whittaker, Fisher Scientific) lacking antimicrobial or antifungal agents. All cell culture processing was performed in a Class II laminar flow hood (Bio-Mat 2). HeLa cells were routinely passaged in 75 cm² tissue culture flasks (Corning) and incubated (37°C; 5% CO₂) to ~80-90% confluence before passaging (1:6 dilution) into a new flask. Passaging involved washing cells twice with sterile phosphate buffered saline (PBS; 137 mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10 mM disodium phosphate and 1.8 mM monopotassium phosphate; pH7.4; Sigma Cat #P5368) before adding trypsin (1 x Trypsin-EDTA [ethylenediamineacetic acid]; Sigma Cat #T4174) to detach cells and resuspending in DMEM/FCS culture media (prewarmed to 37°C) and transferring a sixth to a fresh flask. Frozen stocks were generated by adding a cryoprotectant (DMSO; Sigma Cat #D2650) to a final 10% concentration in a DMEM/FCS/cells suspension (~10⁶ cells/ml) with slow freezing in -80°C freezer before transferring to liquid nitrogen. A test stock culture was thawed out to confirm viability.

2.1.2 Bacterial strains and mammalian cells

Bacterial strains used are listed in the Table 1A-D. Bacteria stocks were stored at -80°C (adding 50% glycerol to final 10% to Luria-Bertani (LB) grown cultures) and, when appropriate, streaked onto LB agar plates supplemented with appropriate antibiotics. Antibiotics used at final concentration of 50, 25, 100, 25, 12 and 25 µg/ml for Nalidixic acid (Nal), Kanamycin (Km), Carbenicillin (Cb), Chloramphenicol (Cm), Tetracycline (Tet) and Streptomycin (Step) respectively. Single colonies were used to inoculate LB broth - supplemented with selective antibiotic(s) when appropriate – for overnight incubation (~16h) at 30 or 37°C with (for molecular biology) or without (for infection studies) shaking.

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Table 1A Strains derived from EPEC E2348/69 (Kenny Lab stock)

Bacterial Strain	Description	Antibiotic	Source
Wild-type (WT) EPEC E2348/69 (0127:H6)	Nal ^R variant of Prototypical EPEC strain E2348/69	Nal ^R	(Levine <i>et al.</i> , 1985)
espZ mutant	Lacks the <i>espZ</i> gene.	Km ^R	Kenny Lab (S Quitard)
cfm14	T3SS-deficient; Transposon in <i>escN</i>	Km ^R	(Donnenberg & Kaper, 1991)
eae	Lacks the Intimin gene	Nal ^R	Kenny Lab (B Kenny)
espB (UMD864)	Effector-delivery system deficient	Nal ^R	(Taylor <i>et al.</i> , 1998)
espA (UMD872)	Effector-delivery system deficient	Nal ^R /Km ^R	(Kenny <i>et al.</i> , 1996b)
espD (UMD870)	Effector-delivery system deficient	Nal ^R /Km ^R	(Lai <i>et al.</i> , 1997)
escD	T3SS-deficient	Nal ^R	This study
cesT_{Km}	Lacks CesT effector chaperone	Nal ^R /Km ^R	Kenny Lab (B Kenny)
sepL	T3SS-deficient	Strep ^R	(Monjaras Feria <i>et al.</i> , 2012)
escK	T3SS-deficient	Strep ^R	(Soto <i>et al.</i> , 2017)
escL	T3SS-deficient	Strep ^R	(Soto <i>et al.</i> , 2017)
escP	T3SS-deficient	Strep ^R	(Monjaras Feria <i>et al.</i> , 2012)
espDB	Lacks EspD and EspB; thus non-functional effector-delivery system	Nal ^R /Km ^R	Kenny Lab (B Kenny)
espAB	Lacks EspA and EspB thus non-functional effector-delivery system	Nal ^R /Km ^R	Kenny Lab (B Kenny)
mz_{Km}	Lacks Map & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
fz_{Km}	Lacks EspF & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
hz_{Km}	Lacks EspH & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
goz	Lacks EspG, Orf3/EspG2 & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
zt_(full)	Lacks EspZ & Tir (missing residues 1-550)	Nal ^R	This study
tb	Lacks Tir & EspB and thus non-functional T3SS	Nal ^R	This study
ez_{Km}	Lacks Intimin & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
etz_{Km}	Lacks Intimin, Tir & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mez_{Km}	Lacks Map, Intimin & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mtz_{Km}	Lacks Map, Tir & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mfz_{Km}	Lacks Map, EspF & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
ftz_{Km}	Lacks EspF, Tir & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mfez_{Km}	Lacks Map, EspF, EspZ and Intimin	Nal ^R /Km ^R	Kenny Lab (S Quitard)
etmz_{Km}	Lacks Intimin, Tir, Map & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
etfz_{Km}	Lacks Intimin, Tir, EspF & EspZ	Nal ^R /Km ^R	Kenny Lab (S Quitard)
fz_{Kme}	Lacks EspF, EspZ and Intimin	Nal ^R /Km ^R	This study
tmfz_{Km}	Lacks Map, EspF, EspZ & Tir	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mfz_{Km}go3	Lacks Map, EspF, EspZ EspG & Orf3/EspG2	Nal ^R /Km ^R	Kenny Lab (S Quitard)
mfz₍₈₁₎go3	As <i>mfz_{Km}go3</i> but <i>EspZ_{Km}</i> is swapped with <i>EspZ₍₈₁₎</i> that is missing 81 of 98 residues	Nal ^R	This study
mfz₍₈₁₎go3e	As <i>mfz₍₈₁₎go3</i> but also lacks Intimin (outer membrane protein)	Nal ^R	This study
mfz₍₈₁₎go3eh_{Km}	As <i>mfz₍₈₁₎go3e</i> but also lacks EspH (marked by Km-encoding gene)	Nal ^R /Km ^R	This study
mfz₍₈₁₎go3h_{Km}	As <i>mfz₍₈₁₎go3</i> but lacks EspH (marked by Km-encoding gene)	Nal ^R /Km ^R	This study
mfz₍₈₁₎go3eh_{Km}b	As <i>mfz₍₈₁₎go3eh_{Km}</i> but also lacks EspB and thus non-functional T3SS	Nal ^R /Km ^R	This study
mfz_(Km)go3t_(full)	Lacks Map, EspF, EspZ, EspG, Orf3/EspG2 & Tir	Nal ^R	This study
Δgo3core	Lacks EspG & Orf3/EspG2 & core region	Nal ^R	Kenny Lab (S Quitard)

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Table 1B Strains derived from EPEC E2348/69 (Japan & Rosenshine Lab stock)

Bacterial Strain	Description	Antibiotic	Source
TOEA7	EPEC E2348/69 (Nal ^S) lacks LEE EspG effector & 14 Nle	None	(Yen <i>et al.</i> , 2010)
TOEA7/pTet	As TOEA7 with pTet plasmid	Tet ^R	This study
TOEA7- <i>ff</i> pTet	As TOEA7/pTet but lacks EspF	Tet ^R	This study
TOEA7- <i>fz</i> pTet	As TOEA7/pTet but lacks EspF & EspZ	Tet ^R	This study
TOEA7- <i>fzm</i> pTet	As TOEA7/pTet but lacks EspF, EspZ & Map	Tet ^R	This study
TOEA7 Δ <i>fzmh</i> _{Km} /pTet	As TOEA7/pTet but lacks EspF, EspZ, Map & EspH	Tet ^R /Km ^R	This study
TOEA7- <i>fzm</i> _(full) /pTet	As TOEA7/pTet but lacks EspF, EspZ, Map & Tir	Tet ^R	This study
TOEA7- <i>fzme</i> pTet	As TOEA7/pTet but lacks EspF, EspZ, Map & Intimin	Tet ^R	This study
TOEA7- <i>fzmeh</i> _{Km} /pTet	As TOEA7/pTet but lacks EspF, EspZ, Map, Intimin & EspH	Tet ^R /Km ^R	This study
TOEA7- <i>fzmth</i> _{Km} /pTet	As TOEA7/pTet but lacks EspF, EspZ, Map, Tir & EspH	Tet ^R /Km ^R	This study
TOEA7- <i>fzmth</i> _{Kme} /pTet	As TOEA7/pTet but lacks EspF, EspZ, Map, Tir, EspH & Intimin	Tet ^R /Km ^R	This study
TOEA7- <i>bl</i> pTet	As TOEA7/pTet but lacks EspB, thus non-functional T3SS	Tet ^R	This study
TOEA7- <i>fzmeh</i> _{Km} <i>bl</i> pTet	As TOEA7- <i>fzmeh</i> _{Km} /pTet but lacks EspB and thus non-functional T3SS	Tet ^R /Km ^R	This study
TOEA7 Δ <i>core</i> _{Km} /pTet	As TOEA7/pTet but lacks LEE 'core' region	Tet ^R /Km ^R	This study
<i>Ler</i>	Lacks EPEC E2348/69 <i>Ler</i> protein (master positive regulator of LEE gene expression)	Km ^R	(Mellies <i>et al.</i> , 2007)

Table 1C: Non-EPEC pathogens

Bacterial Strain	Description	Antibiotic	Source
<i>Yersinia pseudotuberculosis</i> YIII+	Lack genes for most known T3SS Yop effectors	Km ^R /Cm ^R	(Hakansson <i>et al.</i> , 1996)
<i>Yersinia enterocolitica</i> MRS40(pIML421)	Lack genes for most known T3SS Yop effectors	Nal ^R	(Cornelis <i>et al.</i> , 1986)
<i>Edwardsiella tarda</i> (<i>E. tarda</i>)	fish pathogen carrying LEE region that is homologous to the EPEC LEE region	None	(Nakamura <i>et al.</i> , 2013)

Table 1D: Non-pathogenic K12 *E. coli* with or without EPEC factor

Bacterial Strain	Description	Antibiotic	Source
DH10B	Non-pathogenic <i>E. coli</i> K12.	None	Thermo Fisher Scientific
SM10 λ pir	<i>E. coli</i> SM10 (λ pir strain) contains the λ pir gene allows replication of <i>R6K ori</i> -based suicide plasmids.	Km ^R	(Donnenberg & Kaper, 1991)
TOB01	Non-pathogenic <i>E. coli</i> K12 carrying pTOK-01 plasmid encoding <i>bfp</i> and <i>perABC</i> operons. Also has pTOK-02 (no insert).	Km ^R /Cm ^R	(Yen <i>et al.</i> , 2010)
TOB02	As per TOB01 but LEE (from EPEC B171) region on pTOK-02 plasmid.	Km ^R /Cm ^R	(Yen <i>et al.</i> , 2010)
SIEC	Pili/Fimbrin adhesin-deficient K12 (non-pathogenic) <i>E. coli</i> with EPEC LEE1, LEE2, LEE3 & LEE4 operons integrated in chromosome (IPTG inducible)	None	(Ruano-Gallego <i>et al.</i> , 2015)
SIEC-LEE5	As SIEC but also has LEE5 operon	None	(Ruano-Gallego <i>et al.</i> , 2015)
SIEC Δ p1-LEE5	As SIEC-LEE5 but lacks promoter to drive expression of T3SS components needed for the effector-delivery process	None	(Ruano-Gallego <i>et al.</i> , 2015)
SIEC-LEE5 Δ <i>cesT</i> _{Km}	As SIEC-LEE5 but lacks a functional <i>CesT</i> gene	Km ^R	This study

Table 1A-D: Bacterial strains used in these studies providing a brief description, antibiotic selection profile and source. (Km) indicates that associated disrupted gene carries genes encoding kanamycin resistance with (full) indicating that the entire gene – ATG to stop codon -has been disrupted, while (81) indicates that 81 of 98 EspZ residues are missing. Core refers to that the loss of LEE region genes encoding the EspH, CesF, Map, Tir, CesT and Intimin protein.

2.2 Molecular biology

2.2.1 Polymerase chain reaction (PCR) and agarose gel electrophoresis

Polymerase chain reaction was performed to support strain genotype or amplify genes for cloning or DNA sequencing. Oligonucleotide primers (listed in Table 2) were designed using Sigma Life Science program (<http://www.sigmaaldrich.com/catalog/product/sigma/oligo?lang=en®ion=GB>) and obtained from (Sigma-Aldrich-UK). For cloning, oligos were designed to amplify genes with extensions to provide restriction enzyme site or complemented sequence to the vector region as recommended by Gibson assembly protocol (Gibson *et al.*, 2009). *Taq* DNA polymerase (New England Bio-labs: #M0273L) was used for PCR screening programs while Q5 Hot start high-fidelity DNA polymerase (New England Bio-labs; Cat #M0493S) was used for cloning and sequencing. Standard PCR reactions (25 µl) were prepared by mixing the required components (New England Bio-labs) listed in Table 3. For DNA amplification a Thermo-cycling PCR run under conditions given in Table 4. The PCR products were mixed (ratio 1:9) with 10x ficoll gel loading dye (100 mM EDTA, 1.0% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol) and analysed, alongside 1 Kb 2 log DNA ladder (New England Bio-labs; Cat #N3200S), on 0.7-1% TAE (40 mM Tris-acetate, 1 mM EDTA) agarose (MELFORD; #MB1200). The fluorescent nucleic acid stain GelRed™ (Biotium; used 1:25,000) was added to the agarose with gel electrophoresis in TAE buffer at 100 volts with stained DNA visualised via a UV transilluminator (Bio-Rad).

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1	EP- <i>espA</i> FP	GATAACGAGCAGAGGAGTAATAC
2	EP- <i>espA</i> RP	CAGACCTCACAGACTGGATATCG
3	EP- <i>espZ</i> FP	GCCTATGGGATAAATTTGG
4	EP- <i>espZ</i> RP	GCATCCATTACCTCTC
5	EP- <i>eae</i> FP	CATTCTAACTCATTGTGGTGG
6	EP- <i>eae</i> RP	CTAGCTAGAGACTTGATTACC
7	EP- <i>map</i> FP	GTGCTGGAGGAAAAGTTCTG
8	EP- <i>map</i> RP	CAGCGCAGTAAGTTTCC
9	EP- <i>espB</i> FP	GATGTCTGATTCTGCGCGAG
10	EP- <i>espB</i> RP	CACTGCCACAAAGAAACTC
11	EP- <i>espH</i> FP	CCCTTTGGCAACCGTAAAGC
12	EP- <i>espH</i> RP	AAATATCGTCCCCAGAACAG
13	EP- <i>espF</i> FP	ATGGAATTAGTAACGCTGCTTCTACAC
14	EP- <i>espF</i> RP	TTGGTTACCCTTTCTTCGATTGCTCATAG
15	EP- <i>espG</i> FP	ACAAAACTATGGCTGACGCATCAC
16	EP- <i>espG</i> RP	TTCAGCGCATGACATCTCATCCCAG
17	EP- <i>espG2/orf3</i> FP	TAGGTATAACCCTATGCCTGTGTTC
18	EP- <i>espG2/orf3</i> RP	AACAAATTCAGGCTGACACAGTACC
19	EP- <i>escD</i> FP	CCATTAGCCATTGGAACCTCACG
20	EP- <i>escD</i> RP	CGTCGCTAGTATCATTACCC
21	EP- <i>escP</i> FP	CGAACAAAAGAGAATCTGC
22	EP- <i>escP</i> RP	GGTCCGATAACTGGCAAC
23	EP- <i>sepL</i> FP	GGAAGCTGTTGTACAGTAC
24	EP- <i>sepL</i> RP	GGAGATTGCAATAATATCCG
25	EP- <i>escK</i> FP	GCCAATCAACAATGAATCAACG
26	EP- <i>escK</i> RP	CCTCTGGTATGATATCTTC
27	EP- <i>escL</i> FP	CCTTTATGGACCATAGTC
28	EP- <i>escL</i> RP	GCCAATGGTCATTAATTGAG
29	EP- <i>cesT</i> FP	GCTCTAGACAACGTTGCAGCATGGGTAA
30	EP- <i>cesT</i> RP	GCGAATTCTCATGTTTGGGCTCCACCAC
31	ET- <i>tir</i> FP	CGAGATCTGATCGCGAGGATTATG
32	ET- <i>tir</i> RP	GCTCTAGATACTAAGGTGACGTTAC
33	ET- <i>escD</i> FR	CGGGATCCGGCTGCATTGATTATTGC
34	ET- <i>escD</i> RP	GCGTCGACCCGTTGGTTGTGTCAGG
35	ET- <i>gto</i> FP	CGGATATCGATCGCGAGGATTATG
36	ET- <i>gto</i> RP	AAGTCGACGTTTGCCAGCATAAG
37	ET- <i>espB</i> FP	CGGGATCCGGCAATGATCTGGTTCC
38	ET- <i>espB</i> RP	GCGTCGACCTCATCGCCATCAAGTC
39	ET- <i>espD-G</i> FP	CACACCCGTCCTGTGGCAATACCGAACGATTTCC
40	ET- <i>espD-G</i> RP	TCTCAAGGGCATCGGGATACCTGACGCGTTATTC
41	EP- <i>tir-G</i> FP	CACACCCGTCCTGTGGGAACGTGTCAAATTTCTAAATAAAAAG
42	EP- <i>tir-G</i> RP	TCTCAAGGGCATCGGAGTTACCCATGCTGCAAC
43	ET- <i>espA-G</i> FP	GCGACCACACCCGTCCTGTGCAGATGTTATAGATAGAGACTCG
43	ET- <i>espA-G</i> RP	AAGGCTCTCAAGGGCATCGGCCAACGGAGGATATAGTTAAATTAG
44	ET- <i>espADB</i> FP	AAGGCTCTCAAGGGCATCGGCCAACGGAGGATATAGTTAAATTAG
45	ET- <i>espADB</i> RP	TCTCAAGGGCATCGGCATGGCGAAGATGATATCC
46	ET- <i>eae-G</i> FP	GCGACCACACCCGTCCTGTGCTGCGCGTTAGCTACGTCG
47	ET- <i>eae-G</i> RP	AAGGCTCTCAAGGGCATCGGGTACTAACACAAGACAGAATGGC
48	ET- <i>cesT-G</i> FP	GACCACACCCGTCCTGTGCGCCAATTAATATCTCAG
49	ET- <i>cesT-G</i> RP	AAGGCTCTCAAGGGCATCGGGGAATCAGCTTACTATCTTGG
50	ET- <i>tir-G</i> FP	GCGACCACACCCGTCCTGTGCATACTGTATGATCATGGTTG
51	ET- <i>tir-G</i> RP	AAGGCTCTCAAGGGCATCGGGGAGATATTAATTGGCC
52	ET- <i>ler-G</i> FP	GCGACCACACCCGTCCTGTGCGAGAAGTCCATACATTAG
53	ET- <i>ler-G</i> RP	AAGGCTCTCAAGGGCATCGGGTTCAGATGTCGACTGTTC
54	EP- <i>espB_{mid}-G</i> FP	GCCGGGCCTTTGCGGGATAATCTAGACGCGCTCTATTGGTACAAC
55	EP- <i>espB_{mid}-G</i> RP	ACGATGCGTCCGGCGTAGAGTTGAGCTCCTTAATACCCGAAAAGTG
56	ET- <i>escP</i> FP	GCGACCACACCCGTCCTGTGTAACCTCACTAACGGAAG
57	ET- <i>escP</i> RP	AAGGCTCTCAAGGGCATCGGCAACCAATCGATTACAACC
58	ET- <i>escL</i> FP	GCGACCACACCCGTCCTGTGCGTTGCCAGACTATTTG
59	ET- <i>escL</i> RP	AAGGCTCTCAAGGGCATCGGGGCTGACTTCTATAGATATAAG
60	ET- <i>escK</i> FP	GCGACCACACCCGTCCTGTGCATGGTTATCAACATGGAGAG
61	ET- <i>escK</i> RP	AAGGCTCTCAAGGGCATCGGGATCTCACTGGCGCTTAAG
62	ET- <i>sepL</i> FR	GCGACCACACCCGTCCTGTGCTGAGTGCGCTTGTACAG
63	ET- <i>sepL</i> RP	AAGGCTCTCAAGGGCATCGGCTAGGGTACCGTCTACCTG
64	ET- <i>eae</i> FP	CACCGTAATGCGTGATGG (Used for sequencing <i>eae</i> gene)
65	ET- <i>eae</i> RP	CGTATAAGAGGCCGTTTGTG (Used for sequencing <i>eae</i> gene)
66	EP- <i>escD</i> FP	GATGAAGAATCCGGGGCTAC (Used for sequencing <i>escD</i> gene)
67	EP- <i>escD</i> RP	CGTCGCTAGTATCATTACCC (Used for sequencing <i>escD</i> gene)

Table 2: List of primers that is used in this study.

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A) Component	25µl reaction	Final Concentration
10X Taq reaction buffer	1 µl	1x Taq reaction buffer
10 mM dNTPs	0.5 µl	200 µM dNTPs
10 µM forward primer	0.5 µl	0.2 µM
10 µM reverse primer	0.5 µl	0.2 µM
Template bacterial DNA	1 µl	variable
Taq DNA polymerase	0.125 µl	0.625 units
dH2O	Up to 25 µl	

B) Component	25µl reaction	Final Concentration
Q5 reaction buffer	5 µl	1x reaction buffer
10 mM dNTPs	0.5 µl	200 µM dNTPs
10 µM forward primer	0.5 µl	0.2 µM
10 µM reverse primer	0.5 µl	0.2 µM
Template bacterial DNA	1 µl	variable
Q5 Hot start high-fidelity DNA polymerase	0.25 µl	0.02 units/µl
dH2O	Up to 25 µl	

Table 3: Standard PCR reaction mixture using A) Taq DNA polymerase or B) Q5 Hot start high fidelity DNA polymerase. Note, template bacterial DNA was either extracted plasmid or obtained from bacteria by resuspending a bacterial colony in 60µL sterile water and incubation at 100°C for 5 min, using 1µl was in PCR reactions.

A) Step	Cycles	Temperature and Time
Initial denaturation	1	95°C for 30 seconds
Denaturation	30	95°C for 30 seconds
Annealing		53-63°C for 1 min
Extension		68°C for 1 min/kb
Final Extension	1	68°C for 2 min

B) Step	Cycles	Temperature and Time
Initial denaturation	1	98°C for 30 seconds
Denaturation	30	98°C for 10 seconds
Annealing		53-68°C for 30 seconds
Extension		72°C for 30-60 seconds
Final Extension	1	72°C for 2 min

Table 4: Standard PCR reaction condition. A) Taq DNA polymerase and B) Q5 Hot start high-fidelity DNA polymerase. Annealing temp is predicted by sigma software

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2.2.2 Plasmid extraction

To isolate DNA plasmid, the appropriate strain was inoculated into 5-10 ml LB broth, with appropriate antibiotic(s), for overnight growth at 37°C with shaking (~250 rpm). Bacteria were harvested by centrifugation (11752 xg; 3 min; room temperature [RT]) and supernatant discarded. Plasmids were extracted using the Mini plasmid purification kit (Thermo Scientific; Cat # K0503), following the recommended protocol. DNA plasmid concentration was determined (A260 reading) using Nano-Drop 1000 spectrophotometer (Lab-tech) and were typically 0.1-0.2µg/µl.

2.2.3 DNA sequencing

To determine the DNA sequence, the target gene was PCR amplified using Q5 Hot start high-fidelity DNA polymerase (New England Bio-labs; Cat #M0493S) with an appropriate primer (Table 2). PCR products were then cleaned-up using GenElute™ PCR Clean-Up Kit (Sigma Cat #NA 1020-1KT) following the recommended protocol. DNA concentration was determined (A260 reading) using Nano-Drop 100 spectrophotometer (Lab-tech) prior to sending along with primers for sequencing (Source Bioscience, Cambridge, UK). Comparative analysis of resulting sequences with native gene sequence was performed by pairwise alignments programme (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

2.2.4 Preparation of electrocompetent cells

Strains were grown in LB, with appropriate antibiotic(s) at 37°C, unless otherwise indicated, at 225-250 rpm overnight (~16-18h). Bacterial cultures were diluted 1:100 into 100 mL fresh LB broth medium, containing selective antibiotic(s) when appropriate, for growth (37°C shaking) until reached optical density (OD₆₀₀) of 0.6-0.7. Bacterial cultures were cooled down on ice (gentle shaking) prior to centrifugation (12,000 xg for 15 mins at 4°C). The resulting pellet was washed with ice cold sterile water (50 mL) and centrifuged under the same conditions prior to an additional repeat wash and centrifuge. The resulting bacterial pellet was resuspended in sterile ice-cold 15% glycerol (25 mL) and centrifuged using the same conditions. Finally, the pellet was resuspended in sterile ice-cold 15% glycerol (0.5 mL) and divided into 40 µL aliquots for immediate use or snap frozen (liquid nitrogen) for storage at -80°C.

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2.2.5 Electroporation of bacterial cells

10-100 ng of DNA solution (isolated plasmid or ligation mix-Table 5) was added to a freshly made or thawed (on ice) 40 μ l aliquots of electrocompetent bacterial cells for 5 min prior to transferring to an ice-cold 2 mm-gap electroporation cuvette (Cell projects; Cat #EP-102) and electroporating (2KV, 200 Ω , 25mF for 4-5 μ s) using a GenePulser II (BioRad). 1ml of pre-warmed (37°C) SOC media (2% tryptone, 0.5% yeast extract, 10mM sodium chloride [NaCl], 2.5 mM potassium chloride [KCl], 10 mM magnesium chloride [MgCl₂] and 10 mM magnesium sulfate [MgSO₄] pH=7) was gently added immediately with cells incubated for 1h at the appropriate temperature; routinely 37°C. 10, 100 and 890 μ L of bacterial cell suspension was routinely plated onto LB agar plates containing appropriate antibiotic(s) and incubated overnight (16-20h) at an appropriate temperature. Single colonies were streaked to single colonies on fresh selective agar plates and screened for introduction of required gene by colony PCR and the verified correct colony was grown overnight in 3 ml LB broth, with selective antibiotic(s), for use to prepare frozen glycerol stocks (see Section 2.2.4).

2.2.6 Transformation of bacteria using heat-shock procedure

Frozen chemical competent bacteria K12, NEB[®] Turbo Competent *E. coli* (High Efficiency; New England Bio-labs; Cat #C2984H), were thawed on ice for 10 min prior to adding ~5 to 100 ng (2-5 μ l) of plasmid DNA (Table 5) to 25 μ L aliquots, mixed gently, and kept on ice for 30 min. The bacteria were then heat-shocked at 42°C for 30 seconds followed by immediate incubation on ice for a further 5 min. Pre-warmed 37°C of SOC medium (950 μ l) was added to the cells, gently mix, and cells were incubated at 37°C with 225-250 rpm shaking for 60 min prior to plating 50 and 450 μ L onto LB agar containing the appropriate selective antibiotic(s). The plates then were incubated overnight at 37 °C with single colonies screened for introducing of plasmids by PCR. The verified correct colony was grown overnight in 3 ml LB broth, with selective antibiotic(s), for use to prepare frozen glycerol stocks (see Section 2.2.4).

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Plasmid	Description	Antibiotic	Reference
pCR-2.1	Cloning vector	Cb ^R	Invitrogen
pACYC184	Cloning vector	Cm ^R /Tet ^R	(Chang & Cohen, 1978)
pTet	pACYC184 with Cm gene inactivated	Tet ^R	Kenny Lab (B Kenny)
pAC-LacZ-3'gtoe	pACYC184 expressing Tir, CesT and Intimin	Tet ^R	Kenny Lab (B Kenny)
pSK-espH::HA	pACYC184 expressing EspH::HA fusion protein	Cm ^R	Kenny Lab (B Kenny)
pSK-espZ::HA	pSK expressing EspZ::HA fusion protein	Cb ^R	Kenny Lab (S Quitard)
pSK-map::HA	pSK expressing for Map as a Map::HA fusion protein	Cb ^R	(Dean <i>et al.</i> , 2013)
pACYC-espB	pACYC184 expressing EspB	Cm ^R	Kenny Lab (S Quitard)
pBR-espF	pBR expressing EspF	Cb ^R	(Nagai <i>et al.</i> , 2005)
pACYC-eae	pACYC184 expressing Intimin	Cm ^R	Kenny Lab (S Quitard)
pACYC-cesT	pACYC184 expressing CesT chaperone	Cm ^R	Kenny Lab (S Quitard)
pTrc-escL	pTrc expressing EscL	Cb ^R	(Soto <i>et al.</i> , 2017)
pTrc-escP	pTrc expressing EscP	Cb ^R	(Monjaras Feria <i>et al.</i> , 2012)
pTrc-sepL	pTrc expressing SepL	Cb ^R	(Monjaras Feria <i>et al.</i> , 2012)
pET-escK	pET expressing EscK	Cb ^R	(Soto <i>et al.</i> , 2017)
pGEM-espB _{mid}	pGEM vector expressing EspB lacking myosin binding area (AA 159 to 218)	Cm ^R	(Iizumi <i>et al.</i> , 2007)
pACYC-espB _{mid}	pACYC184 expressing for EspB lacking myosin binding area (AA 159 to 218)	Cm ^R	This study
pSK-3'gtHAo	pSK expressing Tir (as a Tir::HA fusion protein) and CesT	Cb ^R	Kenny Lab (S Quitard)
pACYC- espA _{Et}	pACYC184 carrying EspA from <i>E. tarda</i>	Cm ^R	This study
pACYC- espD _{Et}	pACYC184 carrying EspD from <i>E. tarda</i>	Cm ^R	This study
pACYC-espB _{Et}	pACYC184 carrying EspB from <i>E. tarda</i>	Cm ^R	This study
pACYC- espADB _{Et}	pACYC184 carrying EspA, EspB & EspD from <i>E. tarda</i>	Cm ^R	This study
pACYC-escD _{Et}	pACYC184 carrying EscD from <i>E. tarda</i>	Cm ^R	This study
pACYC-ler _{Et}	pACYC184 carrying Ler from <i>E. tarda</i>	Cm ^R	This study
pACYC-eae _{Et}	pACYC184 carrying Intimin from <i>E. tarda</i>	Cm ^R	This study
pSK-tir _{Et} ::HA	pSK expressing Tir from <i>E. tarda</i> as a Tir::HA fusion	Cb ^R	This study
pACYC-tir _{Et} ::HA	pACYC184 carrying Tir from <i>E. tarda</i> as a Tir::HA fusion	Cm ^R	This study
pACYC-gto _{Et}	pACYC184 carrying Tir from <i>E. tarda</i> and its chaperone, CesT	Cm ^R	This study
pACYC-cesT _{Et}	pACYC184 carrying CesT from <i>E. tarda</i>	Cm ^R	This study
pACYC-sepL _{Et}	pACYC184 carrying SepL from <i>E. tarda</i>	Cm ^R	This study
pACYC-escK _{Et}	pACYC184 carrying EscK from <i>E. tarda</i>	Cm ^R	This study
pACYC-escP _{Et}	pACYC184 carrying EscP from <i>E. tarda</i>	Cm ^R	This study
pACYC-EscL _{Et}	pACYC184 carrying EscL from <i>E. tarda</i>	Cm ^R	This study
pSK-map	pSK expressing Map	Cb ^R	(Kenny & Jepson, 2000)
pSK-map(ATG-TAG) E ₇₈ A	pSK expressing Map carrying a E ₇₈ A substitution	Cb ^R	(Dean <i>et al.</i> , 2013)
pSK-map(TAA-TAG) ΔTRL	pSK expressing Map lacking final 3 residues, TRL	Cb ^R	(Dean <i>et al.</i> , 2013)
pSK-3'g5't-ΔMTS-map	pSK expressing a Tir::Map fusion protein (Tir residues 1-101; Map residue 41-206) Map mitochondria targeting sequence (MTS) swapped with the N-terminal of Tir	Cb ^R	(Dean <i>et al.</i> , 2013)
pACYC-3'gtHAo	pACYC184 expressing Tir (as Tir::HA fusion) and CesT	Cm ^R	Kenny Lab (B Kenny) unpublished
pACYC-3'gtHAinto	pACYC184 expressing Tir (with HA tag within Intimin binding domain) and CesT	Cm ^R	Kenny Lab (B Kenny) Unpublished
pACYC-tir _{EPEC}	pACYC184 carrying Tir	Cm ^R	This study
pACYC-tir-ΔSY	pACYC184 expressing Tir carrying S434A; S463A, Y454F; Y474F substitutions	Cm ^R	(Kenny, 1999)

Table 5: plasmids used in this study. Given a brief description of the plasmids and/or cloned gene product alongside with antibiotic selection and source.

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2.2.7 Construction of EPEC gene knockout mutants

Gene knockouts were carried out as previous described (Donnenberg & Kaper, 1991) with available suicide vectors (see Table 6). Briefly, provided SM10 λ pir (Km^R) strains carrying the appropriate, usually pCVD422-based (Cb^R), suicide vector (Table 6) which is carrying the disrupted gene, was co-incubated with the recipient EPEC (Nal^R) strain on LB plate overnight at 37°C. This lead to transfer, via conjugation, the suicide vector to the recipient EPEC (Nal^R) strain. Next day, cells within several confluent areas of the plate were scrapped off into LB broth contains Nal antibiotic and grown to stationary phase before plating onto Nal^R/Cb^R plates selected for transconjugants. The specified single colony was then inoculate into LB broth with appropriate recipient strain selected antibiotic (Nal^R) and grown to stationary phase before plating on LB containing 5% sucrose and Nal antibiotic for 16-20h at 30°C. Growth in the absence of Cb^R selection provided an opportunity for a double cross-over event to remove the gene duplication, While plating on Nal plates containing 5% sucrose leads to kill cells carrying the suicide vector which carries a *sacB* gene and select for strains lacking the suicide vector which can't be maintained without λ pir functions. Single colonies were picked and screened for antibiotic resistance (grow on Nal but not Cb or Km plates). PCR analysis was used to determine the presence of intact or disrupted gene.

Suicide Plasmid	Description	Antibiotic	Source
pCVD442- <i>espZ</i> ₍₈₁₎	Carries disrupted <i>espZ</i> gene (lacks 81 of 98 residues)	Cb ^R	Kenny Lab (S Quitard)
pCVD442- <i>espH</i> _{Km}	Carries disrupted <i>espH</i> gene	Cb ^R /Km ^R	Kenny Lab (S Quitard)
pCVD442- <i>espF</i>	Carries disrupted <i>espF</i> gene	Cb ^R	(Warawa <i>et al.</i> , 1999)
pCVD442- <i>tir</i> _{full}	Carries disrupted <i>tir</i> gene	Cb ^R	(Kenny <i>et al.</i> , 1997b)
pCVD442- <i>cesT</i> _{Km}	Carries disrupted <i>cesT</i> gene	Cb ^R	Kenny Lab (S Quitard)
pCVD442- <i>map</i>	Carries disrupted <i>map</i> gene	Cb ^R	(Kenny & Jepson, 2000)
pCVD442- <i>espB</i>	Carries disrupted <i>espB</i> gene	Cb ^R	(Foubister <i>et al.</i> , 1994)
pCVD442- <i>eae</i>	Carries disrupted <i>eae</i> gene	Cb ^R	(Donnenberg & Kaper, 1991)
pCVD442- <i>escD</i>	Carries disrupted <i>escD</i> gene	Cb ^R	(Ogino <i>et al.</i> , 2006)
pKNG101-core	For deleting LEE core region	Strep ^R	(Ruchaud-Sparagano <i>et al.</i> , 2007)
pCACTUS- <i>espB</i> _{mid}	Delete and swap <i>EspB</i> with <i>EspB</i> -mid	Cm ^R	(Iizumi <i>et al.</i> , 2007)

Table 6: Suicide vectors used in studies with a brief description, antibiotic resistance profile and source reference. (Km) indicated that associated disrupted gene carries genes encoding kanamycin resistance with (full) indicating that the entire gene – ATG to stop codon -has been disrupted, while (81) indicated that the 81 of 98 *EspZ* residues is missing. Core refers to that the core region that has genes encoding *EspH*, *CesF*, *Map*, *Tir*, *CesT* and *Intimin*, has been deleted. Mid refers to that the plasmid expressing for *EspB* protein that missing the myosin binding area (residues from 159aa to 218aa).

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2.2.8 Plasmid construction

2.2.8.1 TA cloning

The TA cloning kit (Invitrogen; Cat #45-0046) was used to insert PCR products directly into the kit provided pCR.2.1 plasmid. However, the PCR product can insert in either orientation so the gene was amplified with restriction enzyme cleaving sites introduced into the oligonucleotides [introduced via PCR amplification] (Table 2). Once cloned to the TA vector (pCR-2.1), gene was digested using flanking unique restriction enzyme sites for introduction into the destination expression vector that was digested with the appropriate restriction enzymes. For example, *Edwardsiella tarda* (*E. tarda*) genes were amplified with oligonucleotides (carrying BamHI/Sall or EcoRV/Sall restriction sites Table 2) using *E. tarda* genomic DNA, kindly provided by Dr Yoji Nakamura, as a template for Q5 Hot start high-fidelity DNA polymerase (New England Bio-labs; Cat #M0493S) and described (Section 2.1) PCR conditions. The reaction was then placed on ice and 1 unit of *Taq* polymerase (New England Bio-labs: #M0273L) was added, mixed and incubated at 72°C for 10 minutes to add a single deoxyadenosine (A) to the 3' ends. PCR reactions were analysed on agarose gels (usually 1% TAE agarose [see section 2.1] to confirm success and estimate DNA concentration. Appropriately 2 µl (50 ng; 20 fmoles) of pCR-2.1 vector and 1-2 µl (~57 ng; 60 fmoles) of insert were added together for ligation (ratio 1:3) in a total volume 10 µl with 1 µl T4 DNA ligase (Invitrogen; Cat # 15224-017), as described by provider, and incubated 16-18 hrs at 14°C prior to transforming 2-3 µl to NEB® Turbo Competent *E. coli* (High Efficiency) competent cells (New England Bio-labs; Cat #C2984H) as described (see Section 2.2.6). Bacterial suspension were plated on LB plate containing Carbenicillin (Cb) and 50 µL of 50 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) (Promega; Cat #V3941), and incubated 16-20 h at 37°C. White colonies were PCR screened for presence of an appropriated insert.

2.2.8.2 Generation of pACYC-*esp*B_{ET}; -*esc*D_{ET} and -*tir_ces*T_{ET} vectors

Cloned inserts were released from the pCR.2.1 recombinant plasmid by digesting with appropriate restriction enzymes (see above) for fragment isolation and cloning into pACYC184 pre-digested with the same restriction enzymes. Briefly, the plasmids were digested with two enzymes (BamHI/Sall or EcoRV/Sall [New England Bio-labs]) and vector/insert fragments isolated (gel extraction Kit [Thermo Scientific Cat #K0691]) following manufacturer's instruction for ligation (3:1 insert to vector ratio), following

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manufacturer's instruction, in total 20 μl volume adding 0.1unit T4 DNA ligase (Invitrogen; Cat # 15224-017) and incubated overnight at room temperature. The product then was introduced into Turbo Competent *E. coli cells* as described above (see Section 2.2.6). Bacterial colonies that grew on chloramphenicol agar plates were PCR screened to confirm presence of cloned gene.

2.2.8.3 Gibson Assembly Cloning

Some genes were cloned using the Gibson Assembly Cloning Kit (New England Bio-labs; Cat #E2621S) which uses recombination to introduce PCR-amplified DNA fragments. This process requires the generation of oligos with 20 bp extensions that correspond to the vector insertion site (Gibson *et al.*, 2009). Briefly, the recipient vector, for example pACYC184, was pre-digested with BamHI and Sall restriction enzymes and gel purified (see above) while the insert with PCR amplified (Q5 Hot start high-fidelity DNA polymerase [New England Bio-labs; Cat #M0493S]), with appropriate oligonucleotide primer sets designed using NEBuilder Assembly construction tool (<http://nebuilder.neb.com/>). The concentration of PCR product and isolated vector were determined (Nano-Drop 100 spectrophotometer [Lab-tech]), following manufacturer's instruction, for addition to Gibson assembly master mix (New England Bio-labs; Cat #E2621S), at a ratio 2:1 [25 ng of vector 2-3 μl + 12 ng of insert 1-2 μl + Mix 5 μl and deionized H₂O to the 10 μl total volume and incubated 12-16 hr at 50°C]. Turbo Competent *E. coli cells* were transformed with 2-5 μl of the reaction mix and processed as described (Section 2.2.6) with PCR screening of colonies for those with the cloned insert.

2.3 Bacterial infection

2.3.1 HeLa cells infection

HeLa cells were seeded 48 hours prior to experimentation to obtain 80-90% confluency on day of infection. Bacterial cultures (in LB containing, when needed, appropriate antibiotics) were grown overnight without shaking at 37°C or, for *Yersinia* strains, 28°C. The Optical Density (OD) was measured at 600nm (UV1101- Biotech photometer; a value of 1 = 1×10^9 bacteria) with mammalian cells infected at a Multiplicity of Infection (MOI) of 40:1 - bacteria to host cells - unless otherwise indicated. Infections usually used LB grown bacteria but sometime bacteria were diluted (1/10) into serum free DMEM (supplemented, when needed, with antibiotic) for 2-3h, before measuring the OD. The infections time is varied (see text and legends), but were undertaken at 37°C in a humidified 5% CO₂ atmosphere, unless otherwise indicated. In some experiments, infected cells were washed (37°C PBS) and incubated with DMEM containing gentamycin (100µg/ml) for 1h to kill extracellular bacteria prior to PBS washing and infecting with a second-wave of bacteria. Following infection cells were placed on ice and washed twice with ice cold PBS [0.01M phosphate buffer saline (0.138 M NaCl; 0.0027 M KCl, pH 7.4 Sigma Cat #P5368)] and processing as given below.

2.3.2 Isolation of cellular protein fractions

The protocol was similar to that described in previous publications from the Kenny laboratory, for example see (Kenny & Warawa, 2001, Kenny & Finlay, 1997). Briefly, ice cold Triton lysis buffer (1% v/v Triton X100 in 50mM PBS [pH 7.5] containing 0.4 mM NaVO₄, 1 mM NaF, and 0.1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma P7626], and protease cocktail [Sigma Cat #P-8340; 1:100 dilution]) was added prior to scraping cells with a rubber policeman (SARSTEDT Cat #83.1830) and transferring to 1.5 ml tubes for incubation on ice 5min. The samples were then centrifuged (5 min; 11752xg; 4°C) and the soluble fraction (contains host cytoplasm and membrane proteins plus delivered effector proteins) transferred to a fresh ice cold tube prior to adding 5X Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 1% w/v SDS, 5% v/v glycerol, 5% v/v β-mercaptoethanol & 0.01 % w/v bromophenol blue) to final 1X concentration. The remaining insoluble pellet (contains host nuclei and cytoskeleton plus adherent bacteria) was washed in ice cold 1x PBS and re-suspending in 1X

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Laemmli sample buffer (Laemmli, 1970). The soluble and insoluble samples were heated (10 min, 100°C), vortexed well and centrifuged (11752×g, 3 min, room temperature) prior to use or storage at -20°C.

2.3.3 Western Blot analysis

Soluble and insoluble fractions derived from $\sim 1 \times 10^6$ infected cells were loaded, alongside protein marker (precision plus standards [BIO-RAD Cat #161-0373]) onto SDS-PAGE gel (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) - usually 10% polyacrylamide resolving gel with 5% stacking gel (Laemmli, 1970). Gels were run in SDS-PAGE running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% w/v SDS; pH 8.3) at 200 volts for 1h using a vertical electrophoresis mini-cell system (Bio-Rad). The gel was placed onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare Cat #G9949937) for protein transfer using the Mini Trans-Blot cell (Bio-Rad) at 110 volts for 60min (Kenny & Finlay, 1997). Transfer efficiency/quality was evaluated by Ponceau S staining (Sigma Cat #P7170-1L) - reversible by washing with 1x PBS – and the membrane blocked overnight (4°C) in PBS containing 5% (w/v) skimmed milk powder (1% Dried skimmed milk-UK).

For detection using alkaline phosphatase-conjugated antibodies. Blocked membranes were incubated with appropriate antibodies (Table 7) in PBS for 1h at room temperature, washed in PBS (3x 5 min), and then incubated with an appropriate secondary antibody (Table 7) in PBS for 1h at room temperature prior to washing twice in PBS and then in developing buffer (0.05 M NaCl, 0.001M MgCl₂ and 0.02 M Tris Base pH 9.5) prior to adding developing agents (NBT [nitro blue tetrazolium; Promega Cat #S380S] and BCIP [5-bromo-4-chloro-3-indolyl-phosphate; Promega Cat #S381S]) in 10mL alkaline phosphatase buffer according to the manufacturer's instructions.

For detection using horseradish peroxidase (HRP)-conjugated antibodies, primary antibodies were incubated 1h in Tris Buffer Saline containing Tween (TBST; 150 mM NaCl, 10m M Tris-HCl, 0.05% v/v Tween-20 pH 7.5) followed by washing (3x 5 min TBST) and incubation with horseradish peroxidase-conjugated HRP secondary antibody (anti-Mouse HRP or anti-Rabbit HRP) in TBST for 1h at room temperature. The blots were then washed (3x 5 min in TBST) prior to overlaying with luminol reagent (1:1 ratio of Luminol Enhancer and Stable peroxide solution; Thermo Scientific, Cat

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#34080) for 5 min with the luminescent signal captured on X-ray film (GE Healthcare Cat #HP35102A). HRP-developed blots were often re-blocked (PBS containing 5% w/v skimmed milk powder) overnight (4°C) and then probed with antibody for detection using alkaline phosphatase-conjugated antibodies as described above.

Primary antibody	Species	Dilution used in		Source
		IF	WB	
Anti-Tir	Rabbit poly Ab	1:2000	1: 5000	Kenny Lab
Anti-EspF	Rabbit poly Ab	NA	1: 2000	Kenny Lab
Anti-EspB	Rabbit poly Ab	NA	1: 2000	Kenny Lab
Anti-EspA	Rabbit poly Ab	NA	1: 500	Kenny Lab
Anti-EspD	Rabbit poly Ab	NA	1: 1000	Kenny Lab
Anti-β-Actin	Mouse Mo Ab	NA	1:10000	Sigma #A2066
Anti-Tubulin	Rabbit Mo Ab	NA	1: 40000	Sigma #T3526
Anti-HA-Taq	Mouse Mo Ab	1:50	1:1000	Sigma
Anti-phospho-tyrosine clone 4G10	Mouse Mo Ab	NA	1:1000	Millipore #05-321

Secondary antibody	Species	Dilution used in		Source
		IF	WB	
Anti-Rabbit IgG-HRP	Goat	NA	1: 5000	Jackson Immuno Research; #111-053-003
Anti-Rabbit IgG-AP	Goat	NA	1: 5000	Jackson Immuno Research; #111-053-144
Anti-Mouse IgG-HRP	Goat	NA	1: 5000	Jackson Immuno Research; #115-035-003
Anti-Mouse IgG-AP	Goat	NA	1: 5000	Jackson Immuno Research; #115-056-068
(TRITC)-phalloidin	NA	1:100	NA	Sigma; #P1951-.1MG
DAPI	NA	1:1000	NA	Invitrogen; #D1306

Table 7: Primary and secondary antibodies with DAPI/TRITC- Phalloidin stains used in this study. Antibodies are used to label and/or detect target protein in western blot or immunofluorescence.

2.3.4 Immunofluorescence microscopy

Hela cells (~1x10⁵) were seeded onto 13mm glass coverslips (VWR Cat #631-1578) in 24 well plates 48h prior to infection to reach 80-90% confluency by infection day. Post-infection, the cells were washed twice with ice cold PBS and fixed with ice cold PBS containing 2.5% paraformaldehyde (Chem Cruz; Cat #sc281692) for 20 min at room temperature. Following two PBS washes the cells were treated with PBS containing 1% (v/v) Triton X-100 - makes the host membrane permeable – containing tetramethyl-rhodamine-isothiocyanate (TRITC)-phalloidin (1µg/ml [1:100; final concentration 10 ng]; binds polymerised actin) and 4'6-diamidino-2-phenylindole

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(DAPI; stains DNA) for 1h. When appropriate, primary antibody(ies) were also present and then, post washing, labelled with an appropriate fluorescent-conjugated secondary antibody (Table 7). PBS washed coverslips were mounted onto 20 µl FluorSave reagent (Millipore Cat #345789-20) prior to examining (Zeiss Axioskop epi-fluorescent microscope) and, when appropriate, images captured using a Hamamatsu C4742-95 charge-coupled device camera and Improvision software.

2.3.5 Lactate dehydrogenase (LDH) cytotoxicity assay

Release of cytoplasmic lactate dehydrogenase (LDH) protein into the cell culture media was detected using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher scientific Cat #88954) following the manufacturer's instructions. Briefly, at the indicated time following infection of Hela cells ($\sim 1 \times 10^5$ per wells of 24 well plate) the extracellular medium was taken and centrifuged (11752 xg, 3 min) with 50 µl aliquots transferred to a flat bottomed 96 well plate (Corning; Cat #3370) adding 50 µl of kit provided reaction mixture for 30 min (in dark at room temperature). The reaction was terminated by adding 50 µl stop solution and the absorbance of the reaction product formazan measured at 490 nm and 680 nm (FLUOstar opitma microplate reader). The percentage cytotoxicity was calculated following subtraction of background level release from uninfected cells from that released in treated samples, this then divided by the value of maximum LDH activity (that released from uninfected cells following addition of kit provided 1x lysis buffer) multiplying by 100.

Chapter 3: Interrogating roles for EPEC T3SS secretion substrates in Tir modification to T⁺ form and membrane insertion processes

3.1 Introduction

Translocated intimin receptor (Tir) is the first example of a bacterial protein delivered into the host cells to act as a receptor for a pathogen (Kenny *et al.*, 1997b). Tir is delivered by the type three secretion system (T3SS) of not only EPEC but also all A/E pathogens where it becomes inserted into the host plasma membrane in a hairpin like conformation, via two transmembrane domains (TMDs), resulting in the central extracellular (Intimin-binding) domain with both terminal domains exposed to the host cytoplasm (Kenny, 1999, de Grado *et al.*, 1999). The insertion mechanism is controversial with studies from our group supporting insertion from the host cytoplasm. Thus, an initial immunoblot-based study linked Tir insertion to a number of kinase-modified intermediates with the first, T' (due to phosphorylation on a serine residue leading to a 5kDa increase in apparent molecular mass) followed by further modification to the T'' form (due to phosphorylation on another serine increasing an apparent molecular mass by another of an 2kDa) linked to insertion and Intimin binding (Kenny, 1999, Warawa & Kenny, 2001). Tir also undergoes host kinase modification on tyrosine residues (without altering apparent molecular mass) linked to subversive activities inducing Intimin-dependent recruitment of the Arp2/3 actin nucleating machinery to generate pedestal-like structures beneath the adherent bacteria (Lai *et al.*, 2013, Campellone *et al.*, 2002, Rohatgi *et al.*, 2001, Gruenheid *et al.*, 2001). In contrast, Gauthier (2000) argues for insertion into the host cell membrane during the translocation process, presumably via an EspB/D pore, with detection of Tir in the host cytoplasm suggested to be due to overloading of the translocation system (Gauthier *et al.*, 2000). However, time course studies revealed the Tir T' form in the cytoplasm prior to T'' in the membrane, arguing for a cytoplasmic intermediate (Kenny, 1999). In addition, introducing Tir into host cells by three different EPEC-independent mechanisms i.e i) transfection, ii) adding purified Tir to detergent permeabilized host cells or iii) delivering Tir by the T3SS of another pathogen (*Yersinia pseudotuberculosis*), led to an only partially modified Tir form (T') which was unable to interact with Intimin (Kenny & Warawa, 2001). This defect in Tir modification could not be restored by co-infection with EPEC missing only one effector, Tir, suggesting that other factors had to be co-expressed and/or delivered with Tir to aid T' to T'' modification and insertion into the host cells membrane in a conformation that could be bound by Intimin (Kenny & Warawa, 2001). In contrast, confocal microscopy examination of EPEC infected red blood cells (RBCs) was suggested to support direct insertion of Tir into the RBC plasma membrane (Shaw *et al.*, 2002). Surprisingly, Tir

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has also been reported to be integrated into host cells membrane in a T3SS-independent manner (Michgehl *et al.*, 2006). Interestingly, this form was detected first in the host cytoplasm before the host cell membrane, supporting the cytoplasmic insertion hypothesis. Crucially, the indirect insertion of Tir into the host cell's membrane is also supported via a transfection study which demonstrated the ability of Tir to insert into the host membrane from the host cytoplasm as Intimin was shown to interact with Tir and trigger pedestal formation in a small percentage of cells. Moreover, targeting Tir to the membrane by replacing the N- terminal domain with a type II transmembrane protein led to increased levels of Intimin-Tir interaction (Campellone *et al.*, 2004a) and presumably Tir insertion, suggesting other factors are needed to aid its insertion.

Importantly, the cytoplasmic insertion mechanism was also supported by the confocal microscopy studies with cultured enterocytes (mimic of EPEC's *in vivo* target), which detected a transient pool of Tir within the host cytoplasm prior to sequestration to the apical membrane (Ruchaud-Sparagano *et al.*, 2011). This pool was evident for a longer time with cells infected with an Intimin-deficient strain, suggesting that Intimin interaction with non-Tir independent receptors, including β -integrin and nucleolin (Frankel *et al.*, 1996, Sinclair *et al.*, 2006) triggers Tir recruitment from the cytoplasm to the host membrane.

Finally, *in vitro* biophysical studies, using a mimic of the human erythrocyte inner leaflet revealed that Tir can be inserted into the host membrane in hairpin like structure following Tir exposure to the erythrocyte inner leaflet (Race *et al.*, 2006), supporting the idea that EPEC Tir is inserted into the membrane from the host cytoplasm. Factors needed for Tir insertion into the host plasma membrane appear to be LEE encoded as introduction of the EPEC E2348/69 LEE region into non-pathogenic *E.coli* provides the capacity to produce actin-rich pedestals (McDaniel & Kaper, 1997), a process dependent on membrane insertion linked to modification of Tir on serine and tyrosine residues

We hypothesise that the LEE region encodes T3SS-dependent factors that need to be co-delivered with Tir to enable the T' to T'' modification process leading to Tir insertion into the host membrane to provide a receptor for Intimin.

3.2 Aim

The aim of experiments described in this chapter was to investigate roles for known LEE-encoded T3SS effectors (Map, EspB, EspF, EspH, EspG, EspZ) and/or translocator (EspA, EspD, EspB) proteins in the Tir T' to T'' modification/plasma membrane insertion processes.

3.3 Results

3.3.1 LEE region sufficiency for Tir functionality

Firstly, we wanted to confirm (McDaniel & Kaper, 1997) that the LEE region encodes all the factors needed to deliver a functional Tir molecule into mammalian cells and to interrogate the prediction of Tir modification to T'/T'' forms. Thus, infection studies were undertaken with available non-pathogenic *E.coli* (K12) strains, TOB01 and TOB02 (Yen *et al.*, 2010). TOB01 carries a plasmid (*pBFP_Per*) encoding the BFP pilus targeting EPEC to enterocytes (Giron *et al.*, 1991) and *Per* promotes LEE gene expression operons from another EPEC strain B171 (Mellies *et al.*, 1999, Mellies *et al.*, 2007). TOB01 also carries an 'empty' (no cloned fragment) plasmid with TOB02 identical to TOB01 except that the EPEC B171 LEE region is cloned into this second plasmid (Yen *et al.*, 2010). Infection studies also included EPEC E2348/69 TOE-A7 mutant strain that lacks genes for 14 non-LEE-encoded effectors (Yen *et al.*, 2010). Therefore, HeLa cells were left uninfected or infected with the various strains before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis as previously described (Kenny & Warawa, 2001).

As reported (Kenny, 1999), Tir was delivered by EPEC as evidenced by T'/T'' forms in the soluble fraction and, due to its interaction with Intimin (Kenny, 1999), the T'' form in the insoluble fraction (Figure 16). Similarly, TOE-A7 delivered Tir into the host cells which was modified to a T''-like form and detected in both soluble and, again due its interaction with Intimin, insoluble fraction. By contrast, Tir was absent from uninfected and TOB01 (LEE-negative)-infected cells (Figure 16). However, TOB02 behaved like EPEC (Figure 16) thereby confirming (McDaniel & Kaper, 1997) that the LEE region encodes all the information needed for Tir delivery, modification to T'/T'' forms and insertion into the host cell membrane for Intimin binding.

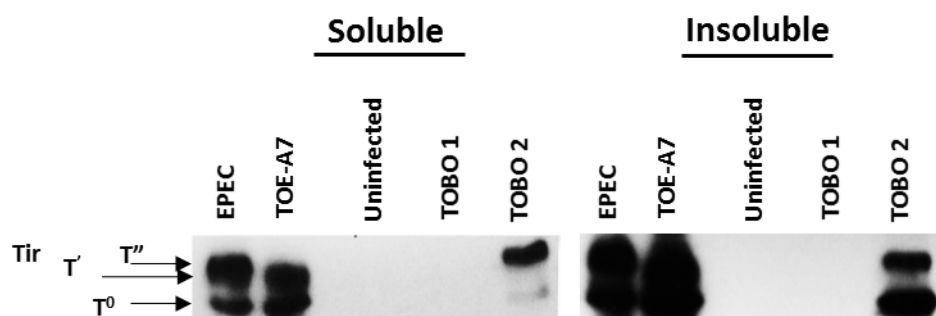


Figure 16: The LEE region encodes sufficient information to enable Tir delivery, modification and insertion into the host plasma membrane for Intimin binding. HeLa cells were left uninfected or infected with indicated strains prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis to probe for Tir. The position of unmodified (T^0) and host kinase modified (T'/T'') Tir forms are indicated (arrows). Strains used were EPEC, TOE-A7 (lacks 14 Nle effectors) mutant and non-pathogenic *E.coli* K12 strains carrying plasmids encoding EPEC BFP/Per operons (TOB01) or BFP/Per/LEE operons (TOB02) from the EPEC strain B171. BFP encodes an adhesin (Bundle Forming Pilus; BFP) while Per provides a positive regulator of LEE gene expression.

3.3.2 *Yersinia enterocolitica*-delivered Tir is only partially modified by host kinases and does not interact with Intimin

Having demonstrated LEE sufficiency for Tir delivery, Kinase modification to T'/T'' forms and Intimin binding, we wished to explore whether the known LEE-encoded effectors (EspG, Map, EspF, EspH, EspZ, EspB), individually or collectively, aid the Tir modification and/or membrane insertion processes. These studies took advantage of the fact that another T3SS-expressing pathogen, *Yersinia pseudotuberculosis* (engineered to lack most *Yersinia* T3SS effectors), can express and deliver Tir into host cells in a manner greatly promoted by co-expressing the Tir chaperone, CesT (Kenny & Warawa, 2001). Thus, we wished to introduce additional LEE effector-encoding plasmids, but this strategy was hindered by the engineering-associated introduction of antibiotic resistance genes (Hakansson *et al.*, 1996). Therefore, studies interrogated if an available *Yersinia enterocolitica* strain, also engineered to lack most *Yersinia* T3SS effectors but without introducing antibiotic resistance genes (Cornelis *et al.*, 1986), hereafter called *Y/ent*, could also deliver Tir. Thus, a plasmid encoding Tir, CesT and Intimin (*p-tir-cesT-Int*) was introduced into *Y/ent* for infection of HeLa cells alongside EPEC and *Y/ent* strains. Western blot analysis of isolated Triton X-100 soluble and insoluble fractions for Tir revealed that, as reported, EPEC delivered Tir

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as evidence by T'/T" forms in the soluble fraction with T" form in the insoluble fraction (Figure 17). As expected, no Tir-related bands are evident in fractions from the *Y/ent*-infected cells (Figure 17) whereas only one Tir modified form (T') was evident in the soluble (not insoluble) fractions of cells infected with the plasmid, *p-tir-cesT-Int*, carrying *Y/ent* strain (Figure 17). This data demonstrates that *Yersinia enterocolitica* can, like *Yersinia pseudotuberculosis* (Kenny & Warawa, 2001), deliver Tir into HeLa cells where it only undergoes partial modification (to T'-like form) and, apparently, is not able to interact with Intimin in a manner that leads to its migration into the insoluble fraction.

A possible reason for the *Yersinia*-delivered Tir T' form not migrating into the insoluble fraction could be a defect in Intimin expression or presentation on the *Yersinia* surface in a conformation that can bind Tir. To examine this possibility, a two-wave infection protocol was carried out in which cells were initially infected with the Intimin-deficient strain (*eae*) which delivers Tir (Kenny *et al.*, 1997b), followed by a second-wave infection with *Y/ent* or *Y/ent* carrying the Intimin-encoding plasmid. As expected, both EPEC and the *eae* mutant delivered Tir which was modified to the T'/T" forms (Figure 18) with the T" form only detected in the insoluble fraction of cells pre-infected with EPEC, not the *eae* mutant (doesn't express Intimin), strains (Figure 18). Importantly, subsequent infection of cells pre-infected with the *eae* mutant was linked to the T" form migrating into the insoluble fraction but only when infecting with the *Y/ent* strain which carried the Intimin-expressed plasmid (Figure 18). This result reveals that *Y/ent* cannot only express Intimin but presents it on the *Yersinia* surface in a conformation that can interact with plasma membrane-inserted Tir. Moreover, the work supports the idea (Kenny & Warawa, 2001) that the *Yersinia*-delivered partially modified (T') Tir form is not inserted in the plasma membrane or is not in a conformation that can stably interact with Intimin.

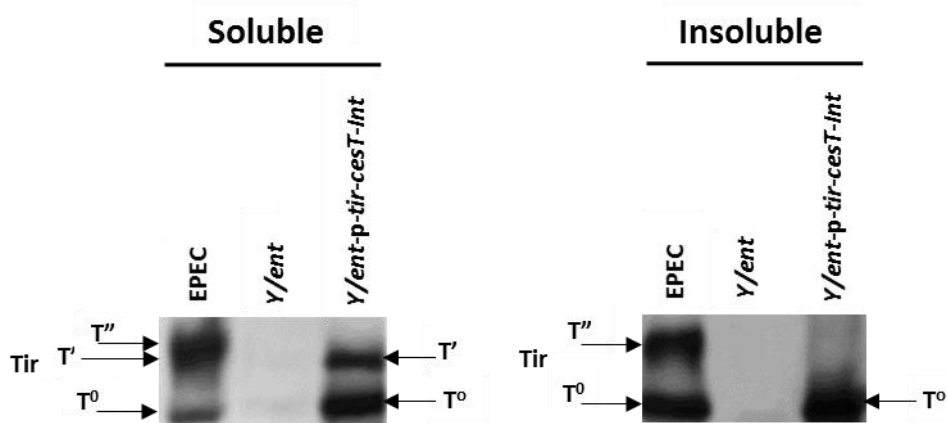


Figure 17: Tir is only partially modified following delivery into HeLa cells by a *Yersinia enterocolitica* (*Y/ent*) strain that lacks most *Yersinia* T3SS effectors. HeLa cells were infected with indicated strains prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis to probe for Tir. The positions of unmodified (T⁰) and modified (T' and T'') Tir forms are indicated (arrows). Strains used were EPEC, *Yersinia enterocolitica* lacking most T3SS effectors (*Y/ent*) or *Y/ent* carrying a plasmid encoding Tir, the Tir chaperone CesT and Intimin (*p-tir-cesT-Int*)

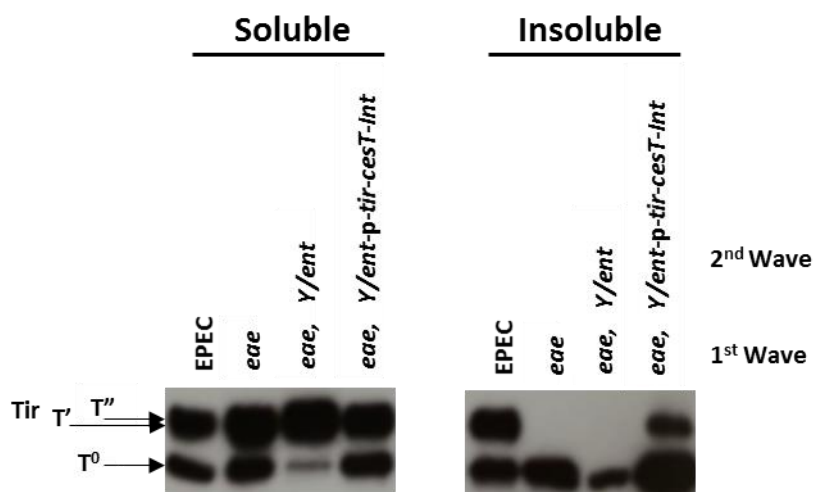


Figure 18: *Yersinia enterocolitica* presents Intimin on its surface in a conformation that can bind fully modified, membrane-inserted, Tir. HeLa cells were infected with EPEC or Intimin-deficient (*eae*) strains for 3hr before gentamycin-killing bacteria and infecting with a *Yersinia enterocolitica* that lacks most *Yersinia* effectors (*Y/ent*) or *Y/ent* carrying the plasmid *p-tir-cesT-Int* (encodes Tir, the Tir chaperone CesT and Intimin). Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions were isolated for western blot analysis probing Tir. The positions of unmodified (T⁰) and modified (T'/T'') Tir forms are indicated (arrows).

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3.3.3 Co-expression of LEE effectors with Tir in *Yersinia enterocolitica* inhibits Tir delivery into HeLa cells

Having developed a model in which *Yersinia* expresses Intimin on its surface and delivers Tir into HeLa cells where it only undergoes partial modification, we wanted to co-express LEE effectors - initially individually - to determine whether any would promote modification to the T' (Intimin-interacting) form. To enable detection, these studies would use available plasmids (See Chapter 2; Table 5) encoding effectors with C-terminal epitope (HA, HSV or Flag) tags. It is important to note that EspF has a dedicated chaperone, CesF (Elliott *et al.*, 2002) – required for efficient *Yersinia* mediated delivery into HeLa cells (A. Al-Layla, unpublished) - EspB has two (CesAB, CesD) chaperones (Wainwright & Kaper, 1998) while Map, EspH and EspZ share the Tir chaperone, CesT (Thomas *et al.*, 2005) with EspG having no known chaperone (Thomas *et al.*, 2005). Thus, initial studies focused on the Map, EspH and EspZ effectors - as they share the Tir chaperone - leading to the introduction of the appropriate plasmids into the *Y/ent p-tir-cesT-Int* (encode Tir, CesT and Intimin) strain. Western blot analysis of the insoluble fraction - containing adherent bacteria - from infected HeLa cells revealed a strong signal for a HA-related protein of the expected molecular mass for Map-HA (Figure 19A) with weak signal for EspH-HA (Figure 19A) and EspZ-HA (not shown; not captured on shown gel due to small, ~10kDa, molecular mass). However, only one protein (Map-HA) was evident, at very low levels, in the soluble fraction (Figure 19A). Probing for Tir, in two independent experiments (Figure 19A and 19B), was suggestive of reduced Tir expression when the strain carried a second plasmid (encoding HA-tagged effector) and failure to deliver Tir into cells (absence of T' form). Given this outcome it was decided to cease with this approach.

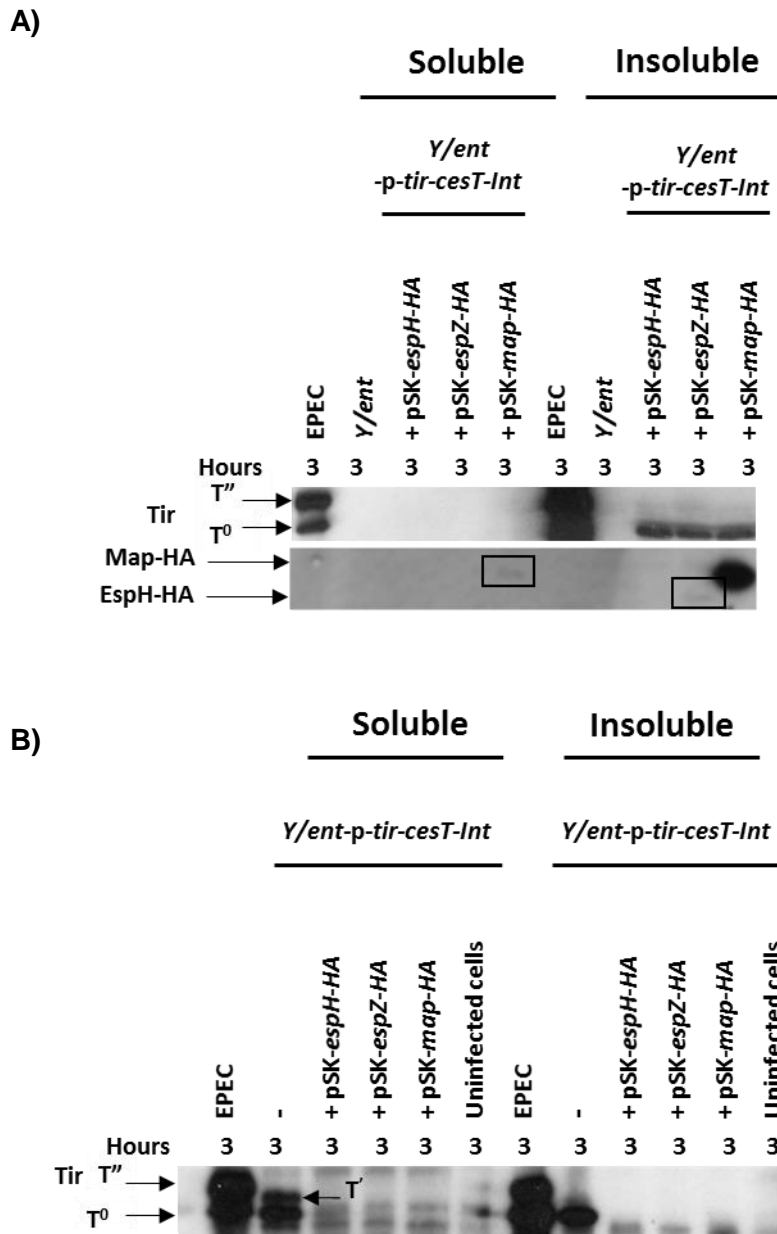


Figure 19: Co-expressing Tir with other LEE effectors in *Yersinia enterocolitica* interferes with the Tir delivery process. HeLa cells were left uninfected or infected (3h) with indicated strains before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for **A)** HA-tagged and Tir or **B)** Tir protein. The position of unmodified (T⁰) and host kinase modified Tir (T[']/T^{''}) forms are indicated as are HA-tagged proteins (arrows). Strains used were EPEC, *Yersinia enterocolitica* lacking genes encoding most *Yersinia* effectors (*Y/ent*) carrying *p-tir-cesT-Int* or, in some cases, an additional plasmid (encoding LEE Map [pSK-*map-HA*], EspH [pSK-*espH-HA*] or EspZ [pSK-*espZ-HA*] effectors as HA fusion proteins). Boxes highlight position of weak HA-related bands.

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3.3.4 Tir does not require Intimin, Map, EspG, EspH, EspF or EspZ activities for host kinase modification to T'' form.

As an alternative strategy to interrogate roles for LEE effector activity in the Tir modification process, a strain unable to express all known LEE effectors, except EspB as it is critical for delivering effector proteins (Kenny *et al.*, 1996, Foubister *et al.*, 1994), was generated (See Chapter 2; Table1). The Intimin gene (*eae*) was also inactivated as Intimin can alter cellular processes through binding Tir and host receptors, such as nucleolin (Kenny *et al.*, 1997b, Sinclair & O'Brien, 2002). The generation of this multi-mutant, *mfz₍₈₁₎go3eh::km*, was supported by PCR analyses (Figure 20) before using it, alongside EPEC, to infect HeLa cells. Western blot analysis of isolated fractions revealed both strains capable of Tir delivery as evidenced by T'/T'' forms in the soluble fraction with T'' form in the insoluble fraction of cells infected with EPEC but, as expected, not the Intimin-deficient *mfz₍₈₁₎go3eh::km* strain (Figure 21). Strain genotype was further supported by the presence (EPEC) and absence (*mfz₍₈₁₎go3eh::km*) of the EspF protein (Figure 21). Examining intermediate strains - *mfz_(km)go3* (generated by Sabine Quitard), *mfz₍₈₁₎go3*, *mfz₍₈₁₎go3e* (See Chapter 2; Table1) - revealed near identical results except that the T'' form was absent from insoluble fractions of cells infected with Intimin-deficient strains and, as before, no strain expressed/delivered EspF (data not shown). This work demonstrates that Tir delivery and modification to the T'' form does not require the activity of the Intimin surface protein or five LEE effectors (EspG, EspZ, EspH, EspF, Map), implicating a role for i) the remaining known effector (EspB), ii) plasma membrane-inserted translocator (EspD, EspB) proteins or iii) unknown LEE-encoded effector(s).

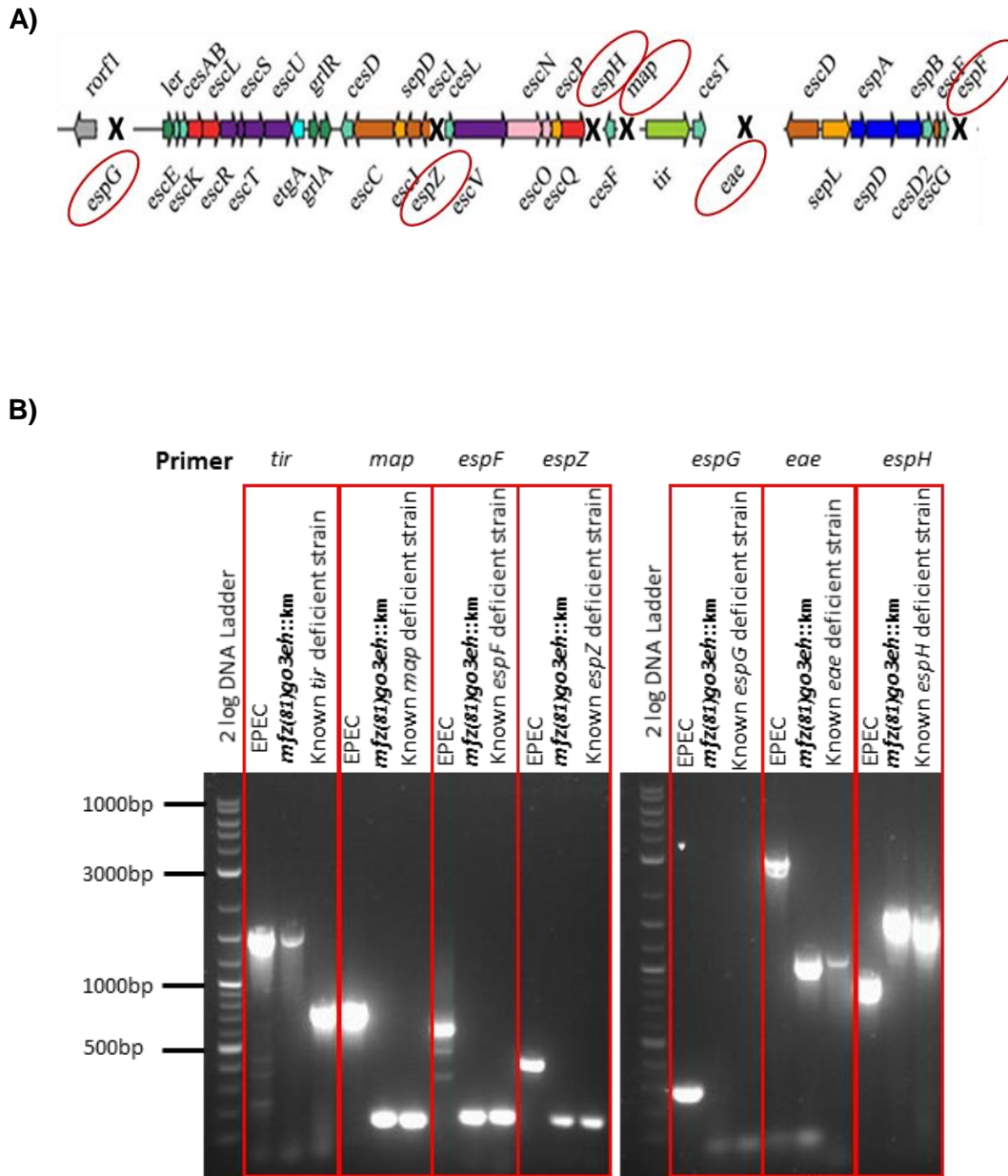


Figure 20: PCR support of *mfz₍₈₁₎go3eh::km* mutant. A) Schematic of EPEC LEE pathogenicity islands with inactivated genes circled (red oval). **B)** PCR reactions were carried out with primer sets designed to specifically amplify the *tir*, *map*, *espF*, *espZ*, *espG*, *eae* or *espH* genes to determine presence or absence from strains. PCR reactions included a positive (EPEC) and negative (strain known to lack the probed for gene) controls. Mutant strain name written in order genes disrupted i.e. *m* (*map*), *f* (*espF*), *z₍₈₁₎* (*espZ*), *g* (*espG*), *o3* (*orf3/espG2*), *e* (*eae* [encodes Intimin]), *h::km* (*espH*). '*z₍₈₁₎*' and '*h::km*' indicate that 81 of 98 EspZ residues are missing with *espH* gene replaced by a kanamycin-encoding gene respectively. PCR products were run, alongside 2 Log DNA ladder marker, on 1% agarose gel containing Gel Red nucleic acid stain.

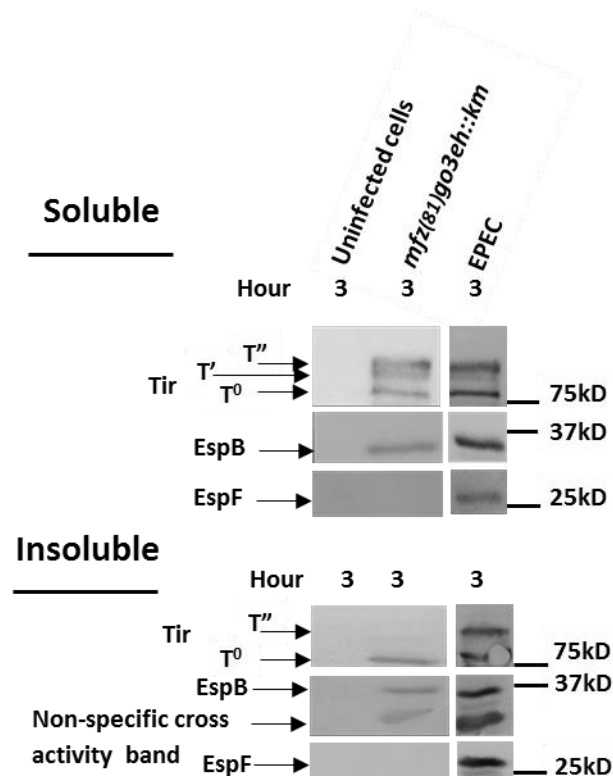


Figure 21: Tir delivered by EPEC lacking Map, EspF, EspZ, EspG, EspG2, EspH effectors and Intimin proteins is modified to T'' form. HeLa cells were left uninfected or infected with indicated strains (3hr) before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for Tir, EspB or EspF proteins. Strains used were EPEC or the *mfz₍₈₁₎go3eh::km* mutant; latter lacks functional *map* (*m*), *espF* (*f*), *espZ* (*z₍₈₁₎*), *espG* (*g*), *orf3/espG2* (*o3*), Intimin (*e*) and *espH* (*h::km*) genes. Where appropriate, immunoblots were cropped and moved for presentation purposes. The position of Tir unmodified (T⁰) and host kinase modified (T'/T'') forms are indicated as well as the positions of EspB and EspF proteins. As indicate is the position of molecular weight protein markers.

3.3.5 EspB-Myc fusion protein lacking EspB residues 159-218 doesn't support Tir delivery into HeLa cells

The *mfz₍₈₁₎go3eh::km* mutant retains one effector, EspB – as it is essential for delivering effector proteins (Kenny *et al.*, 1996, Foubister *et al.*, 1994) - but a variant, EspB-mid (lacks 60 residues; 159-218), reportedly deliver effectors but can no longer efface absorptive microvilli or inhibit phagocytosis (Iizumi *et al.*, 2007). Therefore, we decided to interrogate whether replacing EspB with EspB-mid would impact on the Tir modification to T'' process. To exclude possible redundancy with other LEE effectors and Intimin activities the *espB* gene was inactivated - using an available suicide vector (Donnenberg & Kaper, 1991) - from *mfz₍₈₁₎go3eh::km* generating *mfz₍₈₁₎go3eh::km_b*.

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Plasmids encoding EspB or the kindly provided EspB-mid variant (Iizumi *et al.*, 2007) were introduced into the *mfz(81)go3eh:km_b* mutant strain and used to infect HeLa cells as before.

Western blot analysis of samples following a standard 3h infection failed to detect Tir in cells infected with the EspB-mid complemented strain - linked to a binding defect - leading to extended infection times where a 9h infection was needed to detect Tir delivery (not shown). However, 9hr infections resulted in cell detachment/death but these phenotypes could be, largely, prevented by replacing the media every 3hr. Thus, HeLa cells were re-infected with EPEC, *cfm* or *mfz(81)go3eh:km* for 6h or with *mfz(81)go3eh:km_b* mutant complemented with EspB (pACYC-*espB*) or EspB-mid (pGEM-*espB-mid*) for 9h - with 3 hourly media changes - prior to isolating Triton X-100 soluble and insoluble fractions for Western blot analysis.

As expected, the T3SS mutant strain expressed Tir with no T'/T'' kinase-modified forms detected in the Triton X-100 soluble or insoluble fractions (Figure 22B). By contrast, EPEC infection led to T'/T'' forms in the soluble fraction and, due to its interaction with Intimin, the T'' form in the insoluble fraction (Figure 22B). Not surprisingly, a T3SS mutant Tir profile was obtained with the *espB* mutant while introducing a plasmid carrying the native *espB* gene led to an EPEC-like Tir T'/T'' profile (Figure 22B). However, introducing the EspB-mid encoding plasmid resulted in a T3SS mutant like Tir profile (Figure 22B). Probing for EspB confirmed its expression by EPEC, but not *espB* mutant, as well as EspB complemented *espB* mutant strains but, as expected, the molecular mass was reduced for EspB-mid variant (Figure 22B). However, the reduction in apparent molecular mass was less than expected, as lacks 60 residues (~20% of EspB) a protein band with a predicted migration of ~30kDa (versus 37kDa for EspB) protein. This difference led to the encoding plasmid being isolated for *espB* gene sequencing which revealed the unexpected presence of a C-terminal myc epitope tag revealing that the variant used in co-immunoprecipitation studies (Iizumi *et al.*, 2007) was erroneously provided. As it was possible that this epitope tag compromises EspB's translocator functionality, an alternative strategy was undertaken to examine the impact of replacing EspB with the EspB-mid variant. Thus, the provided suicide vector pCACTUS- Δ *espB*-mid (Iizumi *et al.*, 2007) was used to replace the LEE *espB* gene from EPEC and *mfz(81)go3eh::km_b* strains. While PCR analysis confirmed the presence of *espB*-mid on the provided vector several attempts to generate the required mutant strains were unsuccessful. Thus, we decided to subclone the *espB*-

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mid gene into a bacterial expression vector for complementation studies with EspB-deficient strains.

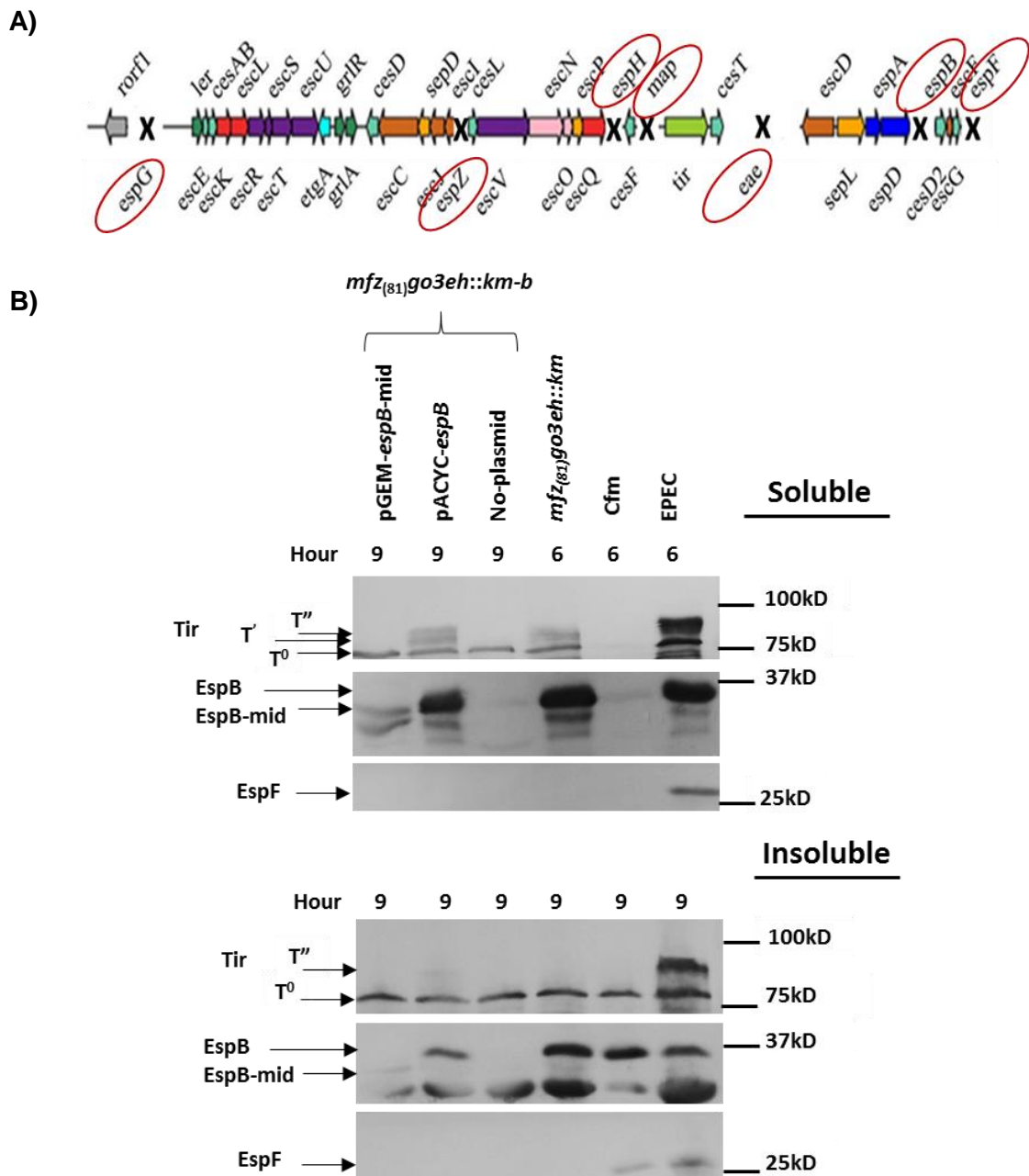


Figure 22: The EspB-mid variant does not support Tir delivery into HeLa cells. **A)** Schematic of LEE pathogenicity islands with effector genes, other than *tir*, and Intimin gene highlighted (red ovals). **B)** HeLa cells were infected with indicated strains for 9hr (replacing media every 3 hours for EPEC and *cfm* infected cells) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) samples for western blot analysis (10% SDS-PA gel) probing for Tir, EspB and EspF. The position of unmodified (T^0) and host kinase modified (T^1/T^2) forms are indicated as are EspB, EspB-mid, EspF and protein marker bands with arrows. Strains used were EPEC, T3SS mutant (*cfm*), *mfz₍₈₁₎go3eh::km* - lacks functional *map* (*m*), *espF* (*f*), *espZ* (*z₍₈₁₎*), *espG* (*g*), *orf3/espG2* (*o3*), Intimin (*e*) and *espH* (*h::km*) gene - *mfz₍₈₁₎go3eh::km_b* as per *mfz₍₈₁₎go3eh::km* but also lacks *espB* gene. Plasmids encoding EspB (pACYC-*espB*) or EspB-mid (pGEM-*espB-mid*) were introduced into *mfz₍₈₁₎go3eh::km_b* mutant.

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3.3.5.1 Cloning and characterization of the *espB*-mid gene

Briefly, the *espB*-mid gene was amplified from pCACTUS- Δ *espB*-mid for introduction into pACYC-184 (pre-digested with restriction enzymes BamHI and Sall) using the Gibson Assembly Kit (Figure 23). The resulting recombinant plasmid, pACYC-*espB*-mid, was shown to have the correct insert by sequencing which revealed the absence of a C-terminal extension and 120bp encoding residues 159 to 218. This plasmid was introduced into the *mfz*₈₁*go3eh::km_b* mutant and used to infect Hela cells.

As initial investigations failed to identify a binding defect, infections were carried out for 4h before isolating soluble and insoluble fractions for western blot analysis. Probing for EspB supported the expression and delivery of EspB-related proteins by all but not the negative control (*mfz*₈₁*go3eh::km_b*) strain. As expected, the EspB protein in cells infected with the EspB-mid complemented strain had a smaller apparent molecular mass, consistent with the absence of 60 residues (Figure 24). Crucially, Tir was evident in its kinase modified T' and T'' forms in the soluble fraction from cells infected with all strains except the EspB-negative control (*mfz*₈₁*go3eh::km_b*) strain (Figure 24). The EspB-mid variant may have a reduced capacity to deliver Tir as possibility less T'/T'' forms detected (Figure 24). As expected, the T'' form was only evident in the insoluble fraction of EPEC infected cells (Figure 24) as the EspB-complemented strains do not express Intimin. These findings confirm that EspB-mid protein retains its translocation activity with residues 159 to 218 not required for Tir modification to T'' form. However, it remains possible that other EspB regions/motifs are needed for the Tir T' to T'' modification process.

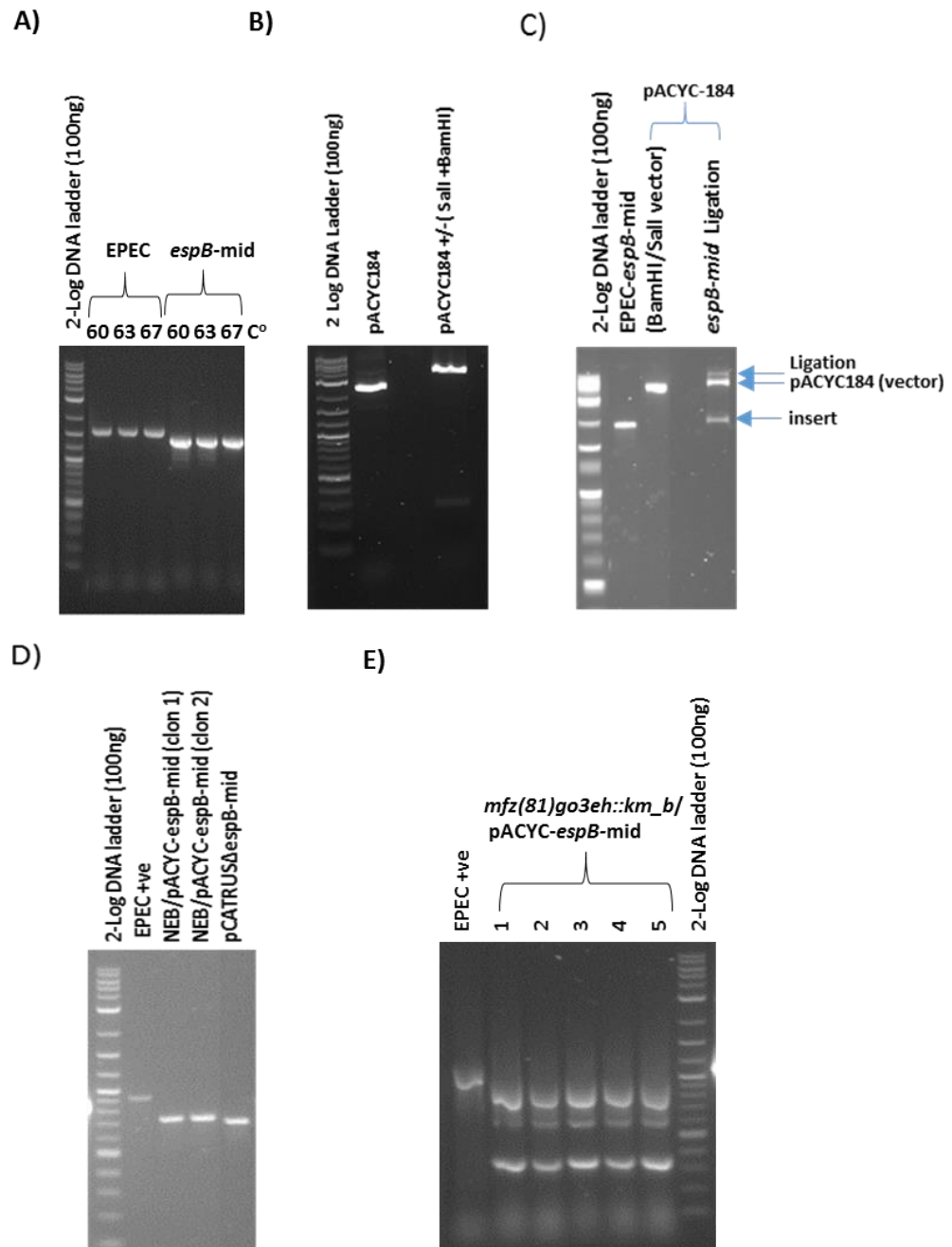


Figure 23: Construction of pACYC-*espB-mid*. **A)** The ability of designed oligonucleotides to PCR amplify *espB* (from EPEC as template) and *espB-mid* gene (from pCACTUS- Δ *espB-mid*) was assessed at different annealing temperatures (60, 63, 67°C). **B)** The expression vector pACYC184 digested with BamHI and Sall restriction enzymes and run, next to uncut pACYC184, on an agarose gel prior to isolating the digested fragment for use with the PCR-generated insert fragment in the Gibson Assembly protocol. **C)** Insertion of PCR fragment into pACYC184 was supported by additional bands. **D)** The ligation product was introduced into NEB chemical competent *E. coli* and colonies PCR-screened, alongside positive (EPEC) and negative (pCACTUS- Δ *espB-mid*) controls, for those carrying the insert. **E)** PCR support for introduction of pACYC- Δ *espB-mid* into *mfz(81)go3eh::km_b*.

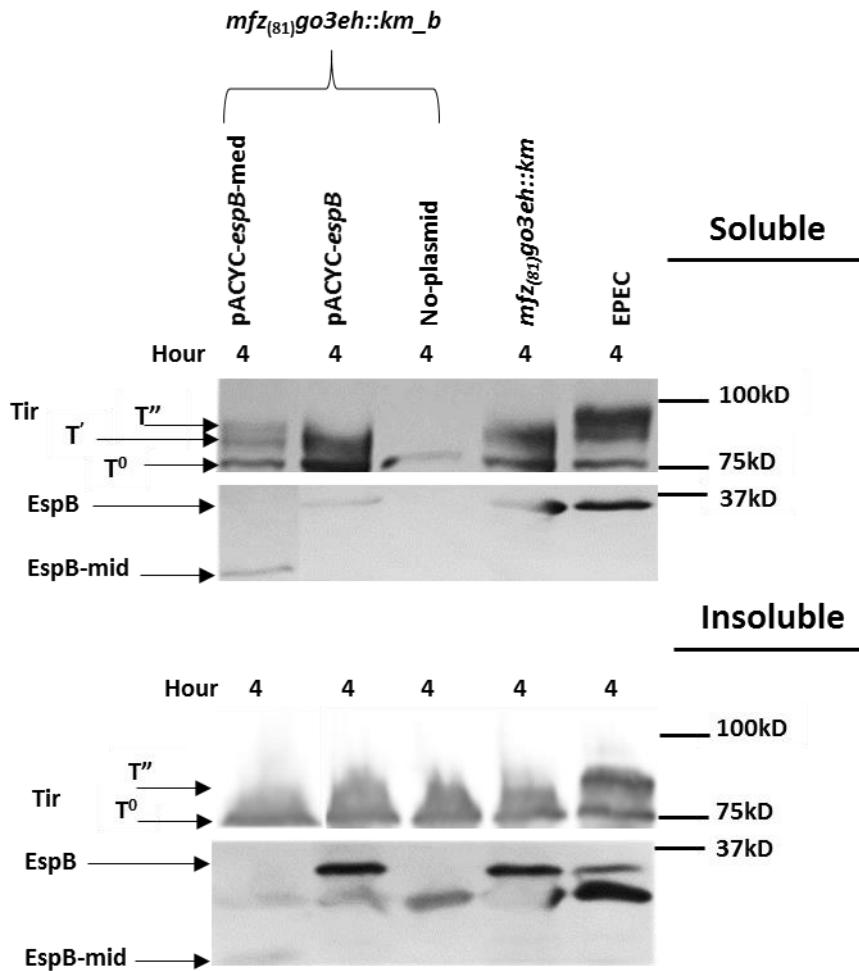


Figure 24: EspB-mid supports Tir delivery and modification to T'' form. HeLa cells were infected with indicated strains for 4h before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for Tir or EspB proteins. Strains used were EPEC, *mfz*₍₈₁₎*go3eh::km* (doesn't express Map/EspF/EspZ/EspG/EspG2/Intimin/EspH proteins) or *mfz*₍₈₁₎*go3eh::km_b* (as *mfz*₍₈₁₎*go3eh::km* but also lacks functional EspB protein) mutants. The latter strain either had no plasmid or carried a plasmid encoding EspB (pACYC184-*espB*) or EspB-mid (pACYC184-*espB-mid*) proteins. Indicated by arrows are the positions of EspB, EspB-mid and Tir (unmodified, T^o and host kinase modified T'/T'' forms) as well as the molecular weight marker proteins.

3.3.6 Probing role for other EspB features by examining functionally interchangeability with *E. tarda* homologue.

Recently a fish pathogen, *Edwardsiella tarda* (*E. tarda*), was reported to carry a LEE region that, theoretically, encodes a T3SS/Esp effector delivery system but lacks genes for all EPEC LEE effectors genes except *tir* (Nakamura *et al.*, 2013). However, this LEE region also encodes the Tir chaperone, CesT, and Intimin protein suggesting that Tir is delivered into the plasma membrane of targeted fish cells to act as a receptor for Intimin to promote pathogen-host cell interactions. Tir delivery into the host cytoplasm by EPEC is proposed to have (Intimin-independent) functions such as interacting with host proteins and/or regulate the activity of co-delivered LEE effectors (Dean *et al.*, 2010a, Jepson *et al.*, 2003, Patel *et al.*, 2006, Goosney *et al.*, 2001). Thus, the absence of Map, EspF-G and EspZ effector-encoding genes on the *E. tarda* LEE region raised the possibility that *E. tarda* Tir may be directly inserted into the plasma membrane and thus lacks a LEE-encoded Tir 'cytoplasm to membrane' insertion mechanism. To interrogate this possibility, we requested the *E. tarda* LEE sequence to examine whether the EspB-encoding gene was intact and, if so, the level of homology with EPEC EspB.

3.3.6.1 Divergence of EPEC and *E. tarda* EspB proteins

Bioinformatics interrogation of the *E. tarda* LEE region sequence revealed a 340aa EspB-like protein which was, thus, slightly larger than EPEC EspB (321AA). Crucially, comparison of the protein homologues revealed low levels of identity/similarity (37.8% & 55.7% respectively) with regions of the *E. tarda* EspB protein containing small in-frame insertion and deletions (Figure 25). Perhaps not surprisingly there was good homology (Figure 25) between i) the N-terminal ~38 residue that provide a T3SS signal sequence, ii) N-terminal region linked to providing a regulated (SepL/SepD) translocation signal iii) the single transmembrane domain and iv) residues implicated in EspB-EspD interaction (Luo & Donnenberg, 2011, Deng *et al.*, 2015). However, it should be noted that features i-iii) were linked to insertions or deletions which may impact/alter their functionality. Interestingly, the coiled-coil feature linked to protein-protein interaction and implicated in EspB's ability to bind myosin protein to inhibit phagocytosis and promote microvilli effacement (Iizumi *et al.*, 2007) is associated to insertions (within or flanking) questioning whether these subversive activities are preserved in the *E. tarda* homologue.

Output from TMpred

2 possible models considered, only significant TM-segments used

-----> Slightly preferred model: N-terminus outside

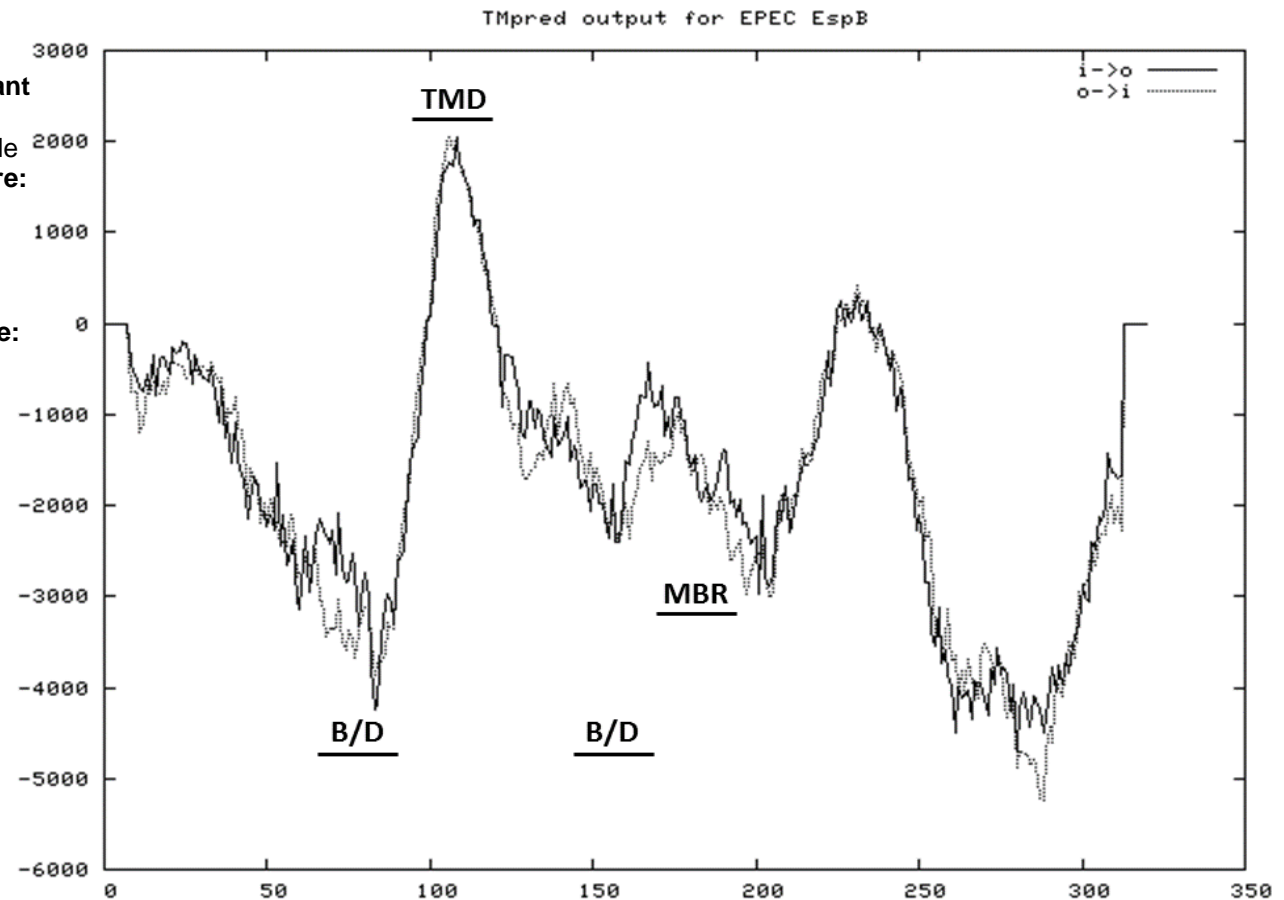
1- Strong transmembrane helices, total score: 2069

#	from	to	length	score	orientation
99	117	(19)	2069	o-i	

-----> Alternative model

1- Strong transmembrane helices, total score: 2021

#	from	to	length	score	orientation
99	119	(21)	2021	i-o	



Output from Tmpred

2 possible models considered, only significant TM-segments used

-----> STRONGLY preferred model: N-terminus outside

1 strong transmembrane helices, total score: 1864

#	from	to	length	score	orientation
93	110	(18)	1864	o-i	

-----> Alternative model

1 strong transmembrane helices, total score: 1760

#	from	to	length	score	orientation
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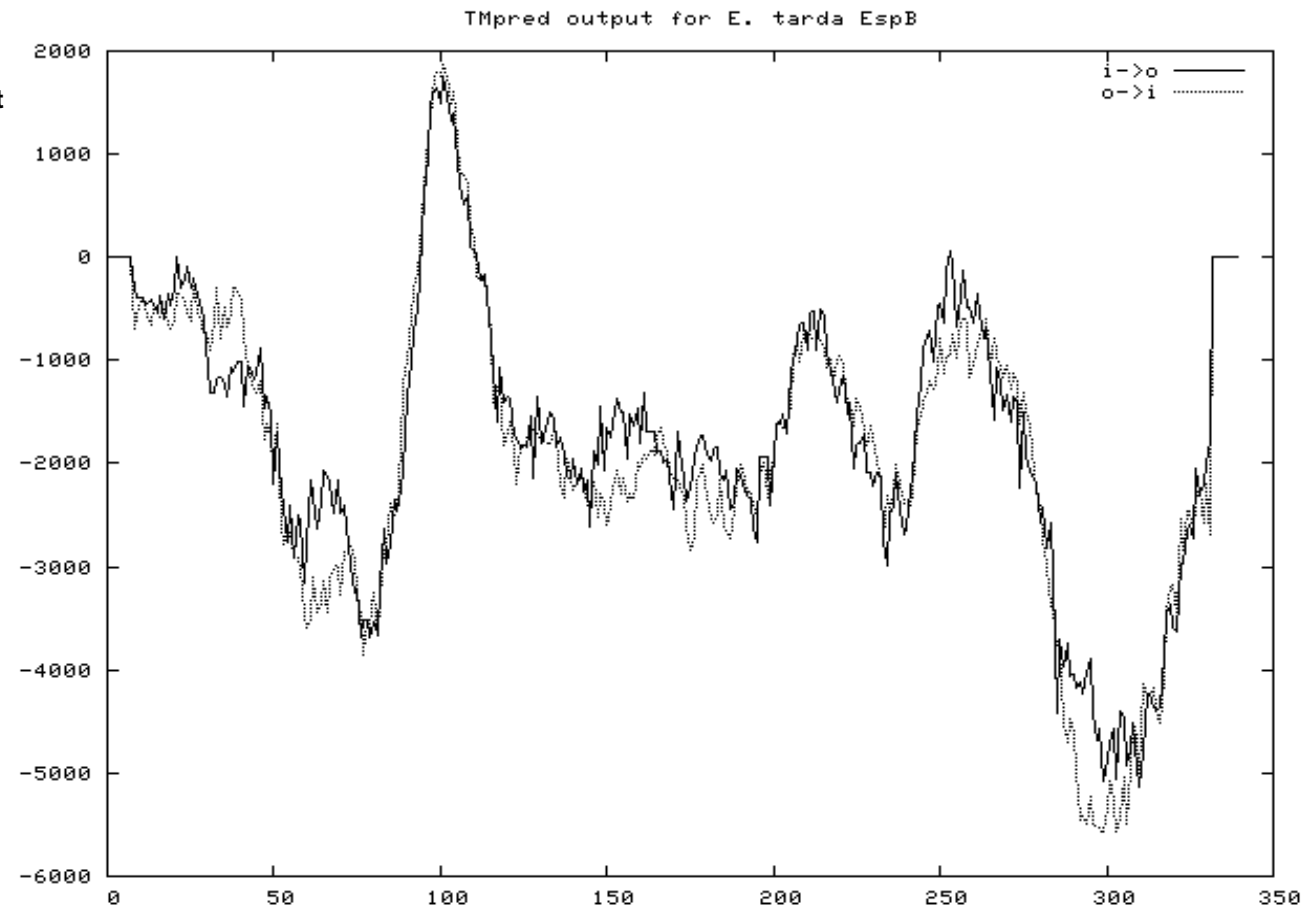


Figure 26: Interrogating the presence of transmembrane domain in *E. tarda* and EPEC EspB proteins. The EPEC and *E. tarda* EspB proteins sequences were interrogated (http://www.ch.embnet.org/software/TMPRED_form.html) leading to the predicted of one transmembrane domain (residues 97 to 120). The program requires setting of minimal and maximal hydrophobic helix length, in this case it was 17 and 33 respectively. Solid line is the prediction for inside to outside TM helix and interrupted line is the prediction for outside to inside TM helix.

3.3.6.2 Cloning *E. tarda espB* into pACYC-184

The *E. tarda espB* gene (*espB_{Et}*) was PCR amplified (Figure 27A) using, kindly provided, *E. tarda* genomic DNA, and oligonucleotides (See Chapter 2; Table 2) designed to introduce flanking restriction enzyme (BamHI/Sall) sites. The PCR product produced a single band of expected size (~1kb) which was ligated into the TOPO TA cloning vector (pCR2.1) before introducing into K12 *E.coli* (Figure 27B and 27C). BamHI/Sall restriction enzyme digestion of pCR2.1-*espB_{Et}* and pACYC184 provided the insert (*espB_{Et}* ~1kB) and pACYC184 (~4kB) vector bands that were ligated together prior to introducing into K12 *E. coli* (Figure 27D-E). The plasmid was isolated with generation of pACYC-*espB_{Et}* supported by restriction digestion and PCR analysis (Figure 27F-G).

3.3.6.3 Functional interchangeability of *E.tarda* EspB

To assess the translocator activity of the *E. tarda* EspB protein, HeLa cells were infected with EPEC or *espB* mutant carrying no plasmid or plasmids encoding EPEC or *E. tarda* EspB proteins prior to isolating Triton X-100 soluble and insoluble fractions for Western blot analysis. As expected, EPEC, unlike the *espB* mutant, delivered Tir as evidenced by detection of modified T'/T" forms in the soluble and T" form in the insoluble fractions (Figure 28). Crucially, the *espB* mutant defect was rescued by introducing plasmids encoding the EPEC or *E. tarda* EspB proteins; latter supported by antibodies raised against EPEC EspB failing to detect the *E. tarda* variant (Figure 28). The delayed and reduced level of Tir (unmodified or modified forms) in fractions from cells infected with the *E. tarda* EspB-expressing strain is suggestive of issues in *E. tarda* EspB protein expression, stability or translocator activity. Nevertheless, this work reveals the *E. tarda espB* gene encodes a protein that can functionally replace the translocator activity of the EPEC EspB protein to deliver Tir which then undergoes host kinase mediated modification to the T" form that can interact with Intimin.

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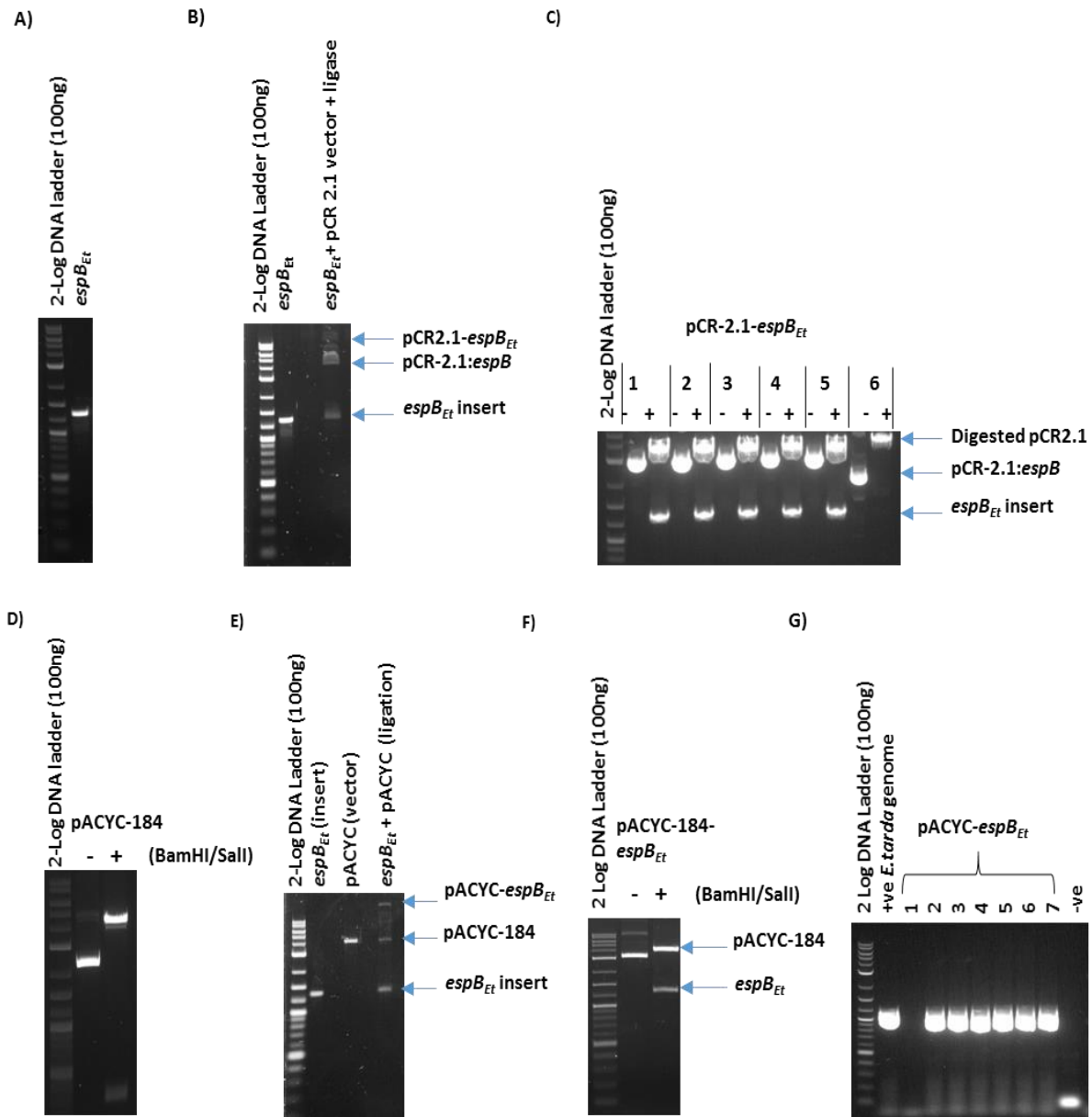


Figure 27: Construction of pACYC-*espB_{Et}*. **A)** The *E. tarda espB* (*espB_{Et}*) gene was PCR amplified providing a single band of expected molecular mass (~1kb). **B)** The PCR product was ligated into TOBO TA cloning vector (pCR-2.1) before introducing into K12 *E. coli* with (C) pCR-2.1-*espB_{Et}* plasmid generation supported by restriction digestion analysis, **D & E)** The pCR2.1-*espB_{Et}* and pACYC184 plasmids were digested with BamHI and Sall restriction enzymes prior to isolating the *espB_{Et}* (~1Kb) and pACYC184 (~4kb) vectors bands for ligation, with success indicate by additional bands. **F)** Generation of pACYC-*espB_{Et}* was supported by restriction digest analysis with **G)** PCR support for the generation of pACYC-*espB_{Et}*.

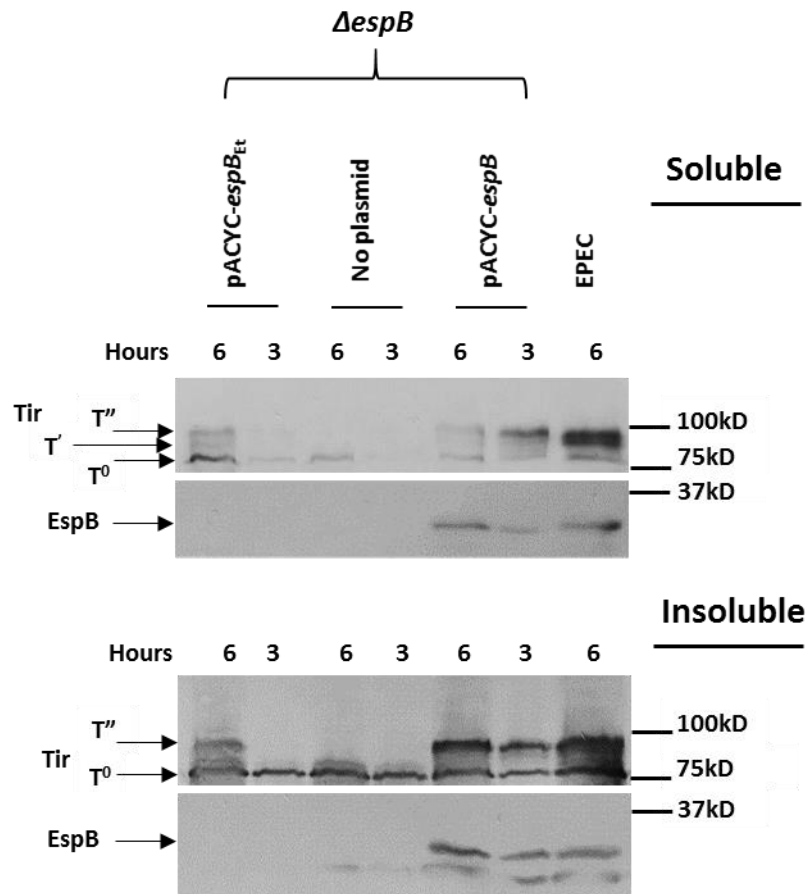


Figure 28: The *E. tarda* EspB protein functionally replaces EPEC EspB to deliver Tir into cells where modified to the T'' form and interacts with Intimin. HeLa cells were infected with indicated strains for 3 and/or 6h before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for Tir and EspB. Strain used were EPEC, the $\Delta espB$ mutant or $\Delta espB$ carrying pACYC184-based plasmids encoding the EPEC (pACYC-*espB*) or *E. tarda* (pACYC-*espB*_{Et}) EspB proteins. The position of unmodified (T⁰) and host kinase modified (T'/T'') forms are indicated alongside the EspB and protein marker proteins.

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3.3.6.4 Actin nucleating functionality of Tir delivered via the EspB_{Et} protein

To interrogate if Tir delivered via the *E. tarda* EspB protein was functional, studies examined for Tir-directed actin nucleation as this event depends on Tir being phosphorylated on tyrosine residues and binding Intimin (Kenny, 1999, Rosenshine *et al.*, 1996). Thus, HeLa cells were seeded on glass coverslips and infected (4h) prior to fixing cells and staining for polymerised actin (Phalloidin-TRITC [Red]) and DNA (host and bacterial; DAPI [blue]) as described (Dean & Kenny, 2004).

Immunofluorescence microscopy evaluation of fluorescent signals revealed that, as reported (Rosenshine *et al.*, 1996, Kenny & Finlay, 1997), EPEC but not the *espB* mutant triggered actin polymerization - i.e. pedestal formation - beneath adherent bacteria (Figure 29). Crucially, pedestal formation was also detected for cells infected with the *espB* mutant carrying the EspB_{Et} encoding plasmid. However, the number of pedestals was significantly lower in cells infected with *espB* mutant carrying the plasmid encoding EspB_{Et}, in contrast to cells infected with EPEC (Figure 29). This data provides further support that the EspB_{Et} protein can functionally replace its EPEC homologue to not only deliver Tir into host cells for kinase mediated modification and insertion into the plasma membrane but places it in a conformation that can be bound by Intimin to trigger subversive signalling activities. Thus, the LEE factor implicated in the Tir T' to T'' modification process either depends on i) features conserved in the EPEC and *E. tarda* EspB proteins, ii) other translocator (EspD; EspA) proteins or iii) other, unknown, LEE encoded effectors.

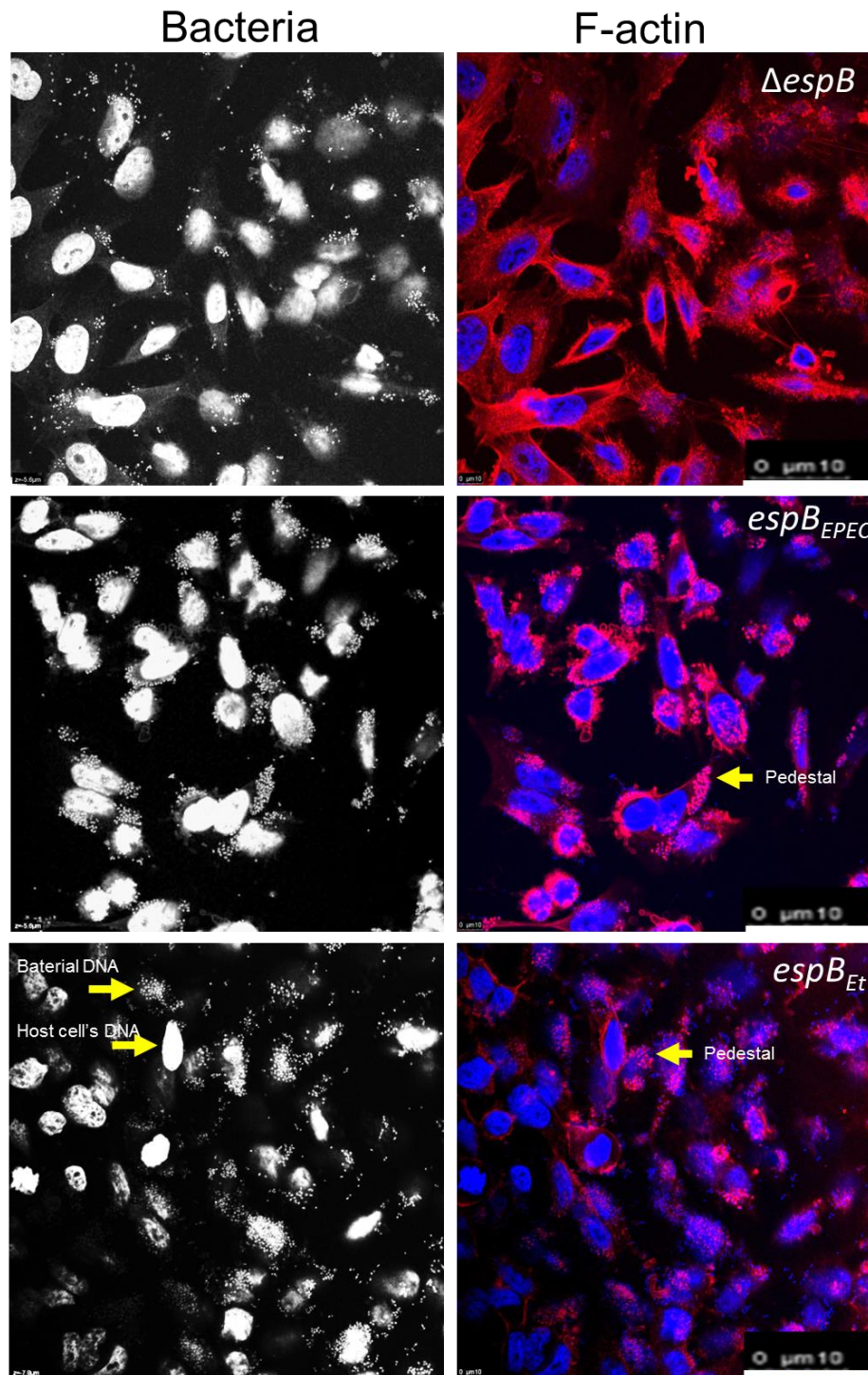


Figure 29: The *E. tarda* EspB protein functionally replaces EPEC EspB to enable Tir/Intimin-dependent pedestal formation. HeLa cells (on glass coverslips) were infected with indicated strains for 4h before fixing cells and staining for polymerized actin (Phalloidin-TRITC [Red]) or DNA (bacterial and host [DAPI; Blue]) that was visualized by fluorescence microscopy. Strain used were EPEC, the *espB* mutant or *espB* carrying pACYC184-*espB_{Et}* (EspB_{Et}). The scale bar represents 10 μ m.

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3.3.7 Examining functional interchangeability of *E. tarda* EspD protein

Studies were also undertaken to examine the possibility that the host membrane-inserted EspD translocator could aid Tir T' to T'' modification and/or membrane insertion processes. To inform of this process, the *E. tarda* LEE region was examined for an *espD* gene to determine if predicted to produce a full length protein and its homology to EPEC EspD.

3.3.7.1 Comparison of *E. tarda* and EPEC EspD proteins

Alignment of the EspD sequences (Figure 30) revealed a slightly larger *E. tarda* protein (385 versus 380 residues) with 50.9% identity (66.9% similarity) to the EPEC homologue. Of note was the presence of in-frame insertions and deletions within the N-terminal region. EspD is reportedly the major component of a 6-7 subunit EspD/EspB complex in the host membrane which provides a pore (2.5-5nm) – linked to EspA via EspD (Ide *et al.*, 2001) - through which effectors are delivered into cells. The key features linked to EspD functionality are highlighted in Figure 30 and include two predicted transmembrane domains – unlike one for EspB – with N-terminal and C-terminal coil-coiled, as well as amphipathic regions linked with EspD-EspD interactions (Dasanayake *et al.*, 2011, Daniell *et al.*, 2001). The final 12 C-terminal amino acid residues of EPEC EspD are essential for its secretion (Deng *et al.*, 2015).

3.3.7.2 Cloning *E. tarda espD* into pACYC-184

To interrogate the functional interchangeability of *E. tarda* EspD and possible role in Tir modification and insertion mechanism, *espD_{Et}* was cloned and used to complement the EspD deficient EPEC strain. Briefly, the *espD_{Et}* gene (~1.3kB) was amplified from kindly provided *E. tarda* genomic DNA and added to isolated pACYC-184 (~4kB) vector band, following digestion with BamHI/Sall restriction enzymes, to generate pACYC-*espD_{Et}* using the Gibson Assembly kit (see Chapter 2; Section 2.2.8). The presence of pACYC-*espD_{Et}* in EPEC EspD deficient ($\Delta espD$) mutant strain was supported by PCR analyses (Figure 31D).

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EPEC-EspD	1	MLNVNNDIQSV-RSGASAATA	TSGINQSEVTSALDLQLVKSTAP-----	43
		:::	: ...:	
<i>E. tarda</i> -EspD	1	MMDTNLTISSVGP	SMALDAVAPGG-----NSLKSSVIPLTQPILQPRA	43
EPEC	44	----SASWTESTALPTPP--AGHSLVTP	SAEDVLSKLFGGISGEV	87
		.: ...: ...: ...: ...: ...: ...: ...:	
<i>E. tarda</i>	44	QVDEGSSSFNSSPLPLPPG	SVAHMVTPSAEDVLDKLFGGISGE-RSGS	92
EPEC	88	EGTEPQRSTQNASSGYPYLSQVNNVDPQAMMMATLLSLDASAQRVASMK		137
		: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	93	DSTGLFADKGEDKTALPI	LARLSQVDP IAMMLMVTSLSMETSTQKIGSLK	142
EPEC	138	NSNEIYADGQNKALDNKTL	EFKQLEEQQAEEKAQKSKI	187
		: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	143	DSNRIYADGQDKALASKLE	EFKQLEEQQAEEKAQKSKVLGQVFGWLGV	192
EPEC	188	AATAIAAIFNPALWAVV	AI SATAMALQTAVDVMGDDAPQALKTAAQAFGG	237
	: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	193	LATAIAALFNPALWVVV	AI SATSMALQTAVDVMGDKAPQALKSAAQVFGG	242
EPEC	238	LSLAAGILTAGIGGVSSLI	SKVGDVANKVGSNIVKVVTTLA	287
		: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	243	ISMAAGLATAGV	GALSSILKSASSVAQKLGETVTKVAVKVFTEKFVENTAA	292
EPEC	288	KISAVANGLTTSSRSIGT	TVLNNDAAAYNVLSQVSAFAVENLTRQSEYLS	337
		: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	293	KVGAIATGLTESSKSI	GTTVLNKESSDALIDSLAAAFSVKLNLDMNYRLMG	342
EPEC	338	QSAKAELEKATLELQ	NQANYIQSASQMSDS	380
		: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...: ...:		
<i>E. tarda</i>	343	ESTGSMLRRAA	DEGNDLVRFLQGTSNVMSD TARVNSRIIRGLA	385

Figure 30: Comparison of *E. tarda* and EPEC-encoded EspD proteins. *E. tarda* and EPEC EspD protein sequences were aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Highlighted (in blue) is the T3SS secretion signal region, (in black) two predicted transmembrane domain, (in red) N and C-terminal coiled coil regions (associated with protein-protein interactions) and (in green) an amphipathic regions.

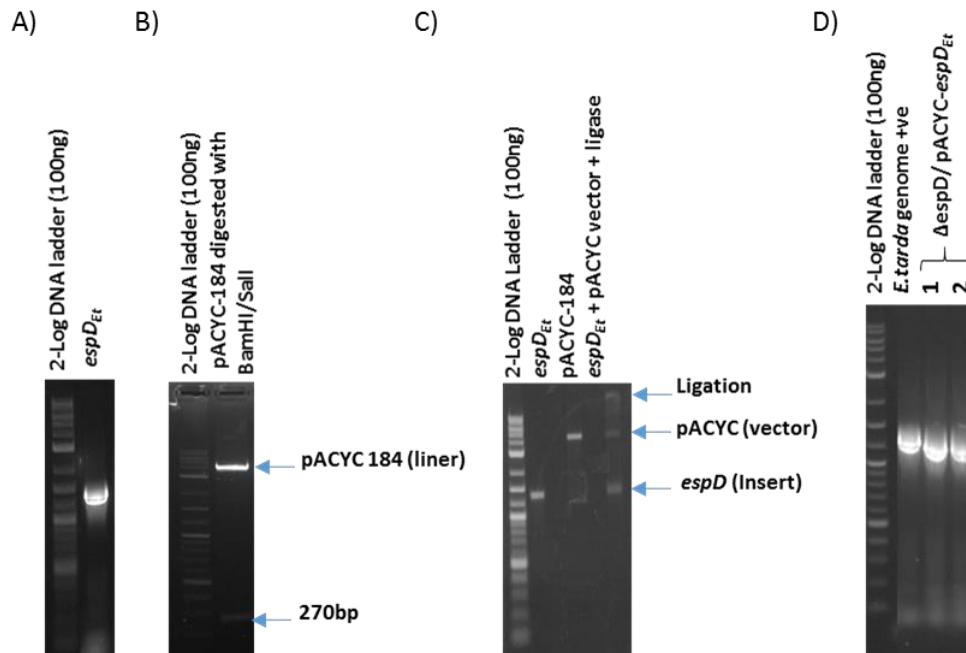


Figure 31: Construction of pACYC-*espD_{Et}*. **A)** The *E. tarda* *espD* (*espD_{Et}*) gene was PCR amplified, providing a single band of expected molecular mass (~1.3kb). **B)** pACYC-184 was digested with BamHI and SalI restriction enzymes prior to isolating ~4kb vector bands for **C)** use with *espD_{Et}* (~1.3Kb) fragment in Gibson Assembly protocol to generate pACYC-*espD_{Et}* with **D)** introduction of pACYC-*espD_{Et}* into the EPEC Δ *espD* mutant supported by PCR.

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3.3.7.3 *E. tarda* EspD weakly rescues the EPEC $\Delta espD$ mutant defect leading to low level of Tir modification to T'/T'' form

To determine whether EspD_{Et} can functionally replace EPEC EspD's translocator functions, studies examined its ability to rescue the Tir delivery defect of the EPEC $\Delta espD$ mutant strain (Lai *et al.*, 1997). As initial studies revealed a binding defect for the $\Delta espD$ mutant when carrying the EspD_{Et} encoding plasmid, cells were infected for up to 9 hr – replacing the media every 3 hrs, as before – prior to isolating Triton X-100 soluble and insoluble fractions.

Western blot analysis of the samples confirmed (Kresse *et al.*, 1999, Lai *et al.*, 1997) that the $\Delta espD$ mutant expresses Tir but can not deliver it into host cells. However, unexpectedly, a single band linked to T⁰ was detected in the soluble fraction of cells infected with $\Delta espD$ mutant (Figure 32). The detection of this band may be related to a cross contamination from bacterial lysis or endocytosis of Tir into such membranous compartments. Importantly, this band (T⁰) was not modified to the T'-like form. In contrast, introduction of the EPEC EspD encoding plasmid restores Tir deliver (evidenced by detecting the T'/T'' forms in the soluble fraction and, due to interaction with Intimin, the T'' form in the insoluble fraction; Figure 32). Interestingly, introducing the EspD_{Et} encoding plasmid restored Tir delivery with only very low levels of modified Tir (T'' form) detected in the soluble (none in insoluble) fraction (Figure 32). Probing for EspB revealed similar signals in the soluble and insoluble fractions of cells infected with the $\Delta espD$ mutant (whether carrying EspD-encoding plasmids or not; Figure 32). This work suggests that the *E. tarda espD* gene encodes a protein that can functional replace the translocator activity of EPEC EspD but negative impacts on the process that modifies Tir to T'/T'' forms and Intimin's ability to stably interact with modified Tir.

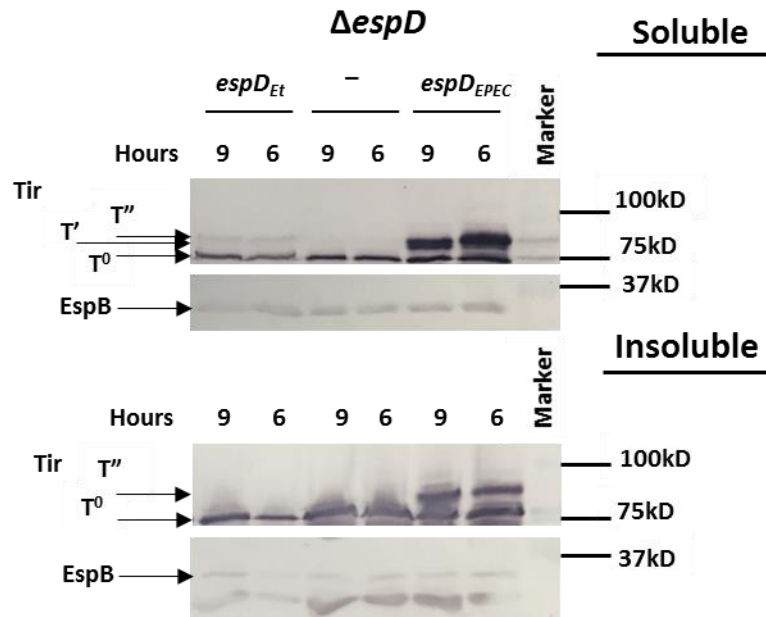


Figure 32: *E. tarda* EspD rescues the EPEC *espD* mutant defect in delivering Tir linked to a defect in Tir modification and Intimin interaction with Tir. HeLa cells were infected for 6 or 9hr with indicated strains before isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for Tir and EspB. The position of unmodified (T⁰) and host kinase modified (T'/T'') forms are indicated (by arrows) as are EspB and protein markers. Strains used were the *ΔespD* mutant that carried no plasmid (-) or plasmids carrying the EPEC (*espD_{EPEC}*) or *E. tarda* (*espD_{Et}*) *espD* genes.

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3.3.8.1 Cloning *E.tarda espA* into pACYC184

The same strategy, as described for *espD_{Et}*, was used to clone *espA_{Et}* into pACYC-184 (Figure 34), with introduction of pACYC-*espA_{Et}* into the EPEC $\Delta espA$ mutant supported by PCR analysis (Figure 34E).

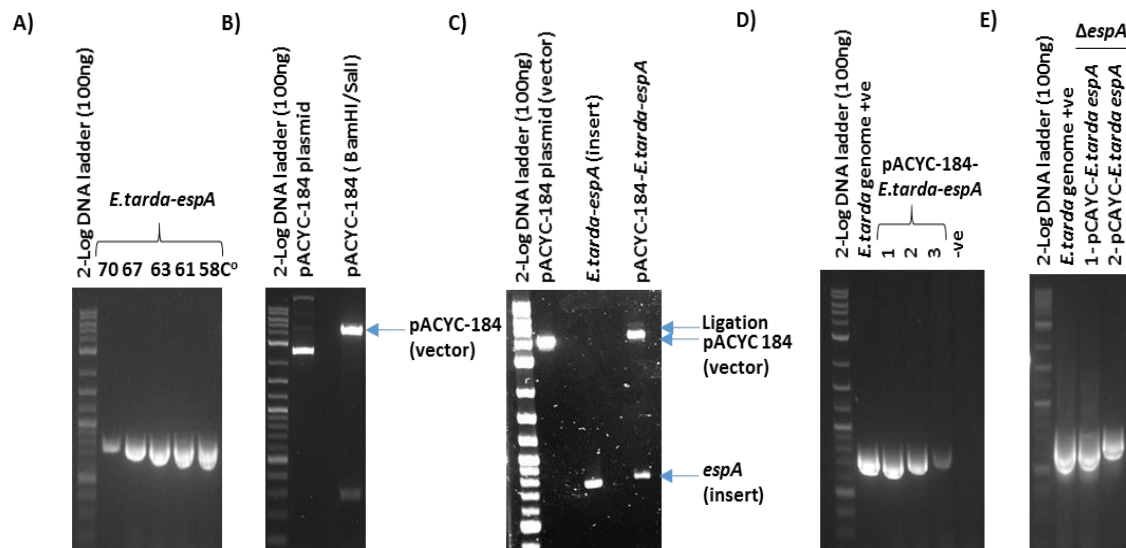


Figure 34: Construction of pACYC-*espA_{Et}*. **A)** The *E.tarda espA* gene was PCR amplified as a single band of expected molecular size (~0.8kb) over a range of annealing temperatures. **B)** pACYC-184 was digested with BamHI and Sall restriction enzymes prior to isolating ~4kb vector bands for **C)** use with *espA_{Et}* (~0.8Kb) PCR fragment in Gibson Assembly protocol to generate pACYC-*espA_{Et}*. PCR analysis supports introduction of *espA_{Et}* carrying plasmid into **D)** non-pathogenic *E.coli* and **E)** EPEC $\Delta espA$ mutant.

3.3.8.2 Similar phenotype for $\Delta espA$ /pACYC-*espA_{Et}* and $\Delta espD$ /pACYC-*espD_{Et}* strains

Preliminary studies also revealed a binding defect for the $\Delta espA$ mutant when carrying the *EspA_{Et}* encoding plasmid, so infections were carried out for up to 9 hr – replacing the media every 3 hrs for control strains – prior to isolating Triton X-100 soluble and insoluble fractions.

Western blot analysis confirmed (Kenny *et al.*, 1996) Tir expression but not delivery by the $\Delta espA$ mutant with plasmid re-introducing the EPEC *espA* gene resulting in an EPEC-like phenotype i.e. similar levels of T'/T'' forms in the soluble fraction and, due to interaction with Intimin, T'' form in the insoluble fraction (Figure 35). Interestingly, while introducing the *espA_{Et}* gene carrying plasmid also restored Tir delivery –

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evidenced by only low level of T^o-like form was evident in the soluble fraction (Figure 35), no T^o form was evident in the insoluble fraction (Figure 35). However, unexpectedly, bands linked to T^o and EspB were detected in the soluble fraction of cells infected with $\Delta espA$ mutant. The detection of these bands may be related to a cross contamination from bacterial lysis or endocytosis of Tir and EspB into such membranous compartments. Importantly, this band (T^o) was not modified to the T^o-like form. Probing for EspB in both soluble and insoluble fractions failed to define differences to explain the different Tir profiles (Figure 35). This work suggests that the *E. tarda espA* gene encodes a protein that can only weakly functional replace the translocator activity of the EPEC EspA protein but, like *E. tarda* EspD, negative impacts on the Tir T^o to T^o/T^o modification process and/or Intimin's ability to stably interact with the Tir T^o form.

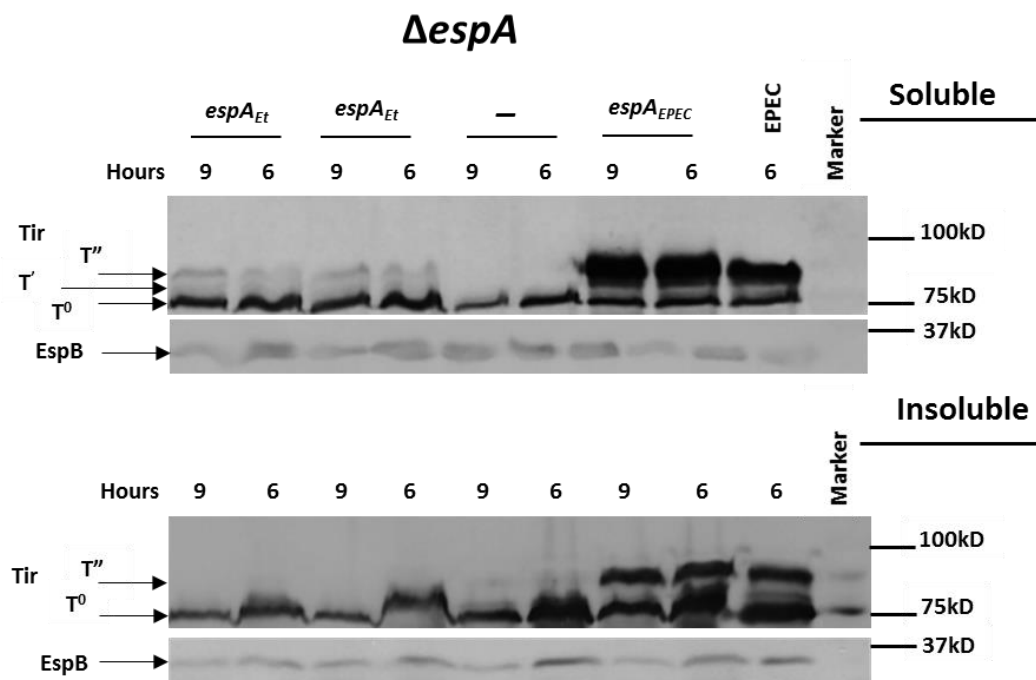


Figure 35: *E. tarda* EspA rescues the EPEC $\Delta espA$ mutant defect in delivering Tir linked to a defect in Tir modification and Intimin interaction with Tir. HeLa cells were infected for 6 and/or 9hr with indicated strains before isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis probing for Tir and EspB. The position of unmodified (T^o) and host kinase modified (T^o/T^o) forms are indicated (by arrows) as are EspB and protein markers. Strains used were EPEC and $\Delta espA$ mutant carrying no plasmid (-) or a plasmid carrying the EPEC (*espA_{EPEC}*) or *E. tarda* (*espA_{Et}*) *espA* genes. This study included two $\Delta espA/pACYC-espA_{Et}$ clones.

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3.3.8.3 The $\Delta espA/pACYC-espA_{Et}$ mutant has a functional Intimin surface protein

The surprising detection of the Tir T'' modified form in the soluble but not insoluble fraction of cells infected with the $\Delta espA$ mutant carrying pACYC- $espA_{Et}$ questioned whether the strain had a functional Intimin protein. To address this possibility, the two-wave infection protocol was undertaken to determine, as before (Chapter 3; Section 3.3.2), whether Tir T'' in cells pre-infected with the Intimin (*eae*) mutant would migrate into the insoluble following a second wave infection with the $\Delta espA$ complemented strains.

Western blot analysis of isolated soluble fractions revealed Tir T'' in the soluble, with a little in the insoluble, fraction of cells pre-infected with the *eae* mutant, with subsequent infection by the $\Delta espA$ complemented (plasmid carrying $espA_{Et}$ or $espA_{EPEC}$ genes) strains clearly increasing the level of Tir T'' in the insoluble fraction (Figure 36). This finding illustrates that the $\Delta espA$ mutant has a surface Intimin protein which can stably interact with the Tir T'' form.

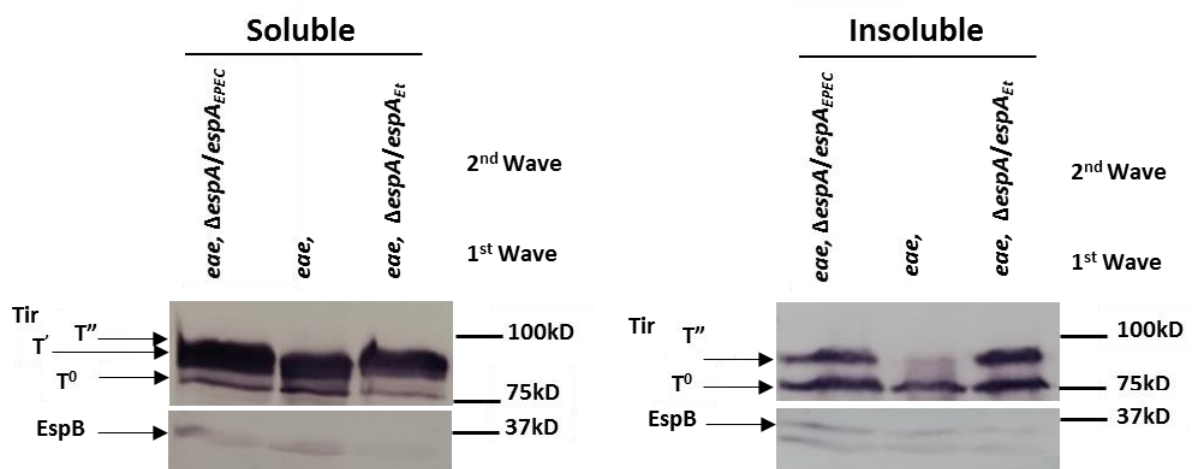


Figure 36: $\Delta espA/pACYC-espA_{Et}$ strain has Intimin on its surface which can stably interact with the Tir T'' modified form. HeLa cells were infected with the Intimin-deficient (*eae*) mutant for 3hr before gentamycin-killing extracellular bacteria and leaving one well uninfected or re-infecting with the $\Delta espA$ mutant carrying either the pACYC- $espA_{Et}$ or pACYC- $espA_{EPEC}$ plasmids. Post-infection cells were washed before isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gel), probing for Tir or EspB. The positions of unmodified (T⁰) and modified (T'') forms are indicated (by arrows) as are EspB and protein markers.

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3.3.9 Rescuing the T3SS defect of EPEC $\Delta espA$, $\Delta espD$, $\Delta espAB$ and $\Delta espDB$ mutants by introducing *E. tarda* region encoding EspA, EspB and EspD

Given that EspA interacts with EspD, but not EspB, while EspD also interacts with EspB (Luo & Donnenberg, 2011) studies investigated the possibility that there are species specific interactions between *E. tarda* and EPEC EspA/EspD proteins for maximal T3SS functionality. To test this hypothesis the *E. tarda* translocator gene region ($espADB_{Et}$) was PCR amplified and cloned into pACYC184 (Figure 37).

The resulting plasmid was initially introduced into the EPEC $\Delta espA$ and $\Delta espD$ mutants for infection studies prior to isolating Triton X-100 soluble and insoluble fractions. Western blot analysis revealed an EPEC like profile for the plasmid complemented strains while the single mutants, as expected, failed to deliver Tir (Figure 38). It should be noted that there was not only greater levels of the Tir T^o form in the soluble fraction but also in the insoluble fraction indicative of stable Tir-Intimin interaction (Figure 38). Indeed, similar results were obtained when the pACYC- $espADB_{Et}$ plasmid was introduced into the $\Delta espA espB$ and $\Delta espD espB$ double mutants (Figure 39). An $\Delta espA espD$ double and $\Delta espA espD espB$ triple mutant was not available. Introduction of pACYC- $espADB_{Et}$ was further supported by probing for EspB as the antibodies (generated to EPEC EspB) don't recognise the *E. tarda* variant and thus failed to detect an EspB protein despite plasmid introduction rescuing the mutant defect.

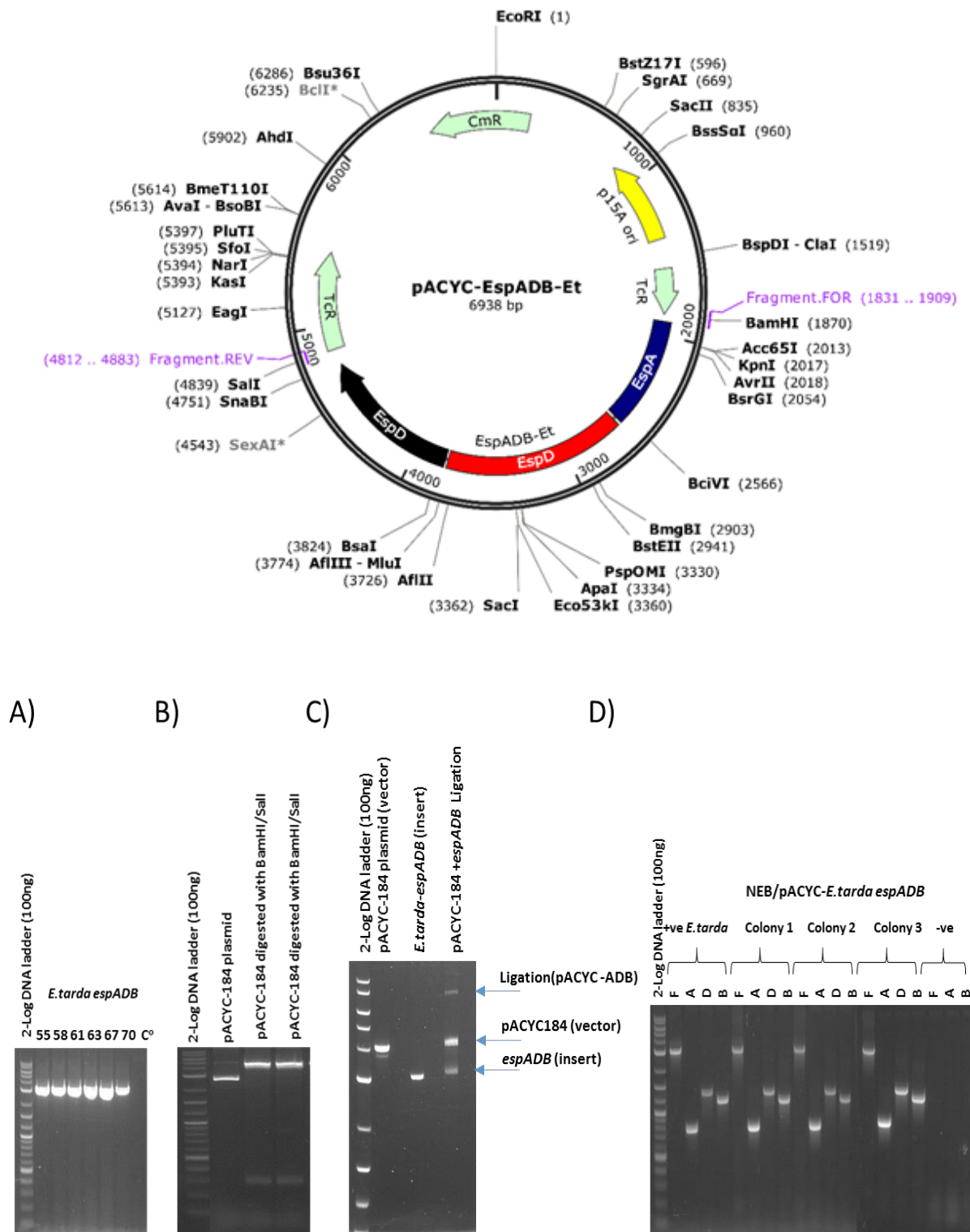
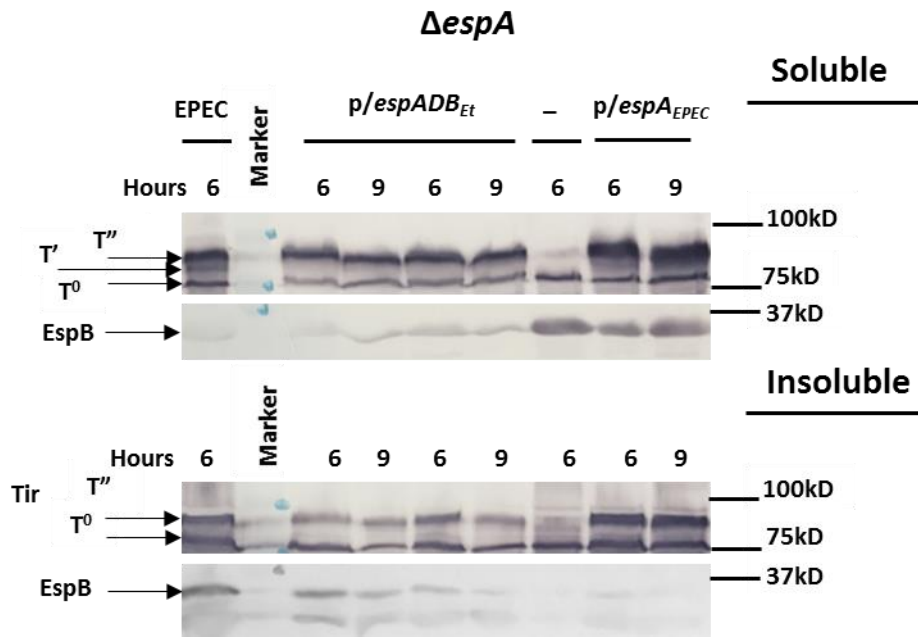


Figure 37: Construction of pACYC-*espADB*_{Et}. Schematic of pACYC184 plasmid carrying *E. tarda espADB* gene region with (A-D) agarose gel data illustrating steps in generating and confirming plasmid construction/introduction into bacteria. **A)** PCR amplification, over a range of annealing temperatures, of single band with expected size for the *espADB*_{Et} gene region, **B)** pACYC184 digestion with BamHI and SalI restriction enzymes to isolate ~4Kb vector fragment for **C)** use with PCR fragment in the Gibson Assembly protocol to produce pACYC-*espADB*_{Et} with **D)** introduction of pACYC-*espADB*_{Et} into *E. coli* supported by PCR analyses using primers specific to *espA*_{Et} (lanes labeled A), *espD*_{Et} (lanes labeled D), *espB*_{Et} (lanes labeled B) and the *espADB*_{Et} region (lanes labeled F). The positive (+ve) and negative (-ve) controls were *E. tarda* genomic DNA and colony that grew in Cm and Tet plates (this indicator for Tet gene was not interrupted by inserted gene) respectively.

A)



B)

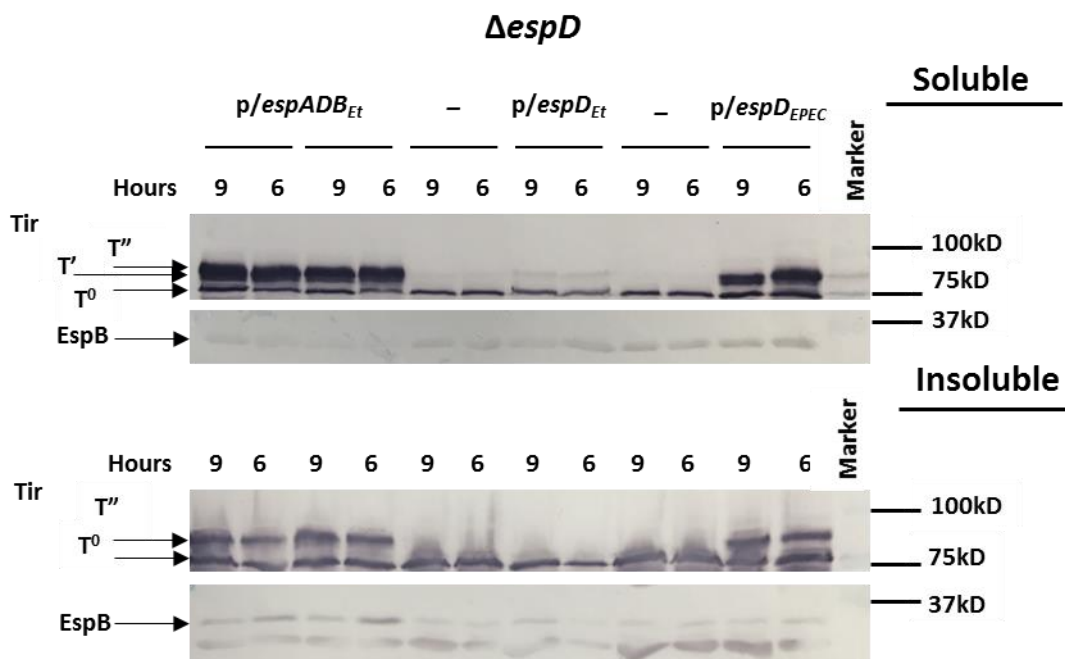


Figure 38: Rescuing the T3SS defect of EPEC *ΔespA* and *ΔespD* mutants by plasmid introducing *pACYC-espADB_{Et}*. HeLa cells were infected 6 or/and 9h with indicated strains prior to isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gel) probing for Tir or EspB. The position of Tir unmodified (T⁰) and host kinase modified (T'/T'') forms, EspB and protein markers are indicated. Strains used were EPEC, *ΔespA* or *ΔespD* mutant with mutant strain carrying no plasmid (-) or plasmid with *espA_{EPEC}*, *espD_{EPEC}* or *espADB_{Et}* genes

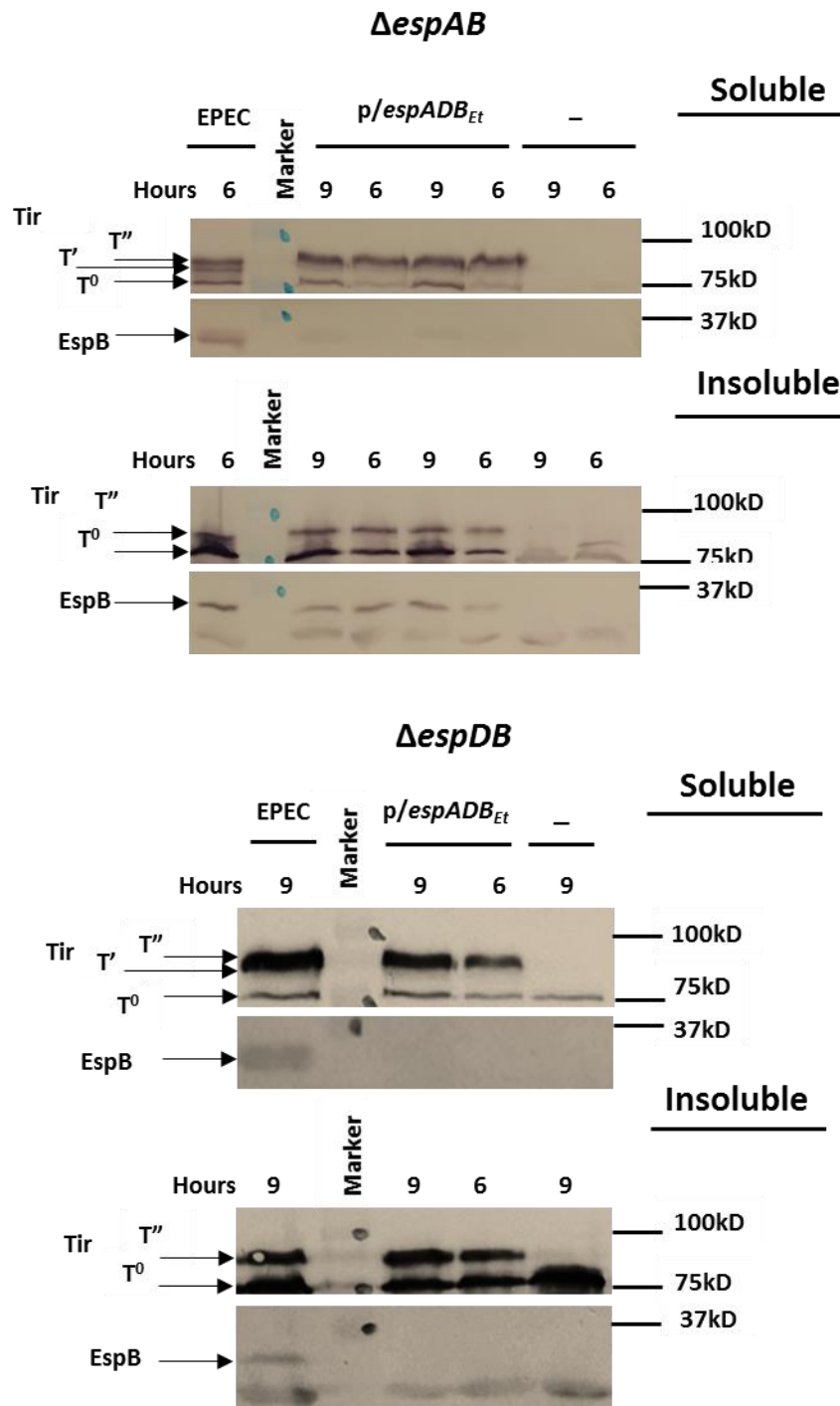


Figure 39: Rescuing the T3SS defect of EPEC *ΔespAespB* and *ΔespDespB* double mutants by plasmid introducing *pACYC-espADB_{Et}*. HeLa cells were infected 6 or/and 9h with indicated strains prior to isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gel) probing for Tir or EspB. The position of unmodified (T⁰) and host kinase modified Tir (T'/T'') forms are indicated alongside the EspB and protein markers bands. Strains used were EPEC, *ΔespAespB* (*ΔespAB*) or *ΔespDespB* (*ΔespDB*) double mutants, with double mutants carrying no plasmid (-) or a plasmid carrying the *espADB_{Et}* genes.

3.4 Discussion

In this study, we investigated the hypothesis that Tir modification to the T^{''}-like form requires the activity of LEE-encoded effector and/or translocator proteins. Firstly, we demonstrated that the LEE region encodes sufficient information for Tir T' to T^{''} modification and ruled out critical roles for all classical LEE effectors (Map, EspF, EspG, EspH, EspZ) and the Intimin protein. However, the work is suggestive of roles for several translocator proteins - EspA and EspD (not EspB which has known effector activities) - to enable Intimin interaction with Tir (T^{''} form). Furthermore, we illustrated that *Yersinia enterocolitica*, like *Yersinia pseudotuberculosis*, can deliver Tir into host cells where it only undergoes partial modification (to T'-like form) but cannot interact with Intimin. Finally, we show (for the first time) that *Yersinia* can present EPEC Intimin on its surface in a conformation that allows it to stably bind plasma membrane-inserted Tir, but not the *Yersinia* delivered Tir T' form.

Previous studies with non-pathogenic *E. coli* carrying the LEE region from either EPEC E2348/69 or B171 strains (Yen *et al.*, 2010, McDaniel & Kaper, 1997) reported LEE sufficiency to trigger actin nucleation beneath the adherent bacteria; a phenotype linked to Intimin interaction with Tir (Kenny & Finlay, 1997). Thus, this finding implies that Tir was delivered, modified, phosphorylated on tyrosine 474 and interacts with Intimin (Warawa & Kenny, 2001, Kenny, 1999). However, the Tir modification profile was not examined in these previous studies (Yen *et al.*, 2010, McDaniel & Kaper, 1997). Here we show, for the first time, that the LEE region from EPEC B171 is sufficient for Tir delivery and host modification to the T^{''} form. It should be noted that these strains carried plasmids encoding an adhesin, BFP, (Giron *et al.*, 1991) and a positive regulator of LEE gene expression, Per (Mellies *et al.*, 1999, Mellies *et al.*, 2007) from the corresponding EPEC LEE-providing strains. While it is possible that these factors may promote the Tir modification process, a recent study with non-pathogenic *E. coli* carrying a mini-LEE region - but lacking BFP and Per (Ruano-Gallego *et al.*, 2015) - triggered Tir/Intimin-mediated actin nucleation beneath the adherent bacteria. Our studies with this strain confirmed its actin nucleation capacity and demonstrated modification of delivered Tir to the T^{''} form (see Chapter 5; Section 5.3.10). It is likely that LEE from EPEC E2348/69 (0127:H6) also contains all the factors required for Tir modification to T^{''} form. The latter is supported by finding that a strain (TOE-A7) lacking most proven (14 of 17) non-LEE effectors delivered Tir which was modified to the T^{''} form and interacted with Intimin.

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Furthermore, we not only confirmed that *Yersinia* (which encodes a T3SS) can deliver Tir into the host cells - where it is only partially modified and cannot interact with Intimin (Kenny & Finlay, 1997, Warawa & Kenny, 2001) - but also show that a *Yersinia enterocolitica* strain can be used instead of *Yersinia pseudotuberculosis*. It should be noted that these strains lack genes encoding the main *Yersinia* encoded (Yop) effectors. We adapted the *Yersinia* Tir-delivery model by introducing a plasmid that not only encodes for Tir and its chaperone CesT but also Intimin in the hope that Intimin would be presented on the *Yersinia* surface in a manner that can bind to membrane-inserted Tir T'' form to aid the screening program. As far as we are aware, we have shown for the first time that *Yersinia* can express and transport Intimin to its surface where it can interact with the plasma membrane-inserted Tir T'' protein. This finding, together with the LEE sufficiency data supports the hypothesis that LEE encoded factors are required to be expressed and/or delivered with Tir to enable modification from T' to T'' form and insertion into the plasma membrane in a conformation that can be stably bound by Intimin.

The initial strategy to screen for LEE effectors aiding Tir modification by introducing plasmids encoding single or multiple LEE effectors into the Tir/CesT/Intimin-expressing *Yersinia* was abandoned as the resulting strains expressed, but no longer delivered Tir. These results are consistent with those of another PhD student (A. Al-Layla; unpublished) where introducing two different plasmids into *Yersinia* sometimes negated delivery of the EspF effector. We suggest that introduction of different combinations of plasmids and/or expressed cloned gene products stress *Yersinia* leading to activation of regulatory circuits that switch-off its T3SS. Further studies are needed to examine this possibility. Nevertheless, the work showed that *Yersinia enterocolitica* can, like *Yersinia pseudotuberculosis*, express Tir and Intimin with the former protein delivered into HeLa cells where modified to the T'-like form while Intimin is correctly presented on the *Yersinia* surface where can stably bind the Tir T'' form, but not the, *Yersinia* delivered, T' form.

An alternative strategy to define LEE factors aiding Tir modification involved using strains lacking multiple LEE genes. This illustrated that the bacterial outer membrane, Intimin and the LEE encoded classical effector proteins (EspG, Map, EspF, EspH and EspZ) play no obvious roles in the Tir expression, delivery, modification to the T'' form or insertion into the host cells membrane processes. Moreover, this strategy showed that the resulting T'' form can interact with the Intimin suggesting that Tir is completely

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functional in the absence of the other, classical, LEE effectors. These results are consistent with a recent study with non-pathogenic *E. coli* carrying a mini-LEE region, from EPEC E2348/69 (Ruano-Gallego *et al.*, 2015) that encodes a functional effector-delivery system plus Tir, CesT and Intimin proteins but lacks genes for the other classical effector proteins (EspG, Map, EspF, EspH and EspZ). Our work has confirmed that this Mini-LEE strain delivers Tir into HeLa cells and illustrates, for the first time, modification to the T^{''} form (see Chapter 5; Section 5.3.10), supporting non-essential contributions for Intimin and all classical LEE effectors in the Tir T' to T^{''} modification process. Collectively, this finding argues for a possible role for the non-classical effector protein, EspB (a translocator), other translocators (EspA; EspD) or other T3SS substrates encoded on the mini-LEE region.

Comparison of attaching and effacing pathogen LEE regions has revealed high homology between most T3SS components with more variation among proteins that interact with host cells i.e. effectors and translocators (Perna *et al.*, 1998). The mini-LEE region encodes 26 proteins (Ruano-Gallego *et al.*, 2015) of which all, but not the translocators and Tir, are in the highly homologous family of proteins. This suggests that the translocators may aid the T' to T^{''} Tir modification and/or membrane insertion processes. However, it is also possible that there are unrecognised Orf (less than scanned for 50AA minimum size) encoding T3SS substrates that mediate and/or contribute to the Tir modification processes. The later idea is not implausible as the LEE encodes small proteins such as EspZ (98AA), the EscF needle protein (73AA) and the EscF chaperone, EscE (72 AA). If such a protein exists, it would be predicted to be shared with a low percentage of homology by all A/E pathogens.

The three translocator proteins (EspB, EspD, EspA) are critical for T3SS functionality (Taylor *et al.*, 1998, Kenny *et al.*, 1996, O'Connell *et al.*, 2004) with only one, EspB, known to possess effector functions (Taylor *et al.*, 1998, Kodama *et al.*, 2002, Iizumi *et al.*, 2007). Crucially, evaluation of these proteins for effector functionality is not possible using a gene deletion strategy. However, an EspB (EspB-mid) variant lacking 60 amino acids was examined as it maintains its T3SS functions but no longer binds host myosin proteins to inhibit phagocytosis (Iizumi *et al.*, 2007). Initial studies with the provided EspB-mid expressing plasmid failed to rescue the *espB* mutant defect linked to the presence of C-terminal myc tag. This myc-tagged EspB variant was used for co-immunoprecipitation studies (Iizumi *et al.*, 2007) and presumably blocks the EspB translocator activity. A key role for the C-terminal domain in the translocation process

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is consistent with the non-functionality of EspB following introduction of a GSK tag (13-amino-acid) after residue 186 or 305 of the 320 residues of EspB protein (Luo & Donnenberg, 2011). However, once we made an untagged EspB-mid variant, our studies confirmed its ability to restore T3SS functionality to an *espB* mutant, leading to Tir delivery and modification to T^o form. However, there was a noticeable reduction in the level of Tir T^o/T^o forms suggesting that EspB-mid may be less capable for delivering effectors. The latter has implications on the conclusion that EspB plays an important, direct, role in anti-phagocytosis and effacement processes (Iizumi *et al.*, 2007) as perhaps the observed defect relates to delayed or reduced delivery of effectors.

The possible role for EspB in the Tir modification process was further investigated by examining the functional interchangeability with a divergent homologue (37.8% identity/55.7% similarity) from a fish pathogen, *E. tarda* FCP503. Crucially, this strain carries a LEE region encoding transcriptional regulators, Intimin, T3SS proteins, chaperones, translocators and Tir, but not other classical LEE effectors (Nakamura *et al.*, 2013). It was postulated that this strain, that lacks other LEE effectors, may insert Tir directly into the membrane of fish cells and thus does not need to bestow translocators or other proteins with functions to aid Tir membrane insertion from the host cytoplasm. However, despite the low level of identity, the *E. tarda* variant (EspB_{Et}) completely restored the capacity of the EPEC EspB deficient mutant to translocate Tir into HeLa cells where modified to T^o form and stably binds Intimin; latter illustrated by detecting pedestal formation (Rosenshine *et al.*, 1996, Kenny, 1999). However, the complemented strain appeared to be less capable of delivering Tir and/or promoting its modification to the T^o/T^o forms but whether this reflects issues with EspB_{Et} expression, translocation - latter perhaps due to different codon usage, instability and/or reduced recognition by EPEC T3SS - or effector functionality differences remains to be determined. Interestingly, the EspB_{Et} chaperone, CesAB, is only 35% identical to its EPEC homologue (see Chapter 4; Table 8). Interestingly, EspB_{Et} shares limited sequence similarity between residues found to be critical to EspB-EspD binding (Luo & Donnenberg, 2011) raising the possibility of reduced EspB_{Et}-EPEC EspD interactions with an impact on effector (Tir and presumably others) translocation levels. Collectively, the work ruled out a key role for the myosin binding region (60 residues) and features not conserved with EspB_{Et} in aiding Tir modification to the T^o form. However, it remains possible that EPEC and *E. tarda* EspB proteins share features that

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aid the Tir modification process but further studies are needed to investigate this. Alternatively, perhaps the Tir modification process is aided by the activity of other translocators or undefined T3SS substrates.

The possible contribution of other translocators (EspD and EspA) in the Tir modification process was investigated by examining the functional interchangeability with the *E. tarda* homologues - EspD_{Et} (50.9% identity) and EspA_{Et} (62.0% identity). Interestingly, complementing the appropriate EPEC single mutant resulted in very small amount of a modified (T^{''}-like) form. However, this Tir species did not appear to interact with Intimin questioned whether it is indeed the T^{''} form. While this defect might be due to poor expression or stability of EspD_{Et} and/or EspA_{Et}, which does not appear to be the case as introducing the EspADB_{Et} operon into the single (or available double; noting triple *espABD* mutant not available) mutants restored Tir delivery with greater level of the T^{''} form. This work demonstrated that the EspA_{Et}/EspD_{Et} homologues can functionally substitute for their homologues but only when co-expressed. The latter finding probably reflects co-divergence of *E. tarda* EspD and EspA proteins, which are known to interact (Luo & Donnenberg, 2011), reducing their ability to work with the EPEC homologues. Strain specific differences are supported by the finding that the PopB/PopD translocators of *P. aeruginosa* are inefficient substitutes for *Yersinia* YopB/YopD translocators unless co-expressed with the hydrophilic translocator PcrV protein (Frithz-Lindsten *et al.*, 1998). While our data has not found a critical role for EspA or EspD proteins in the process that modifies Tir to the T^{''} form, it is suggestive of an unexpected role in the Tir-Intimin interaction. Further studies are needed to confirm this hypothesis and, if appropriate, shed insight on the mechanism.

Chapter 4: Interrogating the functionality of *Edwardsiella tarda* (*E.tarda*) type III secretion system

4.1 Introduction

The prototypic EPEC strain E2348/69 modulates cell traits through a syringe-like apparatus composed of a type III secretion system (T3SS; Esc and Sep proteins) that spans the bacterial envelope with an extension (needle and translocator proteins). The Esc/Sep/translocator proteins are encoded on a 35 Kb-pathogenicity island called the Locus of Enterocyte Effacement (LEE) alongside genes for transcriptional regulators, T3SS substrates (translocator and effector proteins), chaperones (aid T3S substrate secretion) and the virulence-critical Intimin outer membrane protein (McDaniel *et al.*, 1995, Gaytan *et al.*, 2016). LEE encoded regulators include Ler which functions as a master positive regulator while, GrlR acts as a negative regulator of LEE gene expression and GrlA as a positive regulator (Iyoda *et al.*, 2006). The syringe-like apparatus is used to translocate 'effector' proteins into mammalian cells of which the LEE encodes seven: EPEC secreted/signalling protein F (EspF), EspG, EspH, EspZ, EspB, Translocated intimin receptor (Tir) and Mitochondrial-associated protein (Map) (Hueck, 1998, Burkinshaw & Strynadka, 2014). The T3SS/translocator system is composed of ~20 proteins that form a basal body, export apparatus (needle, filament and translocator pore) with roles for cytoplasm-located components (See Figure 40).

4.1.1 Type III secretion system components

4.1.1.1 Export Apparatus

The export apparatus consists of EscR, EscS, EscT, EscU, and EscV proteins (Figure 40) predicted to be expressed immediately after T3SS induction with each being essential for T3SS functionality (Yerushalmi *et al.*, 2014). EscU has two major domains with the N-terminal domain containing four predicted transmembrane regions while the cytoplasmic C-terminal domain undergoes auto-cleavage at a conserved amino acid sequence (Thomassin *et al.*, 2011). This self-cleavage event is required for efficient effector delivery into infected cells (Thomassin *et al.*, 2011). EscU also interacts with two components of the basal body (see below) - EscP 'ruler' protein that measures the length of the EscF needle and EscI which link inner membrane and outer membrane complexes – with this interaction involved in the switching event from early needle (EscI/EscF) to the intermediate translocators (Monjaras Feria *et al.*, 2012). EscV, like EscU, has two distinct domains with the N-terminal region possessing eight transmembrane helices and a C-terminal cytoplasmic domain that is much larger than EscU's (Gaytan *et al.*, 2016).

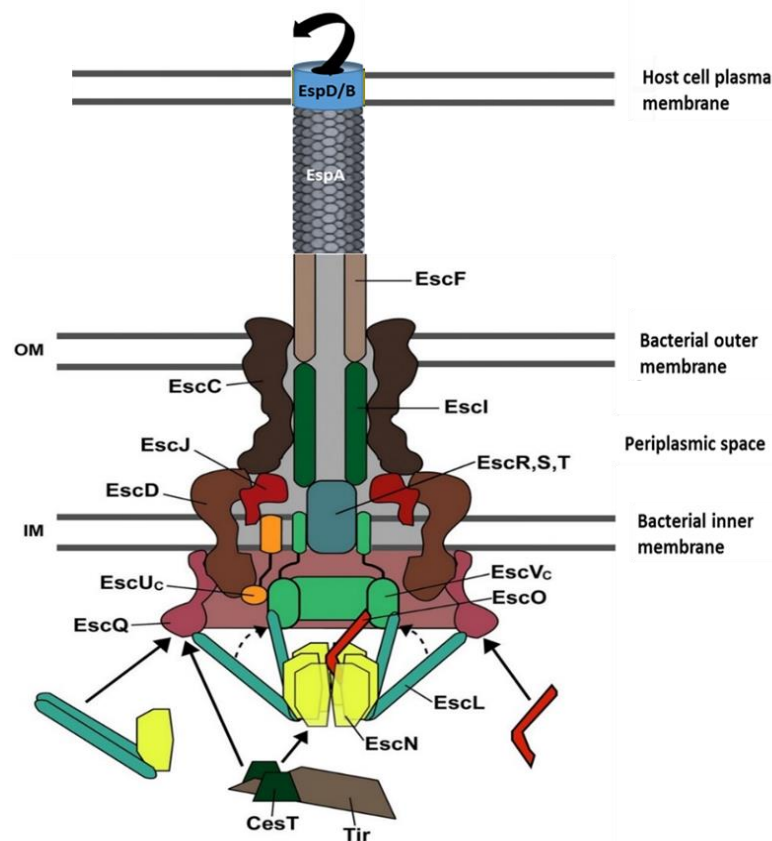


Figure 40: Schematic of EPEC injectisome. The basal body of the T3SS is composed of the inner (EscD and EscJ) and outer (EscC) ring proteins, the export apparatus (EscRSTUV) which connects the inner and outer membrane ring structures, the cytoplasmic components (EscN, EscL & EscO) and C-ring plate sorting platform (EscQ, EscK & EscL). EscF forms the needle which is extended by polymerisation of the EspA translocation protein and tipped by two other translocator (EspB and EspD) protein that can insert in the host cell plasma membrane to form a pore and complete the effector-delivery conduit. ATPase EscN provides the energy to the system, while SepD and SepL act as a molecular switch. Adapted from (Romo-Castillo *et al.*, 2014)

4.1.1.2 Basal Body

The basal body is comprised of four main proteins - EscD and EscJ form the inner membrane ring (IM) with EscC protein the outer membrane ring (OM) while EscI provides a periplasmic inner rod that connects the rings (Figure 40). EscD has a putative transmembrane domain at its N terminus (residues 120 to 141) that may associate with the inner membrane, while a segment of EscD is located in the periplasm interacts with EscC (Ogino *et al.*, 2006). By contrast, EscJ has a sec-dependent signal sequence and interacts with the needle protein, EscF (Crepin *et al.*, 2005, Ogino *et al.*, 2006). EscC has two distinct domains with the N-terminal and C-terminal regions in the periplasm and outer membrane respectively (Figure 40) (Gauthier *et al.*, 2003). In addition, EscC has been shown to interact with both the EscF

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needle protein and EscD and with one of the ATPase complex components, EscO (Creasey *et al.*, 2003a, Ogino *et al.*, 2006, Sal-Man *et al.*, 2012a). EscC, EscD, and EscJ are required for the secretion of effector proteins (Ogino *et al.*, 2006).

4.1.1.3 Cytoplasmic Components

There are five essential cytosolic proteins of which three (EscN, EscL, EscO) form an ATPase complex (Figure 40) critical for T3SS functionality (Romo-Castillo *et al.*, 2014). EscN is an ATPase, and potential source of energy, localized to the inner leaflet of the bacterial cytoplasmic membrane where it energizes the release of chaperones from effectors for subsequent secretion (Gauthier & Finlay, 2003). EscN interacts with a negative regulator, EscL (previously Orf5) (Figure 40), which can inhibit ATPase activity. The formation of an EscN/EscL/EscO complex promotes a conformational change that allows EscO to promote EscN oligomerization and stimulates EscN ATPase activity (Romo-Castillo *et al.*, 2014). EscO (previously Orf15 or EscA) is localized in the periplasm and associated with the inner membrane to interact with the outer membrane OM ring membrane protein EscC (Sal-Man *et al.*, 2012a) and also interacts with the chaperones CesA2 (EscG) and CesL (Mpc) (Lin *et al.*, 2014). The ATPase complex components EscN, EscL and EscO interact with EscQ (previously SepQ) – a cytoplasmic protein that interacts with EscL and EscK (formerly Orf4) to form a sorting platform. The latter platform acts with chaperones and molecular switch (SepL and SepD) proteins to control the correct hierarchy of substrate (early [EscI/EscF], intermediate [translocators] and late [effectors]) secretion (Lara-Tejero *et al.*, 2011, Soto *et al.*, 2017).

4.1.1.4 Extracellular Appendage

The extracellular appendage structure is comprised of the needle protein (EscF), an extension filament (EspA) and pore-forming proteins (EspD and EspB) that connects the bacterial cytoplasm to host cytoplasm (Figure 40) to enable the direct delivery of effector. In EPEC, the EscF needle length is regulated by the EscP ‘ruler’ protein which interacts directly with EscF and measures needle length during the assembly process (Journet *et al.*, 2003). The needle extension is an unusual feature of EPEC, and related pathogens, through the polymerization of EspA subunits with EspB/EspD proteins at the end for insertion to the host plasma membrane to complete the conduit from bacterial to host cytoplasm (Figure 40) (Knutton *et al.*, 1998). Each translocator protein

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is critical for delivery, not extracellular secretion, of effector proteins (Lai *et al.*, 1997, Foubister *et al.*, 1994, Kenny *et al.*, 1996).

The hydrophobic translocators, EspB and EspD, interact with both each other and with hydrophilic EspA (Luo & Donnenberg, 2011). Upon contact with the epithelial cell, EspD is inserted into the plasma membrane of the target cell to form a pore with two predicted transmembrane domains (Wachter *et al.*, 1999). EspD has two coiled coil motifs in its N-terminal to mediate EspD oligomerization, and can interact with itself through a C-terminal coiled-coil domain which is necessary to form a pore (Daniell *et al.*, 2001, Wachter *et al.*, 1999). By contrast EspB is predicted to have one transmembrane domain with an extracellular location of the EspB N-terminal domain and the C-terminal domain exposed to the host cytoplasm to interact with cellular signaling pathways that are important in EPEC-induced cytoskeletal reorganization (Luo & Donnenberg, 2011, Taylor *et al.*, 1998, Kenny & Finlay, 1995). Indeed, while the N-terminal of EspB interacts with the actin-binding protein α -catenin (Kodama *et al.*, 2002), the C-terminal has been found to bind host myosin proteins linked to subverting host cellular processes, leading to microvilli effacement and EPEC phagocytosis inhibition (Iizumi *et al.*, 2007). The pores formed by EspB and EspD are composed of six to eight subunits with a minimal pore size of 3–5 nm (Ide *et al.*, 2001). However, EspD-EspD interaction forming a pore with an inner diameter of 2.5 nm which consist of six to seven subunits (Chatterjee *et al.*, 2015). By contrast, EspB seems to play a secondary role in pore formation since *espB* mutant caused only a slight reduction in haemolysis (Shaw *et al.*, 2001) suggesting a critical role for EspD in pore formation. The T3SS must traverse both bacterial membranes and the peptidoglycan layer. This requires a dedicated peptidoglycan lytic enzyme, EtgA, to locally degrade peptidoglycan (Koraimann, 2003). However, the EtgA PG-lytic activity is enhanced by interacting with the T3SS inner rod component, Escl, (Burkinshaw *et al.*, 2015).

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4.1.2 *Edwardsiella tarda* (*E. tarda*) LEE region

Recently, a fish pathogen *Edwardsiella tarda* (*E. tarda*) was reported to possess a LEE-like region (Nakamura *et al.*, 2013). *E. tarda*, an *enterobacteriaceae* family member, is a Gram-negative intracellular pathogen of fish and mammals (Mohanty & Sahoo, 2007) which can also causes haemorrhagic septicaemia and gastro-intestinal infections in humans (John *et al.*, 2012). The ~30kDa LEE pathogenicity island apparently encodes 29 proteins (of greater than 50AA) and has homologues in the EPEC region (which encodes 41 proteins). The *E. tarda* genes also fall into five operons but the order is different (Figure 41). Interestingly, the 'missing' genes encode all the EPEC effectors, except Tir and EspB, two chaperones (CesF [substrate EspF] and one of two EscF chaperones; EscE], 2 transcriptional regulators (GrIA; GrIR) and a protein of unknown function (rORf1). Thus, the region has genes for Intimin, CesT (Tir chaperone), type three secretion system (T3SS), translocators (EspA, EspB and EspD), other chaperones (CesA, CesD, and CesD2), master transcriptional regulator (Ler) and transglycosylase (EtgA). Indeed, adjacent to the LEE region is a sequence encoding another effector and chaperone similar to those encoded by other enteric pathogens (Nakamura *et al.*, 2013). Thus, the *E. tarda* LEE region may encode an injectisome that delivers Tir, and other effectors, into host cells with Tir serving as a receptor for the Intimin surface protein to promote pathogen-host cell interaction.

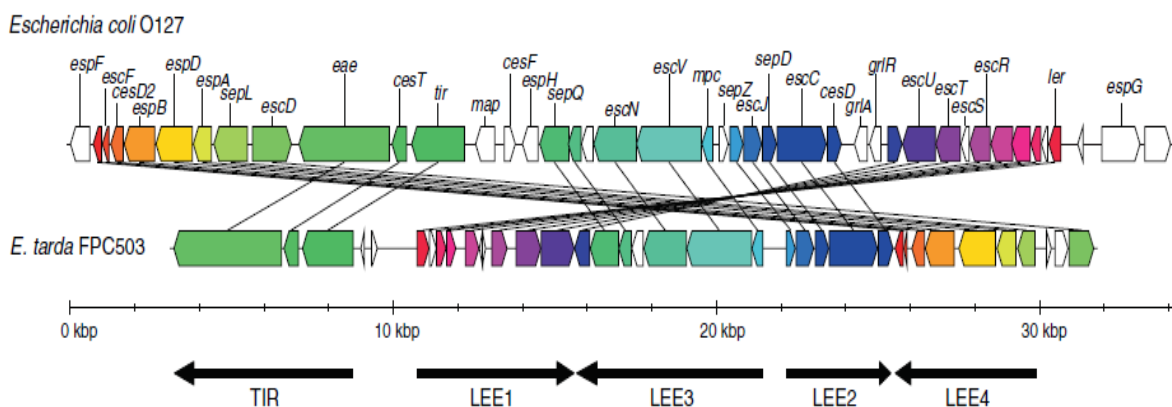


Figure 41: Pathogenicity islands of *E. tarda* genes were compared with the corresponding genes in *Escherichia coli* O127. Taken from (Nakamura *et al.*, 2013).

4.2 Aim

The aim of work presented in this chapter was to use bioinformatics approaches to interrogate the degree of homology and likely functionality of *E. tarda* LEE genes as a possible model for understanding T3SS/translocator functionality in transferring effectors into target cells and mechanism of Tir insertion into the plasma membrane.

4.3 Results

4.3.1 Bioinformatics

4.3.2 *E. tarda* LEE region has an *espZ* gene

The *E. tarda* LEE DNA sequence was kindly provided by Dr Yoji Nakamura enabling bioinformatics to define open reading frames of >50 AA (<http://nc2.neb.com/NEBcutter2/index.php>) for translation to protein sequences and comparison to database proteins (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). These analyses confirmed the absence of genes for homologues of the EPEC LEE EspG, EspH, EspF, Map, CesF, GrlA, GrlR and rOrf1 proteins (Table 8) but, surprisingly, revealed an EspZ-encoding gene. Interestingly the predicted *E. tarda* EspZ protein was larger (129 versus 98 residues) due, mainly, to N and C-terminal extensions. Interestingly, the greatest degree of homology was between the predicted transmembrane domains with least homology in the extracellular domain (Figure 42).

```

EPEC-EspZ      1 -----MEEANLS--PSGAVLPLAA--TINGNNPVDEKTG      30
                |.....:  ||.| ||:.. |....|. |  |
E. tarda-EspZ  1 MLILAALIYDLLEYSAMTTGTIGTLPNSA-LPMRPINTHESTNAV----G      45

EPEC          31 VMQSEG--GTSRSVRILGGVLIGAG---VLAAIGTGIAAMCV---DDPSQ      72
                |:.||. .|||:::| |:::  ||.|||||||... .|...
E. tarda     46 VLNSESSTNTSRTIKI---VTLASSLTLVLGAIGTGIAAKVTGDNGDADS      92

EPEC          73 RTGLGIAAGVLGGVTTVAGGLAMKYA-----          98
                .:.||:|||||.|...|||. ....|
E. tarda     93 SMALGVSAGVLGFVGLGAGVLGSIVAKKLDNNSNNS          129

```

Figure 42: Alignment of *E. tarda* and EPEC EspZ proteins. The *E. tarda* and EPEC EspZ proteins sequences were aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Highlighted in black is the extracellular domain with predicted transmembrane domains in yellow.

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	Protein	EPEC	<i>E. tarda</i>	Identity	similarity	Protein function	Reported
1	rOrf1		Missing			Unknown function	
2	EspG	398aa	Missing			Effector	
3	Ler	129aa	129aa	38.6%	54.5%	LEE encoded the master regulator	✓
4	EscE/Orf2	72aa	76aa	35.1%	51.9%	Class II chaperone for EscF	✓
5	CesAB	107aa	104aa	35.3%	54.6%	Class II chaperone for EspA & EspB	✓
6	EscK/Orf4	199aa	121aa	12.6%	21.4%	A sorting platform protein	✓
7	EscL/Orf5	231aa	215aa	27.3%	53.7%	A negative regulator that inhibits EscN ATPase activity	✓
8	EscR	217aa	218aa	73.4%	88.5%	T3SS core proteins (T3SS export apparatus) contribute to the recruitment and regulation of initial insertion of substrates into the injectisome.	✓
9	EscS	89aa	89aa	63.0%	82.6%		✓
10	EscT	258aa	259aa	56.1%	75.6%		✓
11	EscU	345aa	345aa	58.0%	78.8%		✓
12	EtgA	152aa	158aa	43.2%	62.3%	A peptidoglycan lytic enzyme	✓
13	grIR		Missing			A negative regulator of LEE gene expression	
14	grIA		Missing			A positive regulator of the <i>ler</i> expression	
15	CesD	151aa	153aa	64.3%	79.9%	Class II chaperone for EspD & EspB	✓
16	EscC	512aa	503aa	64.6%	80.3%	T3SS outer membrane ring (IM)	✓
17	SepD	151aa	143aa	39.6%	63.6%	Molecular Switch from translocators to effectors	✓
18	EscJ	190aa	179aa	53.1%	72.9%	T3SS inner membrane ring (IM)	✓
19	EscI/Orf8	142aa	130aa	46.9%	63.3%	The T3SS inner rod that connect OM and IM rings	✓
20	EspZ/SepZ	98aa	129aa	29.9%	38.0%	Effector	
21	CesL/Mpc	117aa	107aa	30.1%	42.3%	A class I chaperone for SepL	✓
22	EscV	675aa	674aa	66.3%	79.9%	T3SS export apparatus	✓
23	EscN	466aa	458aa	62.1%	76.4%	ATPase complex T3SS component	✓
24	EscA/EscO/Orf15	125aa	122aa	25%	54%	AATPase complex component that stimulates EscN ATPase activity	✓
25	EscP/Orf16	138aa	103aa	21%	39%	Regulate EscI secretion/ Molecular ruler/ Molecular Switch (by interacting with EscU)	✓
26	SepQ/EscQ	305aa	297aa	21%	44.1%	C-ring of T3SS that act as sorting platform	✓
27	EspH	168aa	Missing			Effector	
28	CesF	120aa	Missing			Class IA chaperone that binds EspF	
29	Map	203aa	Missing			Effector	
30	Tir	550aa	529aa	24%	36.3%	Translocate intimin receptor	✓
31	CesT	156aa	156aa	60.3%	80.1%	Class IB chaperone for Tir effector and many LEE/non-LEE effectors	✓
32	Intimin/eae	939aa	1117aa	39.6%	51.6%	Extracellular bacterial protein	✓
33	EscD*	406aa	416aa	43.7%	61.0%	The T3SS inner membrane ring (IM)	✓
34	SepL*	351aa	343aa	49.3%	74.4%	Molecular Switch (gatekeeper)	✓
35	EspA	192aa	199aa	62.0%	74.5%	Translocators	✓
36	EspD	380aa	385aa	50.9%	66.9%		✓
37	EspB	321aa	340aa	37.8%	55.7%		✓
38	CesD2	135aa	136aa	53.7%	66.9%	Class II chaperone for EspD	✓
39	EscF	73aa	74aa	67.6%	87.8%	T3SS component (needle protein)	✓
40	EscG/Orf29/CesA2	92aa	89aa	36.8%	56.8%	Class III chaperone of the needle protein EscF and the filament protein EspA	✓
41	EspF	206aa	Missing			Effector	

Table 8: Comparison of reported virulence proteins encoded among *E. tarda* and EPEC LEE region. *E. tarda* and EPEC protein sequences were aligned using the EMBOSS Needle Program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Rows highlighted in green correspond to the missing effectors. While the blue color corresponds to the reported missing protein, red color refers to unreported effectors. EscD* is truncated into two proteins EscD1 (157aa) and EscD2 (259aa). SepL* is truncated into two proteins SepL1 (125aa) and SepL2 (218aa) where the latter beings with the rare codon ATT instead of ATG.

4.3.3 Proteins encoded over two adjacent open reading frames

Comparing the size of predicted *E. tarda* LEE proteins to their EPEC homologues found most to share a similar number of residues with a few notable exceptions (Table 8) i.e. EscL (146 versus 231 residues), EscD (259 versus 406 residues) and SepL (218 versus 351 residues). Further interrogation of the *escD* gene region revealed an *escD*-like sequence (*escD2*) on an adjacent open reading frame, Orf, which if placed in frame with the first Orf (*escD1*) would produce a 416 residue protein sharing good homology, over the entire length, with the EPEC homologue, except for a short N-terminal extension (Figure 43). Indeed, the 'fused' EscD protein shares a similar output with its EPEC homologue when probing for transmembrane domains (Figure 44).

As it is was possible that the two Orfs relate to sequencing errors, oligonucleotides were designed to PCR amplify the out-of-frame region which, surprisingly, failed to define sequencing errors (Figure 45). This finding suggests that EscD is either non-functional or, as reported for some T3SS components (Penno *et al.*, 2006), expressed from two adjacent open reading frames. The latter is associated with regulating protein expression or function with a single polypeptide generated by ribosome slippage or jumping (Atkins *et al.*, 2016, Penno *et al.*, 2006). However, it is also possible that EscD is expressed as two separate polypeptides that can function together and/or individually.

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<i>E. tarda</i> -EscD	1	MLTVLSSYFICLLAAIINMRSCDYKIRFLTWPCLSGRELIVPICESFSIG	50
	 : : . : :	
EPEC-EscD	1	-----MLS-SYKIKLLN-GAMRNRELQLPM-GNLTLG	29
<i>E. tarda</i>	51	KNDDSLITYQLENPCLQNFTLSTNDICDGVWLNTPPTTCFWINGIQTSNCAD	100
		. . : : . : . : : : . : . . : 	
EPEC	30	TEDNDIVYFPLEQG-LNQFLLDIRE-EGVFLSPVE-FWIDGQPTPYEAD	76
<i>E. tarda</i>	101	SILLPLNQPIDIAGCGFICLLSADQETSLSVSLPKRKNACHIDKKNIVV	150
		. . : : : : : : : :	
EPEC	77	K-PLPVGKIIDIAGC---CFIIGDIDHSLPLSDVPERFSAKNQRKKRLIL	122
<i>E. tarda</i>	151	ICASLLFLTLSLS-IFAFFIMIS-----CLISREDVYQSLCKKEKLFIAIV	194
		: : . . . : : : : . . . : . . : . .	
EPEC	123	--ASVIGATFALSGAIGSYVLLSPKAEPPPTFTRADVYQQ-LKENKLHAIT	169
<i>E. tarda</i>	195	CPVWHGSSIALCYGRCEQSSQLQTFNFYLYKNCNIRYINNIICCNDQIIS	244
		. . : . : : . : . : . . .	
EPEC	170	L-VWHGKNIAL-YGRCESTDLTPFFNYL-KEKNIFYNKII-CNNQIIS	215
<i>E. tarda</i>	245	GINDCVLVQYGYDNICDVSYGGSPGFFILSGYIQSPCLQWKKVEGQLCLT	294
		. . : . : . : : . : . . .	
EPEC	216	AIND-VLTEYGYKDII-ITKGNKPGFFLLSGYI-PPSPKWSEVE-NLLLN	261
<i>E. tarda</i>	295	MPGVRGWQCVRNKADAIINCSLVDLSKNKLINKLSIHKRCDAKAIIDGL	344
		. . : . . . : : : . : . : .	
EPEC	262	TPGVAGWE-IHNNSNNKIN-ELASEFKKNKLINYVNIFFKND-VIIVAGE	308
<i>E. tarda</i>	345	VCSAVEEQKIINCIIDRLNNN---KICIFQNIIPPYIPKNLFSGKIIIRCVS	391
	 : . : : . . : : . : :	
EPEC	309	V-SQQNESKIL-AIINAMNKNSNVKI-LFQNIQPYISADIFPGKILR-IS	354
<i>E. tarda</i>	392	GTKNAPMICTLDNGSTLTVCGSALNNGYIICNEISLENGISISRENELIH	441
	 : : . : . . : : : . : .	
EPEC	355	GTMKNPTI-ALDNGTSLGI-GSILKGGYVI-DAIDPKDGINISRPDEYIH	401
<i>E. tarda</i>	442	ICPISYDK 449	
		:	
EPEC	402	I-PLSY-- 406	

Figure 43: Alignment of *E. tarda* and EPEC EscD proteins. The *E. tarda* and EPEC EscD proteins were aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). The combined *E. tarda* EscD1 (1 -259AA) and EscD2 (260-449AA) sequence were combined into a single protein.

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Output from TMpred

2 possible models considered, only significant TM-segments used

-----> STRONGLY preferred model: N terminus inside

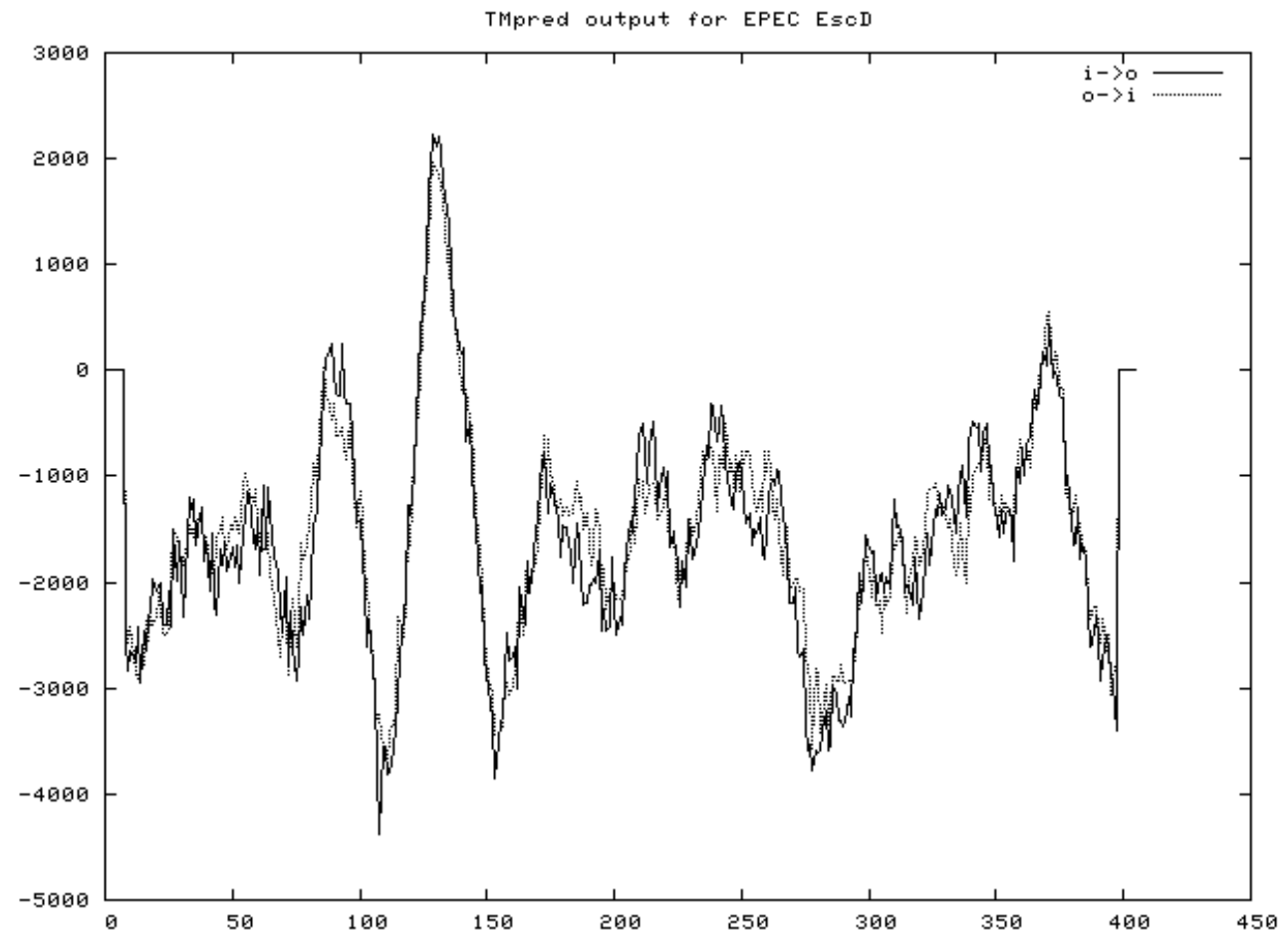
2 strong transmembrane helices, total score: 2798

#	from	to	length	score	orientation
1-	120	142	(23)	2220	i-o
2-	360	382	(23)	578	o-i

-----> Alternative model

1 strong transmembrane helices, total score: 1975

#	from	to	length	score	orientation
1-	120	141	(22)	1975	o-i



Chapter 4 Results II

Output from TMpred

2 possible models considered, only significant TM-segments used

-----> STRONGLY preferred model: N-terminus outside

6 strong transmembrane helices, total score: 7404

#	from	to	length	score	orientation
1-	1	19	(19)	1873	o-i
2-	105	124	(20)	563	i-o
3-	148	171	(24)	2623	o-i
4-	190	208	(19)	932	i-o
5-	262	279	(18)	897	o-i
6-	395	422	(28)	516	i-o

-----> Alternative model

5 strong transmembrane helices, total score: 6871

#	from	to	length	score	orientation
1-	1	17	(17)	1903	i-o
2-	148	171	(24)	2623	o-i
3-	190	208	(19)	932	i-o
4-	262	279	(18)	897	o-i
5-	395	422	(28)	516	i-o

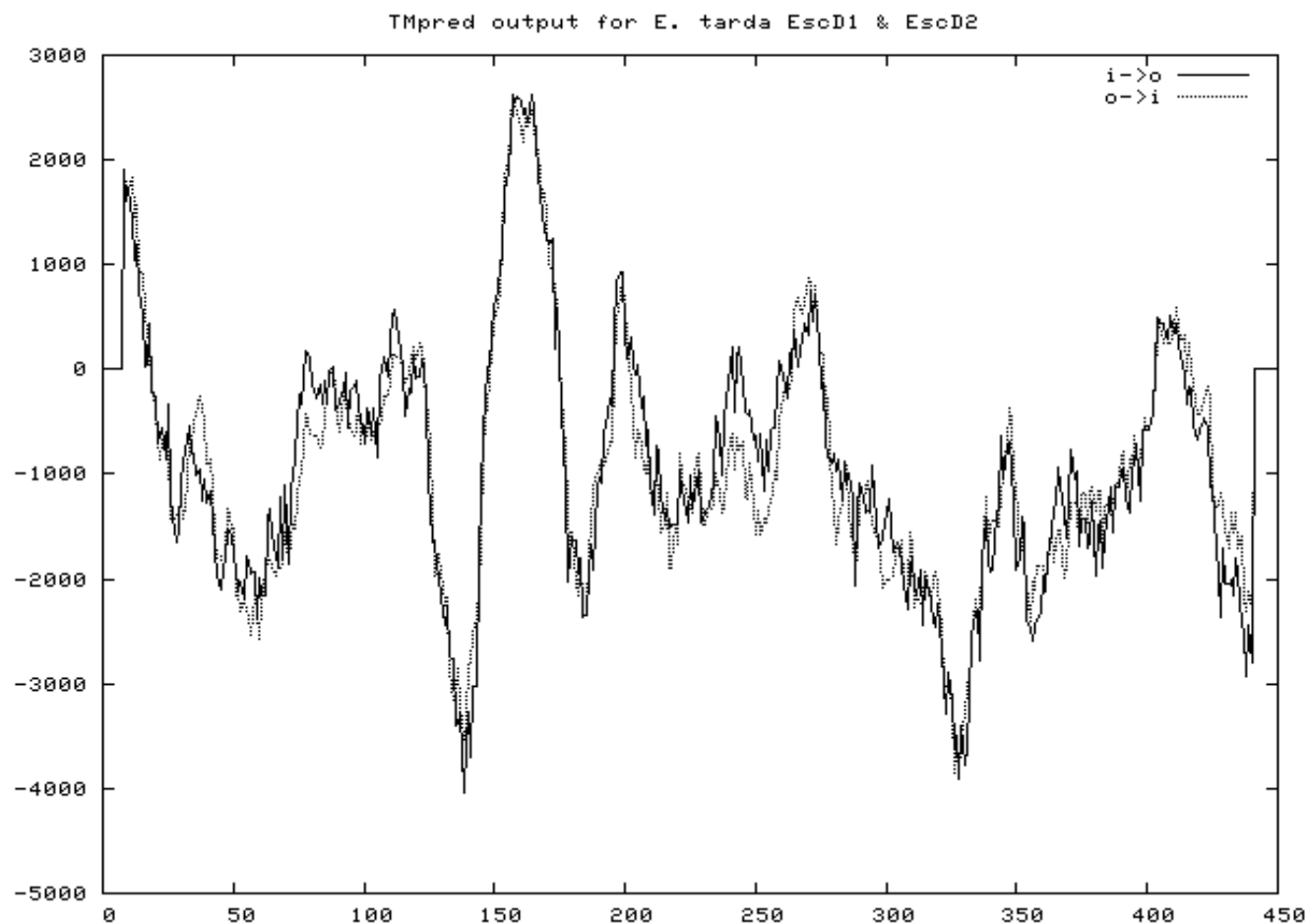


Figure 44: The *E. tarda* and EPEC EscD proteins are predicted to possess one, similarly-located, transmembrane domain. The EPEC EscD and *E. tarda* combined EscD1 (AA 1-259)/EscD2 (AA 260-449) sequence were used to predict transmembrane domains (http://www.ch.embnet.org/software/TMPRED_form.html) revealing one in each protein (residues ~120 to 141 in EPEC EscD; ~140 to 160 in combined *E. tarda* EscD protein). The program requires setting of minimal and maximal hydrophobic helix length, in this case it was 17 and 33 respectively. Solid line is the prediction for inside to outside TM helix and interrupted line is the prediction for outside to inside TM helix.

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ATGCTAACCGTACTATCAAGCTACTTTATACTATTAGCAGCAATCATCAACATGCGATCTGATTATAAAAAAAGATTTCT
 GACTTGGCCACTGAGTGGTAGAGAGTTGATTGTACCTATAGAGTCATTTTCTATTGGTAAAAACGATGATAGTCTCATCA
 CATACCAACTTGAAAATCCATTGCAAAATTTCACTCTTAGCACTAATGATGATGGTGTGGCTAAATACCCCACTACA
 TTTTGGATTAATGGTATTAGACGCTCAACGCTGATTCAATTTTGCTTCCACTCAATCAACCAATTGATATTGCCGGCTG
 CGGATTTATTCTACTTAGCGCAGATCAAGAAACGTCGCTAGTATCATTACCCAAACGAAAAAAAAACGCACATATAGATA
 AGAAAAACATTGTAGTAATCGCATCATTGCTATTTTTAACACTTTCTTTATCGATTTTTTGCTTTTTTTTATATAAAAAAC
AAGATTCTATGATTAGTCTCATCAGCAGAGAAGACGTTTATCAATCACTAAAAAAGAAAAGCTTTTTGCTATTGTACCC
 GTCTGGCATGGGAGTAGCATTGCTTTATACGGTCGATGCGAGCAATCATCTCAATTGCAGACTTTCTTTAATTTTCTTTA
 TAAAAAATAACATAAGATATATAAATAATATTATTTGTAATGACCAAATAATCAGTGGAAATTAATGATGTTTTAGTTCAGT
 ATGGCTATGACAATATAGATGTATCTTATGGTGGTAGCCCCGATTCTTCATCCTATCGGGTTACATTCAGTCCCGCTA
 CAGTGGAAAAAGTAGAAGGCCAGCTCTTAACTATGCCAGGCGTCAGAGGATGGCAAGTACGAAACAAAGCCGACGCCAT
 CATCAATTCATTAGTTGATGAACATCGAAAAACAAATTAATCAACAAGTTAAGCATTATAAGCGTGATAAAGCAATAA
 TCATTGACGGCTTAGTTTCAGCAGTAGAAGAGCAAAAAATAATAAATATTATCGACAGGCTAAATAACAATAAAATAATT
 TTTCAAAATATACCACCCTATATTTCCAAAAACCTATTTTCAGGAAAGATCATCAGAGTGAGTGGAAACAAAAAATGCGCC
 AATGATTACATTAGATAATGGGAGCACTCTCACTGTCCGAGCGCCCTAAACAATGGCTACATAATCAACGAAATCAGCC
 TAGAAAAATGGTATTAGTATCTCGAGGGAAAATGAATTAATCCATATCCCAATCTCTTATGATAAGTAA

E. tarda-escD GCATCATTGCTATTTTTAACACTTTCTTTATCGATTTTTTGCTTTTTTTTATATAAAAAAC
 Sequencing GCATCATTGCTATTTTTAACACTTTCTTTATCGATTTTTTGCTTTTTTTTATAAAAAAAC

E. tarda-escD AAGATTCTATGATTAGTCTCATCAGCAGAGAAGACGTTTATCAATCACTAAAAAAGAAA
 Sequencing AAGATTCTATGATTAGTCTCATCAGCAGAGAAGACGTTTATCAATCACTAAAAAAGAAA

Figure 45: The *E. tarda* EscD protein is encoded on two adjacent open reading frames. The *E. tarda escD* gene region sequence is given with start (ATG) and stop (TAA) codons indicated by red underlined text. *escD1* spans from the first ATG to TAA codon with *escD2* from second ATG to TAA with intervening 14 bases highlighted by blue lettering. Sequencing data from a PCR-amplified fragment confirmed the presence of stop, intervening and start codons between *escD1* and *escD2*.

Closer examination of the *sepL* gene region revealed a premature stop codon with insertion of a single nucleotide restoring a single Orf for a protein similar in size and sequence over its complete length to its EPEC homologue (Figure 46). Indeed, a similar finding was evident for *escL* i.e. addition of extra base restored a single Orf encoding a protein of similar size to the EPEC counterpart with good homology over the whole protein (Figure 47).

Chapter 4 Results II

ATGGCTTCTGGTATTGATTTTAATGTATCTGTAATTCAGTGTTTAACGATATAAATGATTTTTTCGAATAAACAAAATAA
 TAATTTATCGTTAAATACACCATTAGCTAATATGCAAAATGAGTTAGCAATAATCAATGCAGCCTCTATGGCTGAAACGG
 CTGAGGGGATCAGCTTAGGGTTCCTGCGACCTACGGGACGTAGGGTGGAGGAAGATGGGGGTAATGATAATATAATCAAT
 AAAATGCAAGATATCATTAACCTAGCTGGTGGAGGATGCGTTGCAGGATATTGCGTTGCACACCCTATTACAGCTGGATCA
 GCATGACCCCCAGTTGGCGATGTTTGGCGCAATGCCGAAAGCGAGATTATTGCCATTAATATCGTCCCTACTGCATTTG
 AATACGATTACCTTAGCGCAAAAAAAAAAGTACGCCAAATTGCTCTTGGAACTACTCGGTGAAGAGGAATGGGAGCTGGCT
 TTGCTGGCCTGGTTGGGGGTCGGTGGGATAACGCATGAAAACTAAAGAAAATAAAAAACCTCTATCAGAAGGCAAAAGA
 TCAGGAGGATTATGAAGGAAGTACACTGCTTACATGGTCTTAGAGATAAAGGATTTGCCTGATAGAGATAACTACCTTA
 AAGTAATAATTTCGTGCATTGTCATTTGAGCTATCTTATTTACCACAGGTGAGGATAGAGAAAGAACGTCATCTGTCATT
 ACTGATCTGTATAGAATTATAGTTTTTTTTATCATTAATAATATATAGCGAAATAGTTTCTTTATCCTTAAAAAAGGATGC
 TGATATTATTCTTAGTGAGCTGATTAGCACGTTGGAACAACTTGGTTGACCGAAGAGTGGTTTGGGGGAGTCCAAGCC
 GGGTTGGTGTATTGATGGACAGAACTATATTATTATCATTTAATAAAAGATTTTTATCAACGTTGCCCCACTCTTGT
 TTTATGACTGAAGAACAGCGAGAAAGTATTATTAATGGTATTTTCAGATGTTATAGATAGAGACTCGGAGTAA

<i>E. tarda</i> -SepL	1	MASGIDFNVSVIPVFNDIN-DFS NK-----QNNNLSLNTPLANMQNELAI	44
EPEC-SepL	1	MANGIEFNQNPASVFNNSLDFELESQQLTQKNSNTSSPLINLQNELAM	50
<i>E. tarda</i>	45	INAASMAETAEGISLGRFRPTGRRVEEDGGNDNI INKMQDI INLAGEDAL	94
EPEC	51	ITSSSLSETIEGLSLGYRKG SARKEEEGTTIEKLLNEMQELLLTDSDKI	100
<i>E. tarda</i>	95	QDIALHTAIQLDQHDPQLAMFGAMPKGEIIAINIVPTAFEYDYLSAKKKY	144
EPEC	101	KELSLKNSGLLEQHDPTLAMFGNMPKGEIVALISSLLQSKFVKIELKKKY	150
<i>E. tarda</i>	145	AKLLELLGEEEWELALLAWLGVGGITHEKLLKIKNLYQKAKDQEDYEGS	194
EPEC	151	AKLLLDLLGEDDWELALLSWLGVGELNQEGIQKIKKLYEKAKDESENKA	200
<i>E. tarda</i>	195	TLLTWFL EIKDLPDRDNYLKVII RALS FELSYLPQVEDRERTSSVITDLY	244
EPEC	201	SLLDWFMEIKDLPEREKHLKVIIRALSFDLSYMSSFEDKVRTSSIIISDLC	250
<i>E. tarda</i>	245	RIIVFLSLN NYSEIVSLSLKKDADIILSELISTLEQTWLTEEFAGSPSR	294
EPEC	251	RIIIFLSLNNYTDIIAISIKKDKD VILNEMLSII EHVWLTEDWLLES PSR	300
<i>E. tarda</i>	295	VGVIDGQKLYYYHLIKDFYQTLPHSCFMTEEQRESIINGISDVIDRDSE-	343
EPEC	301	VSIVEDKHVYYYFHLLKEFFASLPDACFIDNEQRSNTLLMIGKVIDYKEDV	350
<i>E. tarda</i>	344	-	343
EPEC	351	M	351

Figure 46: The *E. tarda* SepL protein is interrupted by a stop codon. The *E. tarda* *sepL* gene region sequence is given with start (ATG and, rare, ATT) and stop (TAA) codons indicated by red underlined text. A rare start codon (ATT) is part of the premature TAA stop (T in red; AA in black). The two SepL-related open reading frames were spliced together for alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) against the EPEC homologue.

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ATGAAAACAACACGCTACAGAAAAATAAAATTGAGTAATTTTAAATGAAAAAACAACCTTAAGCGCCAGTGAGATCAGT
 ATATTAAATATTGAAAAATAACAACATTAAGAATCTAGAGAAACAGCATGCAATATATTATTATCAGCAAGAAAAAAG
 AAACATCAGATAAAAAGCAAACATTCGCAGAAGACTCGCAATTAGACTATGGAGATATCGTAACAATATTAACACGCAT
 TATCAAATGAGATTTAATGAAAAATAAACCAATCCATTCAACTACTACTCGATACTCAGAATGCTGAGCGTTATCTT
 TTTCTAAAAGCCATAGAGCAGGCGCAGTTATCATTGAGCCGCGCACTCCCTGAACTTTTGGCTAAACGCATCGACTGG
 CCACAAATTCTATCAGAAATAATTAGTGACAAAATTAATAAAAAAaCAAGTTAAATGGAGATATAGAAATAACAAAGAAC
 GAAAATCTCATAATTAATACAGATAGTATTAATGCAACGATTACTAATGATAGCAACATGGACATCGATACCATAGTA
 ATGGAAAATCAATATATAAGAATAACACTATCGCCAAAAAACAAATAGAAAACGCGTTAGCAAACCTCAAACATAAAA
 TATAACACTTTATTAGACGAATAA

EPEC EscL	1	MIYFLTDLIKFYCLMKLYEFKNIEIDLVLTEDIIPEDKLQEIIQSDDIIK	50
		: : : : : : : : : : : : : : : : : : :	
<i>E. tarda</i> EscL	1	-----MKTTRYRKIKLSNFNEKKHLSASEISILNIENNIK	36
EPEC	51	LARKKTYEHLRARRKSKELEKIESRKKIARKMIAMRERIRKNNKIKLDKE	100
		: : : : : : : : : : : : : : : : : : : : : : :	
<i>E. tarda</i>	37	ESRETACNILLSARKKKHQIKANIRRLAIRLWRYRNNINTHYQMRFNK	86
EPEC	101	VNQS IKWVKDIQAI ELVLMQDIMNKVHLSLTNALHSLDTSSRINWDDL N	150
		: : : : : : : . : . . . : . : : : : :	
<i>E. tarda</i>	87	INQSIQLLLDTQNAERYLFLKAI EQAQLSLSRALPEL-LAKRIDWPQILS	135
EPEC	151	EVVRETLSNNNIVGAIKITKNPDIKLDPGEANNIQLINDANTPHNKIIIE	200
		: : : : : . : . : : : : : : : . : . . : . : : : : : : :	
<i>E. tarda</i>	136	EIISDKIKKNKLNLDIEITKNENLIINT-DSINATITNDSNMDIDTIVME	184
EPEC	201	NEYIRITLDPLEQISILLNSFKDNYLSIIQE	231
		: . . : . . : . . : : : .	
<i>E. tarda</i>	185	NQYIRITLSPKKQIENALANFKLYNTLLDE	215

Figure 47: The *E. tarda* EscL protein is interrupted by a stop codon. The *E. tarda* *escL* gene region sequence is given highlighting start (ATG) and stop (TAA) codons with red underlined text. Indicated is position of added base (underlined) upstream of the premature TAA stop to restore an Orf for a full length EscL protein. The protein sequence of the full length EscL and EPEC homologues were aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

4.3.4 The *E. tarda* *escD* gene can functionally substitute for EPEC *escD*

EscD is required for T3SS apparatus functionality and substrate secretion (Ogino *et al.*, 2006). To investigate whether the *escD_{Et}* region encodes a functional protein studies interrogated its ability to functionally replace the EPEC *escD* gene. Thus, the *escD_{Et}* gene region was PCR amplified, from provided *E. tarda* genome DNA (kindly by Dr Yoji Nakamura), and ligated into the AT cloning vector generating pCR-2.1-*escD_{Et}*. The cloned fragment was excised, by digesting BamHI and Sall restriction enzymes (cut at oligonucleotide-introduced restriction enzyme recognition sites flanking *escD*), and ligated in pACYC184 (digested with same restriction enzymes) to generate pACYC-*escD_{Et}* (Figure 48). The presence of the *escD_{Et}* insert was supported by restriction enzyme and PCR analyses (Figure 48). An EPEC *escD* mutant was generated, using kindly provided suicide vector (see Chapter 2; Table 6) before introducing, via electroporation, the pACYC-*escD_{Et}* plasmid (Figure 48).

To examine if *escD_{Et}* could functionally replace EPEC *escD*, HeLa cells were infected for 3 and 6h with the *escD* mutant or two clones of the *escD* mutant complemented with pACYC-*escD_{Et}* plasmid. Following the infection period, the Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions were isolated for Western blot analysis.

Interestingly, the Δ *escD* complemented strain, unlike the EscD deficient mutant (supporting strain genotype), delivered Tir as evidenced by shifts in apparent molecular mass (Figure 49); latter due to host kinase modification. This work suggests that the *E. tarda* *escD* region encodes a functional EscD protein. Importantly, using unique restriction sites in the *E. tarda* DNA to delete most of the *escD1* sequence produced a plasmid that failed to rescue the EPEC Δ *escD* mutant defect (not shown).

A similar approach was undertaken, under my supervision, by undergraduate students (Joe Frost and Shaz Malook), to generate pACYC-*sepL_{Et}* and pACYC-*escL_{Et}* plasmids which both failed to complement the T3SS defect of provided Δ *sepL* (Monjaras Feria *et al.*, 2012) and Δ *escL* mutants (Soto *et al.*, 2017), respectively, unlike plasmids carrying EPEC *sepL* and *escL* genes (not shown).

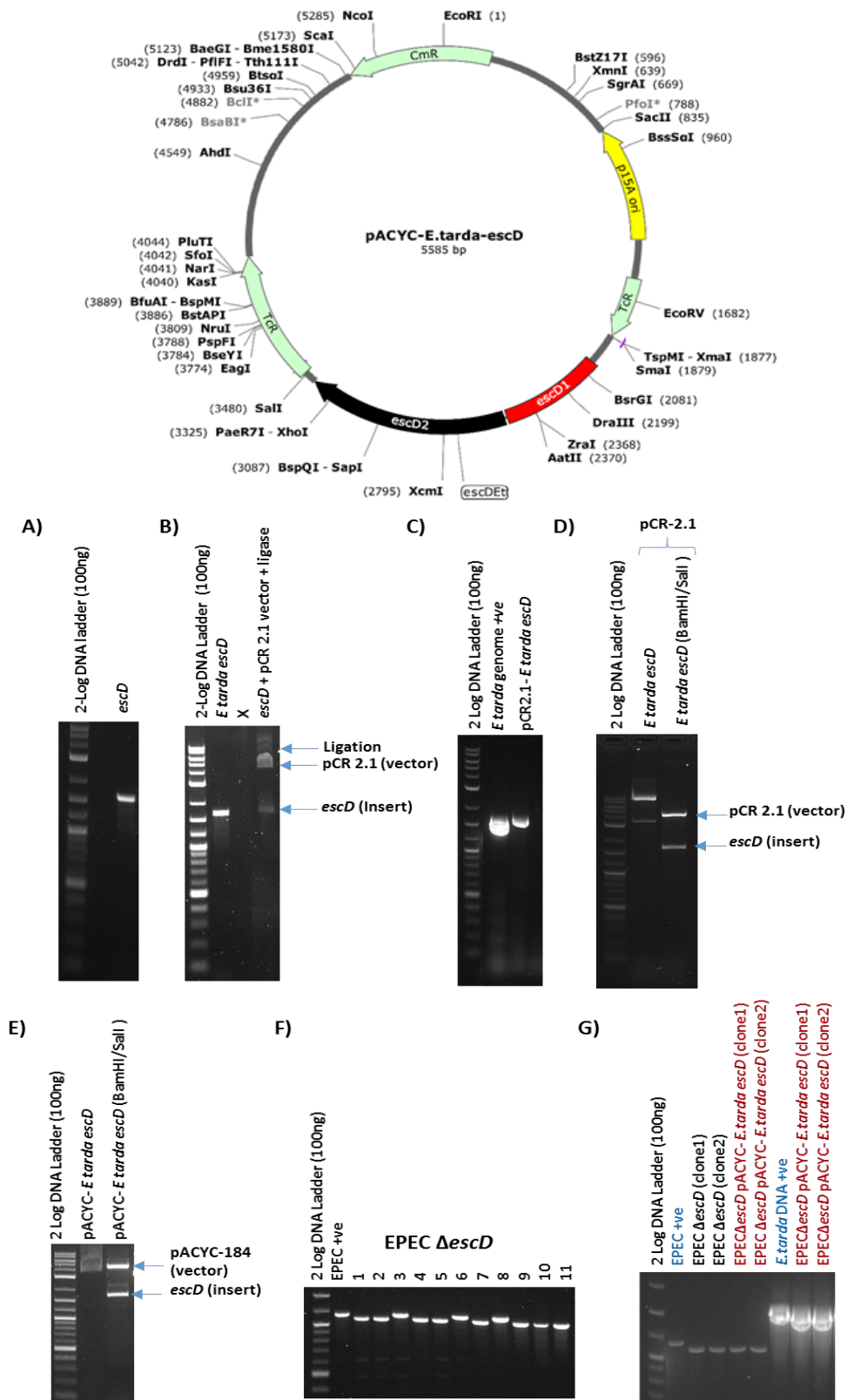


Figure 48: Cloning *E. tarda escD* region into pACYC184 and generating EPEC *escD* mutant. Schematic of pACYC184 plasmid carrying *E. tarda escD* gene region with (A-G) agarose gel data illustrating steps in generating and confirming of pACYC-*escD*_{Et} and EPEC *escD* mutant. **A)** PCR amplification of a single band of expected size for *escD1/escD2* gene, **B)** ligation of PCR fragment into AT vector (pCR-2.1), **C)** PCR support for generating pCR2.1-*escD*_{Et}, **D)** restriction enzyme release of *escD*_{Et} fragment, **E)** restriction enzyme digestion support for *escD*_{Et} gene insertion into pACYC184, **F)** PCR screening supports generation of EPEC *escD* mutant [clones 1, 2, 4, 5, 7, 9-11] and **G)** PCR support for introduction of pACYC-*escD*_{Et} into EPEC *escD* mutant.

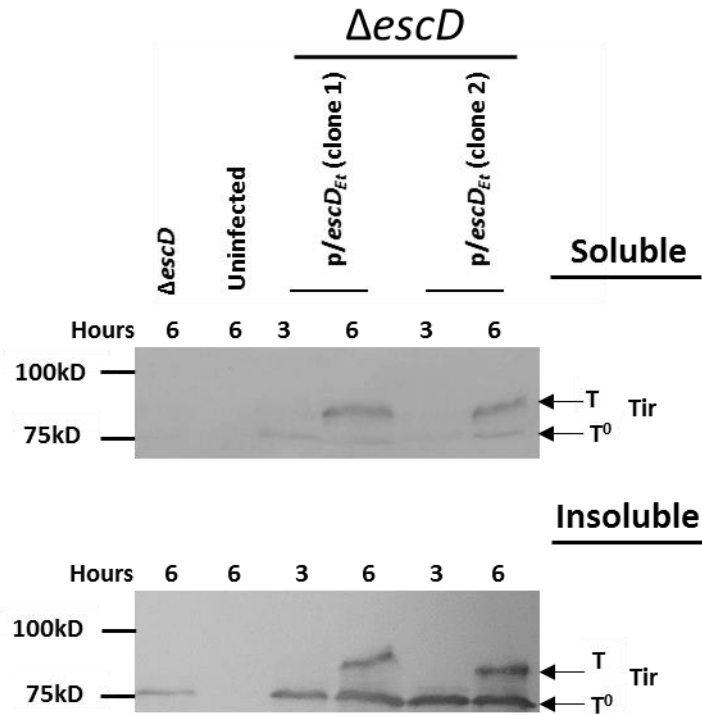


Figure 49: Rescuing the EPEC *escD* mutant T3SS defect by introducing *pACYC-escD_{Et}*. HeLa cells were left uninfected or infected 3 and/or 6h with indicated strains before isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble fractions (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PAGE) probing for Tir. The position of Tir unmodified (T^0) and host kinase fully modified (T) forms, as well as protein markers, are indicated. Strains used were *EscD* deficient EPEC ($\Delta escD$) or $\Delta escD$ complemented with *pACYC-escD_{Et}* (two clones).

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4.3.5 The *E. tarda* *eae* gene encodes for a larger Intimin protein

While bioinformatics revealed the *E. tarda* Intimin-encoding gene (*eae_{Et}*) to encode a protein with a similar number of residues to the EPEC variant (929 versus 937), blast searches revealed an Intimin-like C-terminal domain on an adjacent Orf (not shown). To examine the possibility of a frame-shifting sequencing error, the region of interest was PCR amplified with the sequencing data revealing an additional base (G; Figure 50). Adding this base to the *E. tarda* LEE *eae* sequence restored a single Orf of 1117 residues sharing 39.6% identity (51.6% similarity) to the EPEC homologue (Figure 51). While the EPEC and EHEC homologues are similar sized and highly homology, the *E. tarda* variant has additional sequences throughout its C-terminal domain helping to explain why it shares less homology to the EPEC/EHEC homologues. The cell and Tir binding activity of Intimin maps to the C-terminal 280 amino acids (Frankel *et al.*, 1994) with *E. tarda* Intimin sharing 35.7% identity (51.2% similarity) in this region. Four C-terminal residues (S890, T909, N916 and N927) play critical roles for Intimin binding of Tir (Yi *et al.*, 2010) with Cys937 critical for cell binding (Adu-Bobie *et al.*, 1998). Interestingly, these residues are relatively well conserved between the homologues (Figure 51), suggesting that *E. tarda* Intimin may be able to bind EPEC Tir.

```
E. tarda          83 TGTGACTACGGAGGCTGATGGTACCGCCCGGGTCATTCTGACCAGCGTGA      132
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced        59 TGTGACTACGGAGGCTGATGGTACCGCCCGGGTCATTCTGACCAGCGTGA      108

E. tarda          133 CTGCAGGTCAGGCGACCGTGTCCGCCACTACGGCGGGGGGACGCCAGCG      181
   |||||||||||||||||||||||||||||||||||||||||||||
Sequenced        109 CTGCAGGTCAGGCGACCGTGTCCGCCACTACGGCGGGGGGACGCCAGCG      158

E. tarda          182 ACGAAGACGGTGACATTTAACGCGGTTCTGGAGATCACCGGCGTATTGAT      231
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sequenced        159 ACGAAGACGGTGACATTTAACGCGGTTCTGGAGATCACCGGCGTATTGAT      208
```

Figure 50: Sequence error in *E. tarda* LEE *eae* gene. The nucleotide sequence of the PCR amplified fragment aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) to the corresponding region of the provided *E. tarda* *eae* gene sequence revealed a guanine (G) base is missing in the provided DNA sequence.

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4.3.5.1 *E. tarda* Intimin can bind EPEC Tir triggering its actin nucleation activity

To interrogate functional interchangeability, the *ea_{E_t}* gene was PCR amplified, using *E. tarda* genomic DNA as a template, and ligated into pACYC184 (pre-digested with BamHI/Sall restriction enzymes) using the Gibson Assembly protocol (see Chapter 2; section 2.2.8) generating pACYC-*ea_{E_t}* (Figure 52). PCR analysis was used to support pACYC-*ea_{E_t}* construction and introduction in the Intimin-deficient (Δea_e) mutant (Figure 52).

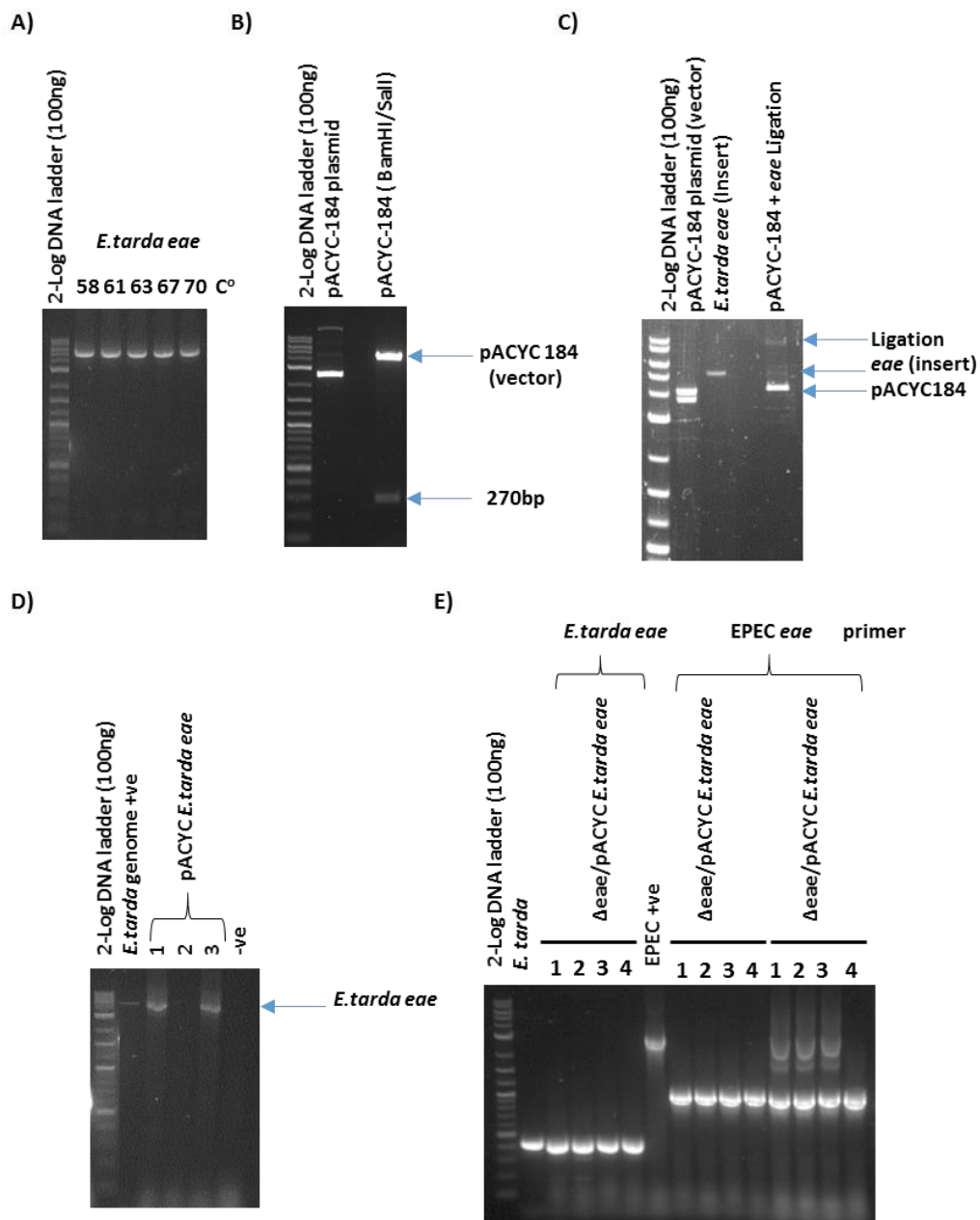


Figure 52: Cloning *E. tarda eae* gene into pACYC184 and introduction into EPEC *ea_e* mutant.

Agarose gel data illustrating steps in generating and/or confirming presence of pACYC-*ea_{E_t}* plasmid **A)** PCR amplification of a single band of expected size of *ea_e* gene, **B)** Digestion of pACYC184 to obtain vector fragment for **C)** use with PCR *ea_e* gene fragment in Gibson Assembly protocol to generate pACYC-*ea_{E_t}*, **D)** supported by PCR detecting the *ea_e* fragment in examined clones (#1, #3) with **E)** providing PCR support for a disrupted *ea_e* gene in the EPEC *ea_e* mutant (4 colonies examined) and pACYC-*ea_{E_t}* introduction into EPEC *ea_e* mutant (3, of 4, examined colonies).

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HeLa cells were then infected to confirm Tir delivery - linked with kinase modification to T'' form - and determine if there was an Intimin-dependent migration of the T'' form into the insoluble fraction. As expected, EPEC and *eae* mutant delivered Tir which was modified to T'' form and detected in the soluble fraction but only the insoluble fraction of cells infected with EPEC, not the *eae* mutant (Figure 53). However, introducing a plasmid carrying the EPEC or *E. tarda eae* genes rescued this defect (Figure 53). Importantly, probing insoluble fractions (contains proteins from adherent bacteria) with antibodies raised against EPEC Intimin detected Intimin in samples from cells infected with EPEC or *eae* mutant carrying the plasmid encoding the EPEC, but not *E. tarda eae* gene (Figure 53). These findings show that the *E. tarda eae* gene encode a functional Intimin protein that can bind the membrane-inserted EPEC Tir effector.

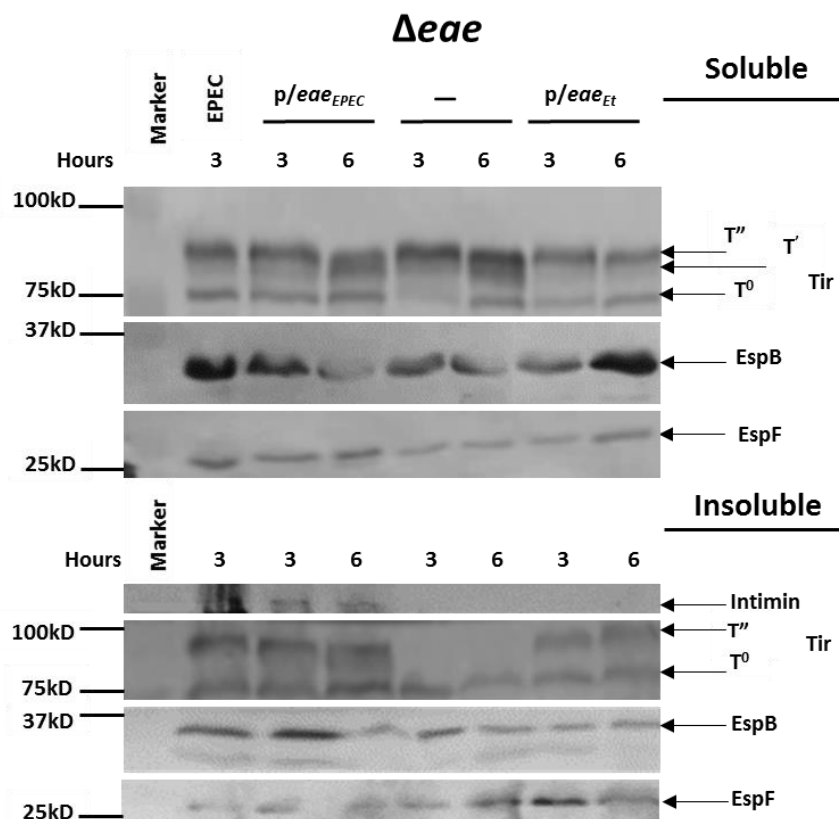


Figure 53: Rescuing the EPEC *eae* mutant defect in binding membrane-inserted Tir by introducing *E. tarda eae* gene. HeLa cells were left uninfected or infected 3 and/or 6h with indicated strains before isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble fractions (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gels) probing for EspB, EspF, Tir and/or Intimin. The position of Tir unmodified (T⁰) and host kinase fully modified (T'/T'') forms are indicated as well as, Intimin, EspF, EspB and protein markers. Strains used were EPEC, Intimin deficient EPEC mutant (*eae*) or *eae* mutant complemented with plasmid carrying EPEC (*eae*_{EPEC}) or *E. tarda* (*eae*_{Et}) *eae* genes.

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4.3.6 *E. tarda* LEE proteins of highest, medium and lowest homology to EPEC counterparts

Interrogating the 'identify/similarity' data (Table 8) revealed great variability between homologues with 9 proteins (27%) sharing the highest level (60-73% identity); 15 (45%) the lowest level (~12-39% identity) with the remaining 8 (24%) intermediate levels (~40-59% identity) compared to their corresponding EPEC homologue.

Members of the 'highest level' homology (60-73% identity) group are four core T3SS components (EscR, EscS, EscC, EscV), the needle protein (EscF), ATPase (EscN), a chaperone of middle (CesD) and late (CesT) substrates and the translocator (EspA) which failed (Chapter 3; section 3.3.8) to functionally replace its EPEC homologue, unless co-expressed with *E.tarda* EspD and EspB (Chapter 3; section 3.3.9).

The 'intermediate level' homology (40-59% identity) group members are five core T3SS components (EscT, EscU, EscJ, EscI, EscD), the peptidoglycan lytic enzyme (EtgA), molecular switch protein (SepL), a translocator (EspD) and chaperone for a middle (CesD2) substrate. Interestingly, the previous functional interchangeability studies included two of these proteins with one (EscD unlike SepL) functionally interchangeable for its EPEC homologue.

The final group (lowest level homology[(12-39% identity)]) contains the master regulator (Ler), sorting platform/molecular switch proteins (EscK, EscL, SepD, EscQ), chaperones for early (EscG, CesL, EscE) and middle (CesAB, EscA) substrates, ruler protein (EscP), a translocator (EspB), two effectors (Tir, EspZ) plus Intimin. Two members of this group (EspB and Intimin) have already been shown to functionally interchangeable in contrast to the third examined protein, EscL

4.3.7 *E. tarda* Ler protein replaces EPEC Ler regulatory activity

As it was not feasible to examine the functional interchangeability of all *E. tarda* LEE proteins, studies focused on those proteins in the lowest homology group focusing on proteins linked to delivering effectors i.e. Ler, EscK, EscP and Tir.

The *E. tarda ler* gene (*ler_{Et}*) was PCR amplified, using *E.tarda* genomic DNA as a template, and inserted into pACYC184 (pre-digested with BamHI/Sall) using the Gibson Assembly protocol (Figure 54). Construction of pACYC-*ler_{Et}*, and its introduction into the provided Ler deficient EPEC, Δler (Mellies *et al.*, 2007) was supported by PCR analysis (Figure 54) before HeLa cell infection. Western blot analysis on isolated Triton X-100 soluble and insoluble fractions confirmed that the *ler* mutant does not express Tir, EspB or EspF proteins (Figure 55) (Yerushalmi *et al.*, 2008). Crucially, introducing pACYC-*ler_{Et}* led to Tir, EspB and EspF expression and delivery as evidenced by host kinase modification of Tir to T'/'T" forms (Figure 55). Thus, the *E. tarda ler* gene encodes a protein which can functionally replace the EPEC Ler protein to induce EPEC LEE protein expression and produce a functional effector delivery system.

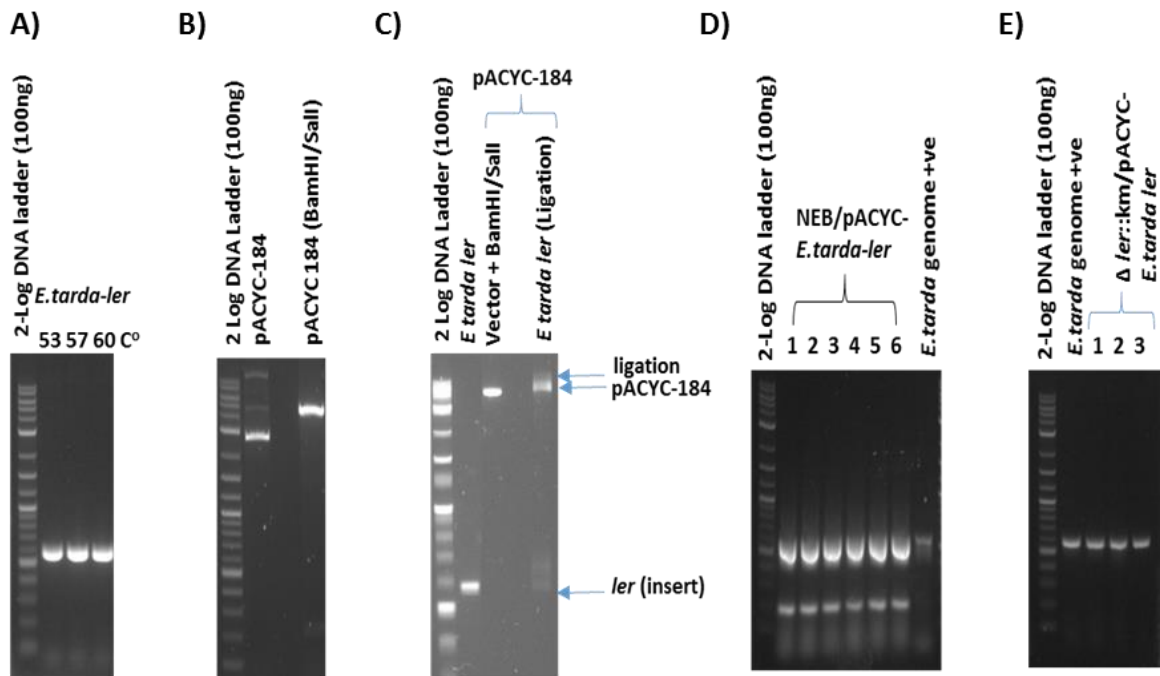


Figure 54: Cloning *E. tarda ler* gene into pACYC184 and introduction into EPEC *ler* mutant.

Agarose gel data illustrating steps in generating and/or confirming presence of pACYC-*ler_{Et}* plasmid **A)** PCR amplification of a single band of expected size of *ler_{Et}* gene, **B)** Digestion of pACYC184 to obtain vector fragment for **C)** use with PCR *ler* gene fragment in Gibson Assembly protocol to generate pACYC-*ler_{Et}*, **D)** supported by PCR detecting the *ler* fragment in all examined clones with **E)** providing PCR support for pACYC-*ler_{Et}* introduction into EPEC *ler* mutant.

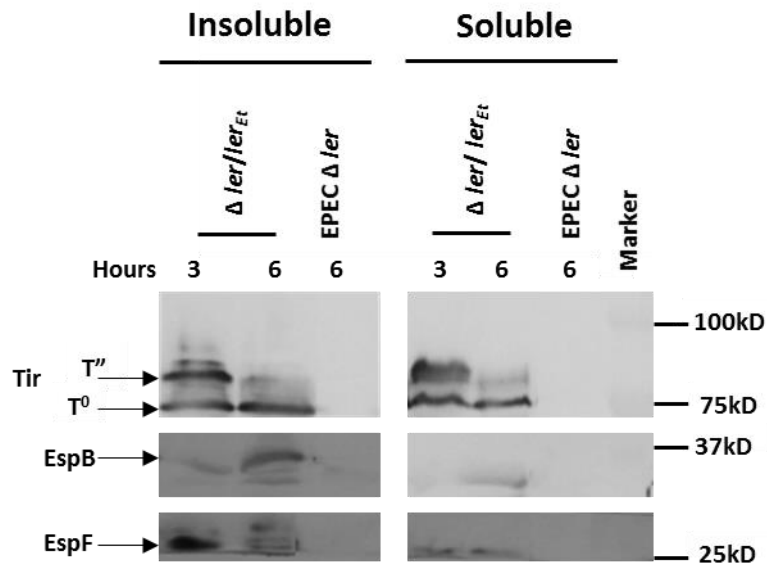


Figure 55: The *E. tarda ler* genes restores the ability of the EPEC *ler* mutant to express and deliver LEE effector proteins. HeLa cells were infected 3 and/or 6h with indicated strains prior to isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effectors) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gels) probing for Tir, EspB and EspF. The position of Tir unmodified (T^0) and host kinase fully modified (T'') forms and indicated as are the EspB, EspF and protein markers. Strains used were EPEC Δler mutant and *ler* mutant carrying the pACYC-*ler_{Et}* plasmid (carries *E. tarda ler* gene)

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4.3.8 *E.tarda* Tir protein lacks key EPEC Tir-related subversive features can be delivered by EPEC in a CesT/T3SS-dependent manner without evidence of modification to T” form.

While the predicted *E. tarda* Tir protein is similar in size to EPEC Tir, aligning the sequences revealed many unexpected differences (Figure 56) that helps explain the low homology (24% identity; 36% similarity [Table 8]). Thus, the N-terminus (19.7% identity/34.6% similarity) contains some small deletions and several large insertions in contrast to high similarity (66.7%) between the transmembrane domains and Intimin binding region (Figure 56). The shared presence of residues K289, G305 and N306 (Figure 56) required for EPEC Tir-Intimin binding (Ross & Miller, 2007) suggests that the *E. tarda* Tir variant may interact with EPEC Intimin. As expected, examining *E. tarda* Tir for transmembrane spanning regions found ones flanking the Intimin binding region (Figure 57) supporting that, like EPEC Tir (Kenny, 1999, Kenny *et al.*, 1997b), it is inserted into the host plasma membrane in a hairpin-like conformation. A polyproline rich region is one of the few N-terminal features with a defined role in EPEC Tir's subversive activities - recruits host tyrosine kinases to modify Tir residues (Bommarius *et al.*, 2007) - with this feature highly degraded in *E. tarda* Tir (Figure 56). Surprisingly, the C-terminal domain (10% identity; 17% similarity) which contains most features linked to EPEC Tir's subversive activities (Kenny, 1999, Kenny & Warawa, 2001, Allen-Vercoe *et al.*, 2006, Yan *et al.*, 2013) is truncated in *E. tarda* Tir and thus lacks all these important EPEC Tir features (Figure 56).

The absence of residues that are substrates for host kinase phosphorylation predicted that *E. tarda* Tir, if delivered, may not undergo shifts in apparently molecular mass and, if inserted into the plasma membrane, would interact with EPEC Intimin without triggering actin nucleation (depends on kinase modification of Tir tyrosine residues).

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<i>E. tarda</i> Tir	1	MKLESAAN----SPLINPYAPSESGTQNTA-----NLGRLGDRAI-	36
EPEC Tir	1	MPIGNLGNVNGNHLI PPAPPLP SQTDGAARGGTGHLISSTGALGSRSLF	50
<i>E. tarda</i>	37	--AVNADSSARDGGQLPGLPSSPVALQPSRGS DPVYRHTNVYTAQDSFEA	84
EPEC	51	SPLRNSMADSVDSRDI PGLPTNPSRLAAA-----TSETCLLGGFEV	91
<i>E. tarda</i>	85	I--KGMSLNMLFERVGNATFSRRTTPEGNVEAVSTRGQHEMAVLLPQDEQ	132
EPEC	92	LHDKG-PLDILNTQIGPSAFRVEVQADGTHAAIGEKNGLEVSVTLSPQEW	140
<i>E. tarda</i>	133	YALNSLSPDPEANFVFIG--GSRSHPLVSLAGESSEV-ISVRQTLERGGQ	179
EPEC	141	SSLQSIDTEGKNRFVFTGGRRGSGHPMVTVASDIAEARTKILAKLD----	186
<i>E. tarda</i>	180	LSDRPGGVSPLDSSSLFRGQLEHSLGNRTGVDREMTGGTGLHSSHSELGA	229
EPEC	187	-PDNHGGRQPKD-----VDTRSVG-----	204
<i>E. tarda</i>	230	TGQTDMRGSATTQALAGQADTINQGPQIDGGERLRELTHPQLVEINNYIA	279
EPEC	205	-----VGSASGIDDG-----VVSEHTST	223
<i>E. tarda</i>	280	VNPPAMSRSHLYVALGTAAVGLTAL-ATGLSQAFAITPAPDDALVVDPN	328
EPEC	224	TNSSVSRDPKFWVSVG-AIAAGLAGLAATGI AQALALTPPEPDDPTTDPD	272
<i>E. tarda</i>	329	QAQQDAVTNTRNELTSEALKDPANQ KVEVDEMGN QRPTGILLDNVAAEIE	378
EPEC	273	QAANAESATKDQLTQEAFKNPENQKVN T DANGNAIPSGE LKDDIVEQIA	322
<i>E. tarda</i>	379	VKAQEAGEAAKQQAITDNAVAQERHDTQQEKRDKELAISGGAGYG-AAAL	427
EPEC	323	QQAKEAGEVARQQAVESNAQAQQRYEDQHARRQEELQLSSG IGYGLSSAL	372
<i>E. tarda</i>	428	IGAGGF TAAGV---LYRRNKETHEEAMELHRALPQAEPVGNRTETG---	471
EPEC	373	IVAGGI-GAGVTTALHRRNQPAEQTTTTTHTTVVQQQTG-GNTPAQGGTD	420
<i>E. tarda</i>	472	-TRSERFTFPRAVLDQPPREP GAVGGIYQGLQRGEWSAASSTW-DSASQ	519
EPEC	421	ATRAEDASLNR-----RD S QGSVASTHWSDSSE	449
<i>E. tarda</i>	520	TSLAGNVTLV-----	529
EPEC	450	-----VVNP V AEVGGARN S LSAHQPEEHI V DEVAADPG S SVIQNFSG	491
<i>E. tarda</i>	530	-----	529
EPEC	492	SGPVTGRLIGTPGQGIQST V ALLANS GGLR LGMGGLTSGGESAVSSVNAA	541
<i>E. tarda</i>	530	-----	529
EPEC	542	PTPGPVRFV	550

Figure 56: Comparison of *E. tarda* and EPEC Tir proteins. *E. tarda* and EPEC Tir sequences were aligned (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Key features linked to EPEC Tir functionality are highlighted: polyproline rich motif (Red), Intimin binding area (Green), residues important for Intimin binding (Blue), kinase modified residues (single residues; dark green) and arginine finger motif (Yellow).

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Output from TMpred

2 possible models considered, only significant TM-segments used

-----> STRONGLY preferred model: N-terminus inside

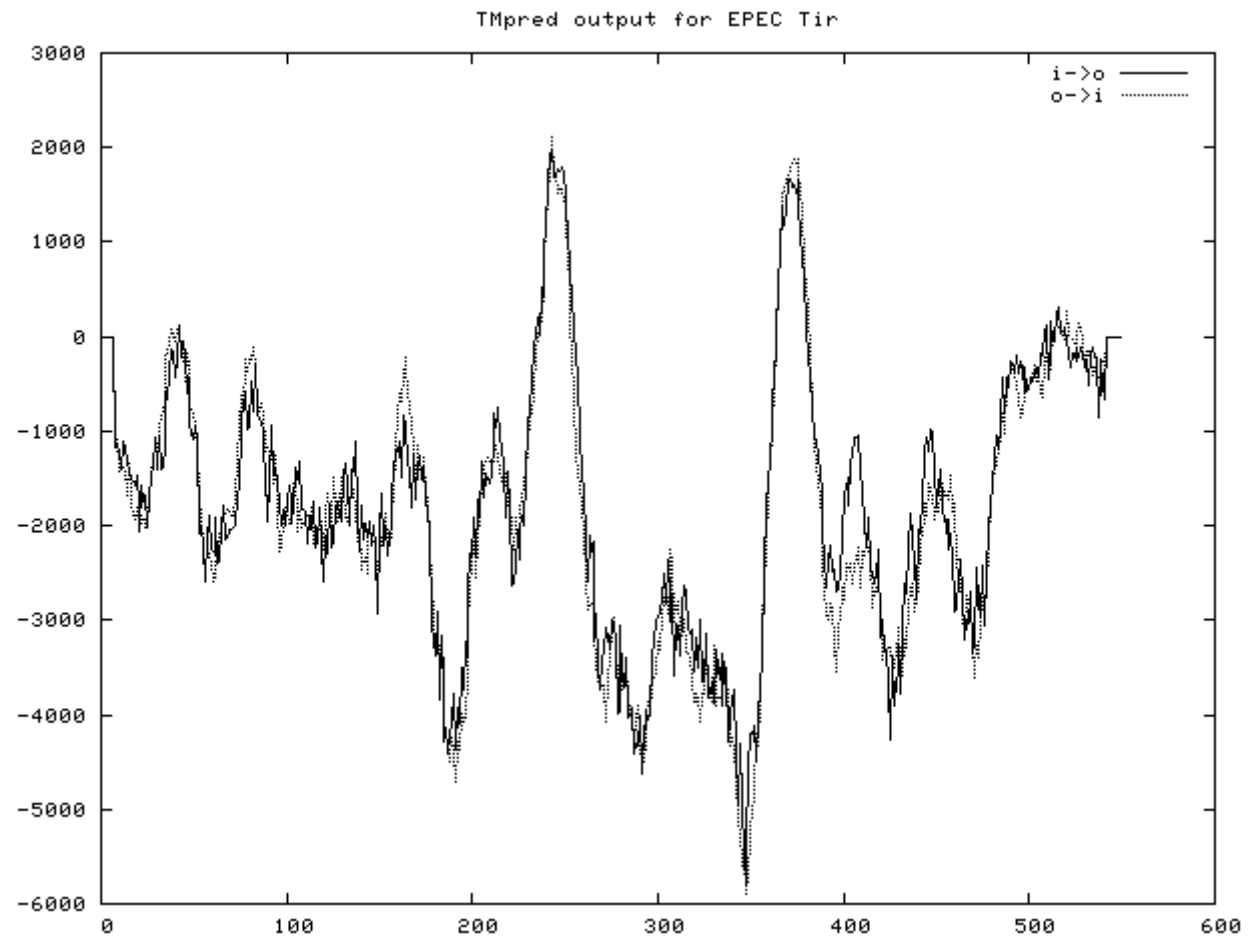
2 strong transmembrane helices, total score: 3875

#	from	to	length	score	orientation
1-	234	253	(20)	1981	i-o
2-	364	386	(23)	1894	o-i

-----> Alternative model

2 strong transmembrane helices, total score: 3779

#	from	to	length	score	orientation
1-	234	253	(20)	2104	o-i
2-	363	382	(20)	1675	i-o



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Output from TMpred

2 possible models considered, only significant TM-segments used

-----> STRONGLY preferred model: N-terminus inside

2 strong transmembrane helices, total score: 3678

#	from	to	length	score	orientation
1-	290	309	(20)	1722	i-o
2-	418	440	(23)	1956	o-i

-----> Alternative model

2 strong transmembrane helices, total score: 3304

#	from	to	length	score	orientation
1-	290	309	(20)	1659	o-i
2-	421	440	(20)	1645	i-o

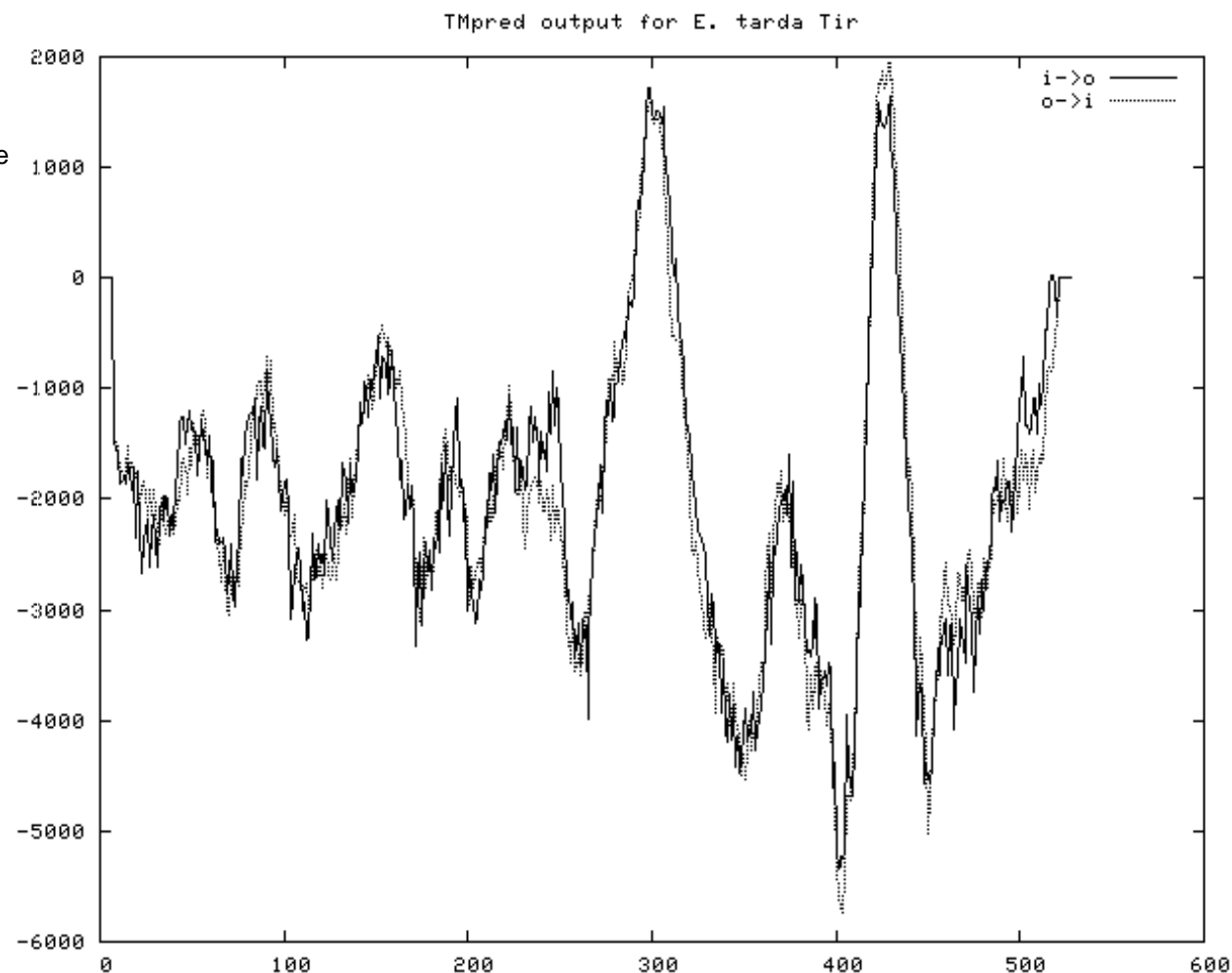


Figure 57: The *E. tarda* and EPEC Tir proteins have two putative transmembrane domains. The *E. tarda* and EPEC Tir protein sequences were examined for the presence of transmembrane domains (http://www.ch.embnet.org/software/TMPRED_form.html) and found to possess two in similar locations (234-253 & 363-382 EPEC Tir; 290-309 & 421-440 *E. tarda* Tir) flanking the Intimin binding region. The program requires setting of minimal and maximal hydrophobic helix length, in this case it was 17 and 33 respectively. Solid line is the prediction for inside to outside TM helix and interrupted line is the prediction for outside to inside TM helix.

4.3.8.1 Generating an *E. tarda* Tir-HA fusion protein

Given the low degree of Tir homology, it was not surprising that plasmids carrying the *E. tarda* gene failed to produce a protein that could be detected with antibodies raised against EPEC Tir. Adding epitope tags, including HA, to the C-terminus of EPEC Tir does not interfere with its known subversive functions (Kenny *et al.*, 1997b). Thus, a plasmid encoding an *E. tarda* Tir-HA fusion protein (TirHA_{Et}) was generated (see Chapter 2; Section 2.2.8). Briefly, *tir*_{Et} was amplified with flanking unique restriction enzyme sites and inserted into the TA cloning vector (pCR-2.1) prior to subcloning into a pSK-based plasmid (carrying EPEC *tirHA* and *cesT* genes) replacing the EPEC *tir* fragment (Figure 58). PCR analysis supported the generation of pSK-*tirHA*_{Et} *cesT*_{EPEC} (Figure 58).

To assess TirHA_{Et} fusion protein expression and delivery, HeLa cells were infected with EPEC or the *tir* mutant carrying no plasmid or a plasmid encoding EPEC or *E. tarda* TirHA fusion proteins prior to isolating Triton X-100 soluble and insoluble fractions for Western blot probing for HA tagged proteins. This analysis confirmed expression and delivery of EPEC TirHA into host cells (kinase modified T” form in soluble and insoluble fractions; not shown) but only a very weak band of the expected molecular mass for the *E. tarda* TirHA protein was evident in the insoluble, but not soluble, fraction (data not shown).

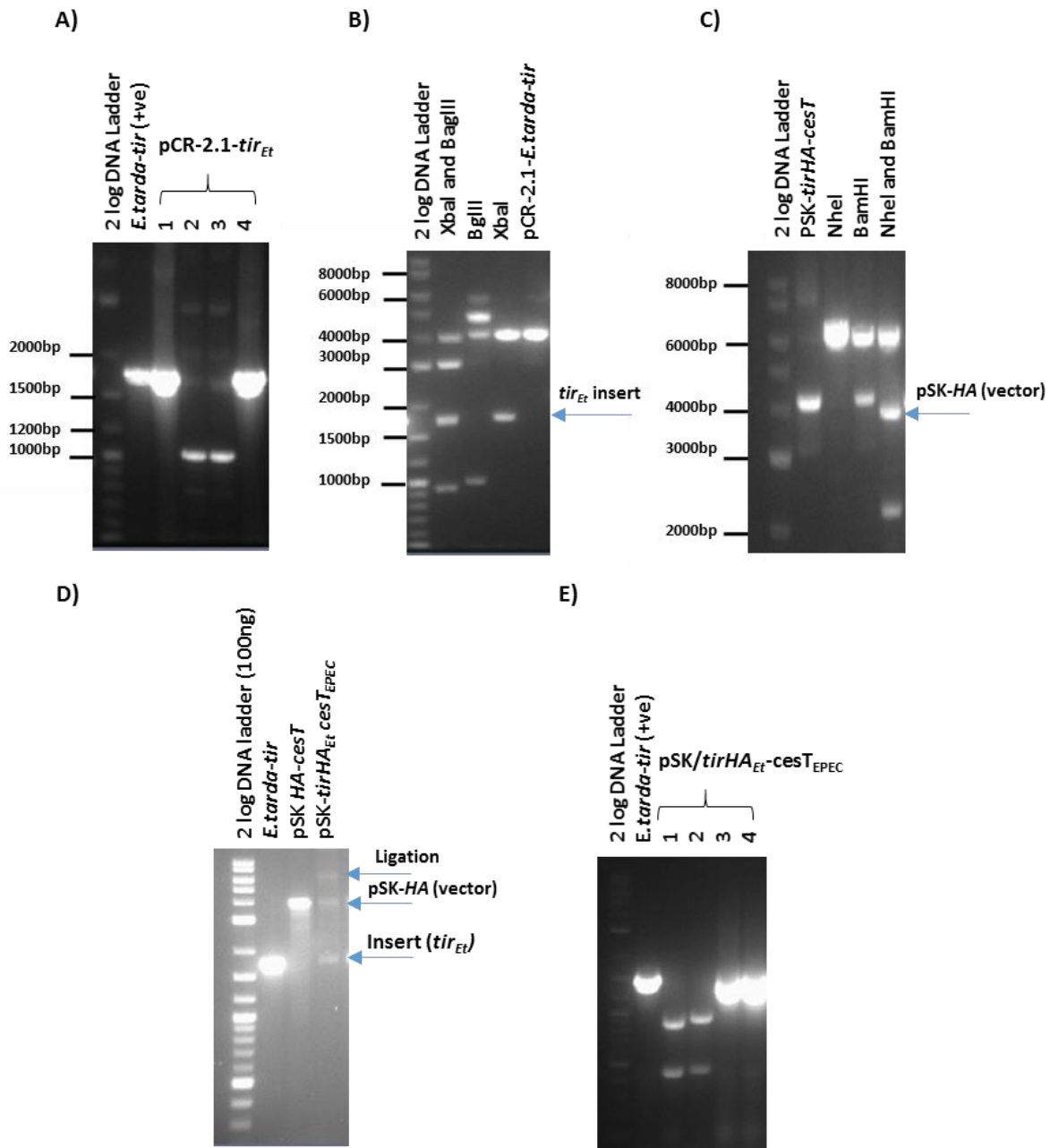


Figure 58: Construction of pSK-*tirHA_{Et} cesT_{EPEC}*. Agarose gel data illustrating steps in generating and/or confirming presence of pSK-*tirHA_{Et} cesT_{EPEC}* **A)** PCR amplification of a single band of expected size of *tir_{Et}* gene and support for introduction into pCR-2.1 plasmid **(B&C)** Restriction digestion release of *tir_{Et}* from pCR-2.1 *tirHA_{Et}* and *tir_{EPEC}* from pSK*tirHA cesT* to isolated fragments for **D)** ligation to generate pSK-*tirHA_{Et} cesT_{EPEC}* **E)** supported by PCR detecting *tir_{Et}* in two of 4 screened clones (#3, #4)

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4.3.8.2 Generating pACYC-*tir*HA_{Et}

It was possible that this expression problem may relate to cloning the *E. tarda* gene into a high copy number plasmid and/or co-expression with CesT_{EPEC}, so it was decided to subclone *E. tarda tirHA* gene to pACYC184 as used in previous complementation studies (and medium copy number).

Briefly, pSK-*tir*HA_{Et} CesT_{EPEC} was digested XbaI/BglII - releases *E. tarda tirHA* fragment and 5' end of EPEC *cesT* gene - and ligated into XbaI/BamHI sites of pACYC184. Generation of pACYC-*tir*HA_{Et} and its introduction into the EPEC Δtir mutant strain was supported by PCR analyses (Figure 59). Studies also examined whether CesT_{EPEC} is needed for *E. tarda* Tir stability and/or T3SS-dependent delivery into cells by introducing the plasmid into the CesT/Tir-negative $\Delta go3core$ mutant and EspB/Tir-negative Δtir_espB mutant (see Chapter 2; Table 1).

Western blot analysis of Triton X-100 insoluble fractions from infected HeLa cells revealed TirHA_{Et} to be expressed by all the Tir negative (Δtir , Δtir_espB and $\Delta go3core$) strains with reduced levels in the CesT-deficient ($\Delta go3core$) mutant indicative of CesT_{EPEC}-dependent stability (Figure 60). Probing for EspB confirmed its expected absence from the Δtir_espB mutant. Crucially, probing soluble fractions for HA proteins failed to detect a HA protein in Δtir_espB mutant infected cells in contrast to low and high levels of a band of Tir_{ET} expected molecular mass in $\Delta go3core$ and Δtir mutant-infected cells, respectively (Figure 60). This data is consistent with EspB/T3SS-dependent delivery of *E. tarda* Tir by EPEC with efficient transfer requiring CesT_{EPEC} expression. Crucially, detecting only a single band suggests that, as predicted, *E. tarda* Tir does not undergo host kinase modifications that trigger shifts in apparent molecular mass.

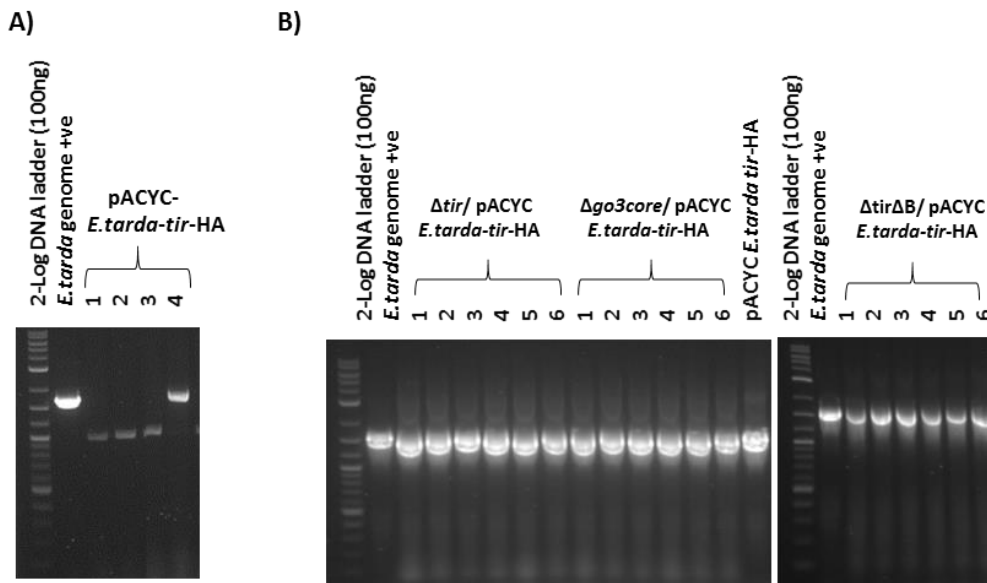
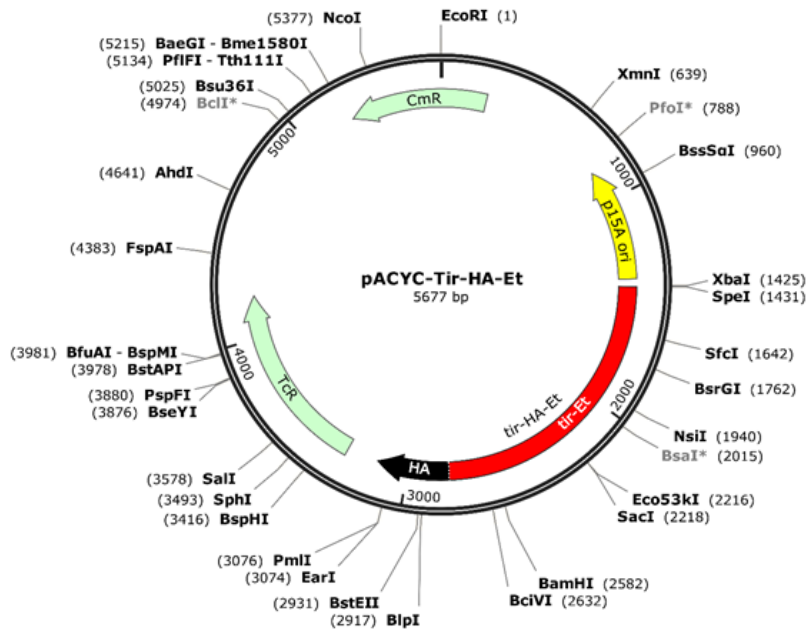


Figure 59: Schematic of pACYC-*tir*HA_{ET} and PCR support for introduction into EPEC strains. Plasmid map of pACYC-*tir*HA_{ET} with PCR data **A)** supporting *E. tarda tir* gene in pACYC184 and **B)** introduction into EPEC Δtir (lacks functional Tir protein); EPEC Δtir_espB (lacks functional Tir and EspB [translocator/effector] proteins) and $\Delta go3core$ (missing genes for 5 effectors [LEE EspG, Tir, Map, EspH, Orf3/EspG2], Intimin and 2 chaperones Cest/CesF) mutants.

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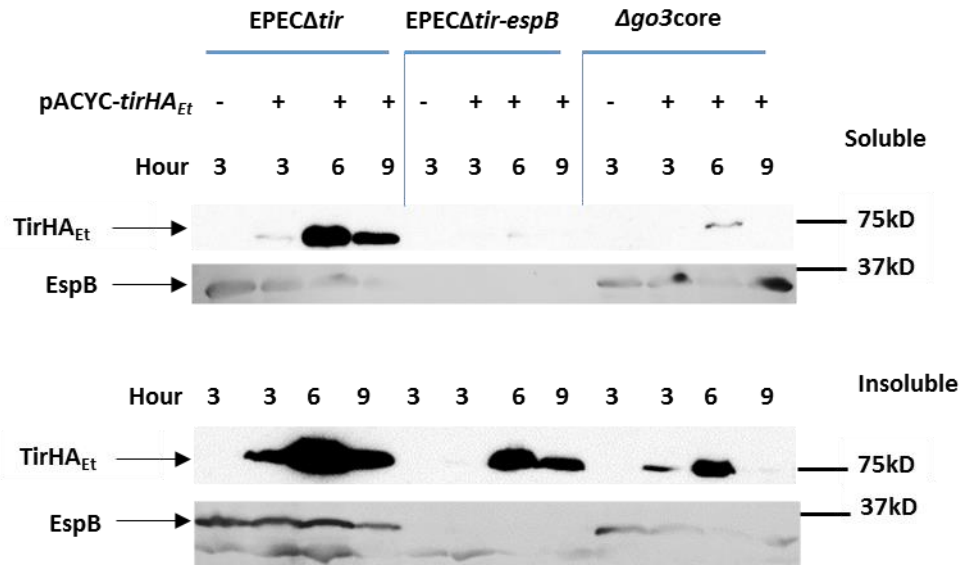


Figure 60: EPEC T3SS-dependent delivery of *E. tarda* Tir into HeLa cells promoted by EPEC CesT. HeLa cells were infected for 3, 6 and/or 9h with indicated strains before isolating Triton X-100 soluble (containing host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (containing host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for western blot analysis (10% SDS-PA gel) probing for EspB or HA proteins. The positions of Tir-HA, EspB and protein markers are indicated. Strains used lacked Tir (Δ tir), Tir and EspB (Δ tir_espB) or the CesT/CesF chaperones, Intimin, EspG, EspG2, EspH, Map, Tir (Δ go3core).

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4.3.9 The *E. tarda cesT* gene produced a protein that weakly substitutes for $CesT_{EPEC}$ in promoting Tir_{EPEC} delivery into HeLa cells

$CesT_{EPEC}$ promoting *E. tarda* TirHA stability in EPEC and efficient delivery into HeLa cells made it likely that the *E. tarda* *CesT* homologue (~60% identity; ~80% similarity) would be functionally interchangeable. To interrogate this prediction *cesT_{Et}* was cloned into pACYC184 (see Chapter 2; Section 2.2.8). Briefly, *cesT_{Et}* was amplified, using *E. tarda* genomic DNA as a template, and ligated into pACYC184 (predigested BamHI/Sall) using the Gibson Assembly protocol (Figure 61). Generation of pACYC-*cesT_{Et}* was supported by PCR analyses (Figure 61).

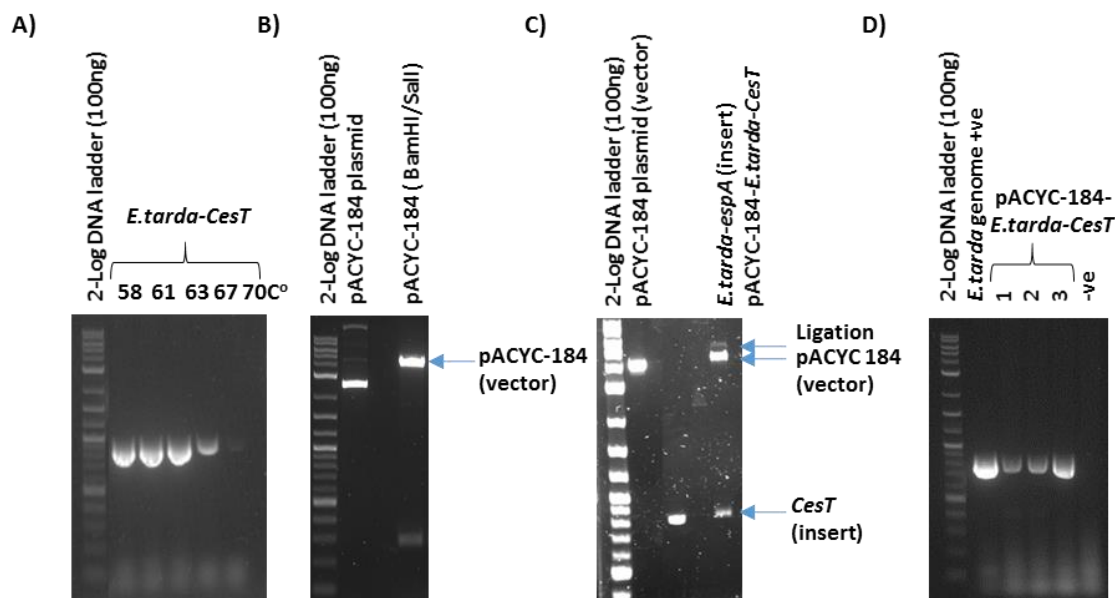


Figure 61: Construction of pACYC-*cesT_{Et}*. Agarose gel data illustrating steps in generating and/or confirming presence in strains **A)** The *E. tarda cesT* gene was amplified as a single band at most assessed temperatures for use with **B)** pACYC184 digested with BamHI and Sall restriction enzymes. **C)** for linking together using the Gibson Assembly protocol with introduction of the generated pACYC-*cesT_{ET}* **D)** confirmed in all 3 examined clones.

Probing western blots of fractions isolated from HeLa-infected cells revealed the expected $\Delta cesT$ mutant phenotype – i.e. little Tir expression/no detectable delivery - with introduction of *cesT_{EPEC}* rescuing the defect as evidenced by detecting kinase modified T” from in soluble and insoluble fractions (Figure 62). Interestingly, introducing the *cesT_{ET}* plasmid also rescued the $\Delta cesT$ mutant defect but to a much less extent, especially in the soluble fractions. Thus, the Tir level in the insoluble fraction was reduced - relative to samples from cells infected with the *cesT_{EPEC}*

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complemented strain - at 3, not 6, hour post-infection time point in contrast to both time points for the soluble fraction samples (Figure 62). The presented data implies that *E. tarda* CesT is less capable than EPEC CesT at chaperoning EPEC Tir to the EPEC T3SS for transfer into host cells. It was also noted that plasmid expressing *E. tarda* CesT was associated with higher EspB protein levels in the insoluble fractions (Figure 62).

Examining the CesT alignment data (Figure 63) revealed differences in features linked to EPEC CesT chaperone activity. The N-terminal region (65% identity) of EPEC CesT is linked to chaperone dimerization (Delahay *et al.*, 2002) while the C-terminal domain (51% identity) has an amphipathic α -helical regions; linked to EPEC CesT substrate binding (Delahay *et al.*, 2002). Residues E142, V116, V126, H128, N129 and S147, along with the final 11 amino acids, are important for EPEC CesT functionality in secreting NleA and Tir (Ramu, 2013). Interestingly, there are residues differences in the amphipathic α -helical with the *E. tarda* variant lacking E142 and S147 and 4 of the last 11 residues are different to the EPEC homologue.

4.4 Discussion

Type III secretion systems (T3SS) play a critical role in the pathogenesis of many bacterial pathogens that infect humans, animals and plants (Galán *et al.*, 2014, Buttner & He, 2009). Thus, it is important to understand the functionality of the ~20 proteins that make up T3SSs which serves to deliver effector proteins into infected host cells (Buttner, 2012, Cornelis, 2006, Cardenal-Munoz *et al.*, 2014). The T3SS components of A/E pathogens, including EPEC, EHEC, *Citrobacter rodentium*, are highly homologous with many displaying much more diversity to corresponding proteins from other pathogens such as *Yersinia*, *Salmonella* and *Shigella* species (Diepold & Wagner, 2014). Recently, a fish pathogen, *Edwardsiella tarda* (*E. tarda*), was reported to carry a LEE region (Nakamura *et al.*, 2013) – key virulence determinant of A/E pathogens – encoding proteins for a T3SS, transcriptional regulator, Intimin surface protein and one effector, Tir. Here, we report on our bioinformatics analysis of the *E. tarda* LEE region and ability of *E. tarda* proteins to functionally replace their EPEC homologues. The work reveals issues with the original bioinformatics analysis and argues that *E. tarda* encodes a functional T3SS to deliver, under specific conditions, two LEE (Tir, EspZ) and, probably, non-LEE-encoded effectors into fish cells. Importantly, finding little or no functional interchangeability between some components provides an opportunity to gain insights on the structure and/or function of T3SS proteins from A/E, and possibly other, pathogens.

Although *E. tarda* was reported to share 29 out of 42 EPEC LEE open reading frames, Orfs (Nakamura *et al.*, 2013), EPEC only has 41 recognised Orfs (Elliott *et al.*, 1998, Deng *et al.*, 2004) with the additional one - between *ler* and *espG* genes – not considered to produce a functional protein. Interestingly, the *E. tarda* LEE region appears to have 37 Orfs and thus 8 proteins with no EPEC homologous. However, six of these new Orfs are in regions where *E. tarda* LEE genes (*escD*, *sepL*, *escL*) are significantly smaller than their EPEC homologues or replacing the *escE*, *escA* and *escS* genes while the final two are in a region where the ‘missing’ EPEC effector genes would be expected to be located. However, most of these new genes appear to encode very small proteins with our analysis revealing the *E. tarda* *escE*, *escA* and *escS* genes in the same area as that they might be there with another three likely not to be expressed as our bioinformatics analyses revealed ‘missing’ portions of the *escD*, *sepL* and *escL* genes on open reading frames in the regions that encode these non-EPEC LEE proteins.

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Sequencing the 'out of frame' region for three genes (*escD*, *sepL*, *escL*) revealed the two *escD* Orfs to be separated by a 14bp gap while the *SepL* and *EscL* Orfs are distinct due to the apparent absence of a single base. While such findings are suggestive of these T3SS critical proteins no longer being functional, this was not supported, at least for *EscD*, by finding that the effector delivery defect of an EPEC *escD* mutant (Ogino *et al.*, 2006) could be rescued by introducing both, not one, *E. tarda* *escD* Orfs. The two *EscD* Orfs produce a protein of similar size to EPEC *EscD*, 10 residues larger, but with many differences (~44% identity/61% similarity). This finding suggests that *E. tarda* *EscD* is expressed either as i) two independent polypeptides or ii) as one Orf - via, for example, ribosome hopping; a process linked to expressing T3SS proteins, from two Orfs, in other pathogens in relation to specific environmental signals such as stress (Penno *et al.*, 2006, Atkins *et al.*, 2016, Namy *et al.*, 2004). The latter mechanism is supported by the presence of a hungry Ile (AUA) codon (Gallant & Lindsley, 1998) – linked to ribosome stalling (Keiler, 2015) and hopping (Lovett & Rogers, 1996) - prior to the stop codon of the first *escD_{Et}* orf. *EscD_{Et}* functionally interchangeable, but only when both Orfs were introduced, supports a critical role for each domain in accordance to the finding that T3SS functionality depends on the N-terminal domain interacting with *EscJ* while the C-terminal domain interacts with *EscC* and *EscF* proteins (Ogino *et al.*, 2006). Crucially, *EscD* functional interchangeability supports the idea that the *E. tarda* LEE region might produce a functional effector delivery system. Further studies will be needed to elucidate the exact mechanism that underpins the functional interchangeability of *E. tarda* *EscD* encoded over two adjacent, but non-overlapping Orfs.

By contrast, similar complementation studies with the *E. tarda* *SepL* and *EscL* proteins failed to rescue the effector delivery defect of EPEC *espL* and *escL* mutants, respectively, though the *E. tarda* *SepL* protein appeared to partially rescue T3SS functionality (data not shown). It is predicted that these T3SS critical proteins, like *EscD*, are under some form of regulatory control at the protein translation level (i.e. ribosome frame-shifting to generate a single Orf) with the failure to functionally replace the EPEC homologue likely to reflect differences in protein sequences (~49% identity/~74% similarity between *SepL* proteins and ~27% identity/~54% similarity between *EscL* proteins). *SepL* and *EscL* are components of a sorting platform with *SepD*, *EscP*, *EscK*, *EscA* and *EscN*. It is possible that co-expressing these proteins with each other or other sorting platform proteins could restore functionality in line with our findings with translocator proteins i.e. *E. tarda* *EspA* and *EspD* proteins had little functional

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interchangeability until co-expressed. Moreover, complementation studies with two other platform proteins, EscK (~13% identity/~21% similarity) and EscP (~21% identity/~39% similarity), found that only the latter could substitute for its EPEC homologue. Further studies should examine the functional interchangeability of other platform proteins: SepD (~40% identity/~64% similarity), EscA (~25% identity/~54% similarity) and EscN (~62% identity/~76% similarity) with the definition of features/residues differences that hinder complementation, perhaps, providing important new insights on how these proteins contribute to T3SS functionality.

It is worth pointing out that T3SS components from different A/E pathogens are usually highly homologous while proteins exposed to the host immune system (i.e. translocators, effectors and Intimin proteins) are generally more divergent - due to selective pressure to avoid immune recognition (Perna *et al.*, 1998). However, T3SS components of different pathogens can either have low or high levels of conservation (Diepold & Wagner, 2014) with the former presumably reflecting species specific alterations that promote the pathogen's lifecycle. Indeed, the *E. tarda* T3SS proteins, in general, have a low degree of conservation to homologues of other A/E pathogens suggesting that *E. tarda* has been undergoing a distinct evolutionary pathway that presumably reflects its role in aiding the lifecycle of this invasive fish pathogen.

Crucially, while our data supports (Nakamura *et al.*, 2013) *E. tarda* LEE lacking genes for multiple effectors (Map, EspF, EspG, EspH), a chaperone (CesF; needed for efficient EspF delivery) (Elliott *et al.*, 2002) and two transcriptional regulators (GrlA, GrlR), it uncovered genes for proteins critical for i) T3SS functionality (i.e. EscA [ATPase complex component], EscS [T3SS core protein] plus, as mentioned, EscE [EscF chaperone]) and ii) A/E pathogen virulence i.e. the EspZ effector (Deng *et al.*, 2004, Shames *et al.*, 2010). It is not obvious why these small proteins (122, 89, 78, 129AA for EscA EscS, EscE and EspZ respectively) were missed and why the authors did not comment on the implications of this (as well as truncated EscD, SepL, EscL proteins) on T3SS functionality. By contrast, our findings provide further support for the *E. tarda* LEE region encoding a functional T3SS that serves to deliver EspZ and Tir into host cells to promote cell survival (EspZ) and provide a receptor (Tir) for the Intimin surface protein to promote pathogen-fish cell interaction. Interestingly, our analysis revealed a region flanking LEE to carry genes for an effector - 25.7% identical (39.4% similar) to the *Yersinia* tyrosine phosphatase YopH (Guan & Dixon, 1990, Rosqvist *et al.*, 1988) – and a chaperone,

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SycH ~30% identical (~54% similar) (Woestyn *et al.*, 1996). This finding raises the possibility that the LEE T3SS may also deliver other, non-LEE-encoded, effectors into fish cells. However, it must be noted that this LEE carrying *E. tarda* strain (FPC503) is an atypical family member, as carries an additional T3SS and two T6SS (Nakamura *et al.*, 2013). Further studies are needed to determine which system transfers which, if any, identified effectors into host cells. By contrast, the absence of *GrlR* and *GrlA* regulators should have no impact on T3SS functionality as they are linked to regulating the activity of the LEE encoded master regulator, *Ler*, to environmental cues (Barba *et al.*, 2005, Jimenez *et al.*, 2010) while a recently published non-pathogenic *E.coli* strain (SIEC), lacking *grlR* and *grlA* genes, produced a functional T3SS when transcriptional activation was no longer under their control (Ruano-Gallego *et al.*, 2015). Interestingly, while the *Ler* proteins are only ~39% identical (~59% similar) the *E. tarda* variant could functionally replace the EPEC homologue to induce T3SS expression and effector delivery. Thus, the *E. tarda* LEE region is undoubtedly under transcriptional control of the *Ler* protein which is presumably controlled through the activity of non-LEE encoded proteins, equivalent to the plasmid-encoded regulator (*Per*) in EPEC, to induce T3SS production under a specific set of environmental conditions (Gomez-Duarte & Kaper, 1995).

Interestingly, while the initial analysis suggested that the *E. tarda* Intimin protein is larger than the EPEC homologue our analysis uncovered a slightly smaller protein (929 versus 939 residues) with an Intimin-like sequence on an overlapping reading frame. Moreover, DNA sequencing revealing an error which, when corrected, provides a gene encoding an 1117 residue protein that is quite dissimilar to its EPEC homologue (~40% identity; 52% similarity). Importantly, the *E. tarda* protein could functionally substitute for EPEC Intimin, at least in binding the EPEC delivered Tir protein and triggering Tir's actin nucleating activity. The latter result was, perhaps, not too unexpected as *E. tarda* Intimin has residues reported to be critical for binding Tir (Yi *et al.*, 2010).

Perhaps not too surprisingly, the *E. tarda* LEE Tir and EspZ effectors shared low homology with their EPEC counterparts (~24-30% identity/~36-38% similarity) though this is greater than some *E. tarda* T3SS components such as *EscK* (~13% identity/~21% similarity). Tir from another A/E pathogen, enterohemorrhagic *E.coli* O157:H7 (EDL993 strain), shares one of the lowest level of identity (66%) with its EPEC homologue linked to divergent evolution and EHEC Tir nucleating actin by a different, Intimin-dependent, mechanism (Perna *et al.*, 1998, Diepold & Wagner, 2014, DeVinney *et al.*, 1999,

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Campellone *et al.*, 2004b). The corresponding EPEC/EHEC EspZ proteins share 70% identity (Perna *et al.*, 1998) and thus *E. tarda* provides the best example of a LEE carrying pathogen with strain-specific divergence in effector (and other T3SS-related proteins). Interestingly, much of the EspZ variance relates to N- and C-terminal extensions with highest homology between two predicted transmembrane domains and the small, possibly extended (10-14 residue) extracellular domain. It would be interesting to examine the functional interchangeability of *E. tarda* EspZ protein for the EPEC homologue both *in vitro* and *in vivo* models. By contrast, Tir divergence was linked to a smaller *E. tarda* protein (529 versus 550 residues) with absence of features linked to EPEC Tir's known subversive activities (through residues differences, insertions and deletions; latter includes loss of most of C-terminal domain) with, as per EspZ, the most homology related to the two predicted transmembrane regions and extracellular (Intimin binding) domain. Indeed, these differences are presumably responsible for the failure of the anti-EPEC Tir polyclonal antibodies from recognising *E. tarda* Tir - a finding in line with the failure of anti-EPEC EspB polyclonal antibodies detecting *E. tarda* EspB – prompting studies with a HA tagged Tir variant. Interestingly, this variant was expressed by EPEC and, apparently, delivered into HeLa cells (expression/delivery were reduced when the EPEC Tir chaperone, CesT, was absent) but there was no evidence for host kinase modification-induced shifts in molecular mass. However, the latter was not surprising given that *E. tarda* Tir lacks serine and tyrosine-related motifs linked to host kinase modification-induced shifts in molecular mass and, following Intimin binding, actin nucleation respectively. The latter findings, makes it very difficult to assess whether *E. tarda* Tir is truly delivered into cells and/or becomes inserted into the plasma membrane and, if the latter occurs, whether during the transfer process or via a cytoplasmic intermediate. Future studies are needed to address these questions. By contrast, preliminary studies to determine whether *E. tarda* can deliver EPEC Tir (as a TirHA fusion protein) into cells for host kinase modification, insertion into the plasma membrane and Intimin binding are suggestive of low level delivery and kinase modification (but apparently not Intimin binding). However, the transfer process may relate to the second T3, or either T6 secretion systems. The low deliver levels may relate to issues including failure to define conditions that maximally induce *E. tarda* LEE T3SS expression and/or functionality and the finding that the *E. tarda* CesT protein could only weakly substitute for the EPEC homologue (despite being ~60% identical/~80% similarity). Interestingly, the *E. tarda* CesT variant did not appear to have a defect in promoting Tir stability within EPEC but was associated with reduced delivery levels suggesting that differences

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between these variants either i) impact on CesT docking with the T3SS, ii) Tir release from CesT and/or iii) Tir transfer through the T3SS/translocon systems. Interestingly, there are differences in residues linked to transfer efficiency so, undoubtedly, domains swaps and residue substitutions experiments would identify the responsible residues/features and promote studies to define how they impact on Tir deliver levels.

It should be noted that we only tested the functional interchangeability of 12 (~36%) of *E. tarda* LEE proteins with six (EscD; Ler; Intimin; EspA; EspD and EspB) appeared to fully substitute for their homologues (though two [EspA and EspD need to be co-expressed]) while 3 displayed low levels of functional interchangeability (SepL; CesT and EscP) with the remaining three (EscL; EscK and Tir) not able to replace their EPEC homologues. It is possible that the complementation defects related to issues in the expression (i.e codon usage), stability (i.e defects in binding chaperones) or sequence divergence of *E. tarda* proteins, with the latter group the most important for future studies to provide insights on their roles in T3SS functionality and/or pathogen virulence. Undoubtedly, the remaining 21 *E. tarda* LEE proteins should also be examined for functional interchangeability, and the *E. tarda* LEE region cloned into non-pathogenic *E. coli* to define conditions to induce expression and test its ability to deliver EPEC, *E. tarda* and/or hybrid Tir proteins into host cells to investigate the direct or indirect membrane insertion mechanisms.

Chapter 5: The enteropathogenic *E. coli* (EPEC) delivered EspZ effector prevents host cells cytotoxicity in response to the Tir-Intimin interaction

5.1 Introduction

The virulence of enteropathogenic *Escherichia coli* (EPEC) depends on a functional T3SS, Tir, Intimin and EspZ proteins (Wilbur *et al.*, 2015, Marchès *et al.*, 2000, Coburn *et al.*, 2007). While Tir and Intimin's virulence critical role is linked to their interacting together to mediate intimate host-pathogen interaction (Kenny *et al.*, 1997b, Kenny, 1999) that of EspZ is associated with preventing infected cells from undergoing a cytotoxic response (Shames *et al.*, 2011, Berger *et al.*, 2012, Roxas *et al.*, 2012). EspZ, is a small (9kDa) effector predicted to be integrated, like Tir, in the host cell plasma membrane in a hairpin-loop topology, via two transmembrane domains, with a small (10AA) extracellular loop (Kanack *et al.*, 2005). EspZ is reported to be, after Tir, the next most-abundantly delivered effector with some localising to mitochondria (Shames *et al.*, 2011, Mills *et al.*, 2008).

The ability of EspZ to stop cells cytotoxicity was initially linked to its interacting with a transmembrane glycoprotein, CD98 (Shames *et al.*, 2010) triggering increased focal adhesion kinase (FAK) activity to enhance pro-survival signalling (Shames *et al.*, 2010). However, this hypothesis was questioned by deletion of CD98 not promoting cell cytotoxicity (Roxas *et al.*, 2012). A second hypothesis stemmed from finding EspZ interacts with translocase of inner mitochondrial membrane 17b (TIM17b) promoting maintenance of mitochondrial membrane potential and thus protecting against cell death during EPEC infection (Shames *et al.*, 2011). However, depletion of TIM17b also decreased survival of uninfected cells (Garabedian *et al.*, 2011, Sinha *et al.*, 2014). As two EPEC effectors (Map and EspF) target the mitochondria, via mitochondrial targeting sequences [MTS], linked to loss of mitochondrial membrane potential (Nagai *et al.*, 2005, Kenny & Jepson, 2000, Papatheodorou *et al.*, 2006), it has been suggested that EspZ may protect against cell death by inhibiting the deleterious impact of these effectors on mitochondrial function (Shames *et al.*, 2011, Roxas *et al.*, 2012). A third possibility relates to finding that EspZ also interacts with a translocator protein, EspD (Creasey *et al.*, 2003a), suggesting that the EspZ might regulate translocation of EPEC effectors into the host cells (Berger *et al.*, 2012). Thus, it is proposed that when EspZ is absence there is continuous translocation of subversive effectors which overloads the cells, leading to permeabilization of plasma membrane and, thus, cytotoxicity (Berger *et al.*, 2012). It is also worth mentioning that EspZ is also reported to interfere with apoptosis – programmed cell death (not cytotoxic) - by inhibiting the intrinsic apoptotic pathway as *espZ* mutant infected cells had higher levels of cytosolic

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cytochrome *c* and increased activation of caspases 9, 7, and 3 (Roxas *et al.*, 2012). Interestingly, EPEC infection of epithelial cells activates the epidermal growth factor receptor; EGFR, which induces cell survival signalling (Roxas *et al.*, 2007) with EspZ found to regulate EGFR stability by protecting against EspF-dependent cleavage thereby promoting host cell survival (Roxas *et al.*, 2014).

Our previous work (Chapter 3) revealed that a *mfz*(~~81~~)*go3* mutant (lacks LEE Map, EspF, EspZ & EspG effectors plus the non-LEE-encoded Orf3/EspG2 effector) could deliver Tir into HeLa cells where it was modified to the T^Y form and could be bound by Intimin. However, extending the infection time (from 3 to 6hr) revealed a number of phenotypes: loss of i) Tir T^Y form, ii) host and Tir tyrosine phosphorylated proteins and iii) host cytoplasmic tubulin and actin proteins (not shown but see below). These phenotypes were, presumably, due to cell cytotoxicity as the strain does not express the pro-survival EspZ effector. Surprisingly, these phenotypes were not observed following infection with a similar multi-mutant, *mfz*(~~81~~)*go3e*, which differs only by the additional absence of the Intimin encoding gene, *iae*. This observation questioned the idea about EspZ's anti-cytotoxicity activity relates to it limiting the level of effector delivery, counteracting Map/EspF's deleterious impact on mitochondrial function or preventing EspF cleavage of EGFR unless these are Intimin-dependent events.

5.2 Aim

The aim of this chapter was to investigate the hypothesis that EspZ protects infected cells from Intimin-dependent cytotoxicity and, if true, to provide insight on the process.

5.3 Results

5.3.1 *mfz*₍₈₁₎*go3* mutant phenotypes linked to absence of EspZ delivery

To investigate whether loss of i) T'' form, ii) tyrosine dephosphorylated Tir and host proteins and iii) cytoplasmic tubulin and actin proteins were due to EPEC failing to deliver EspZ, HeLa cells were infected over a 9h period with EPEC, *espZ* and T3SS mutant strains before Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effectors) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions were isolated for Western blot analyses.

The T3SS mutant strain expressed Tir (Figure 64A, insoluble fraction) but did not, as expected, deliver it (or EspB or EspF) into host cells (Figure 64A, soluble fraction). Similar gel loadings and absence of cytotoxicity over the first 7 hours of infection was supported by probing for tubulin (mostly in soluble fraction) and actin (~70% in soluble fraction) with the 9h infection period linked to tubulin/actin signal loss (Figure 64A). Probing for tyrosine phosphorylated proteins revealed a single prominent band in the insoluble fraction linked to the EPEC protein Ep85 (also known as Etk) (Rosenshine *et al.*, 1992, Kenny & Finlay, 1997, Ilan *et al.*, 1999) with infection linked to dephosphorylation of host tyrosine phosphorylated proteins in the soluble fraction by 7h post-infection. The latter findings reveals a T3SS-independent mechanism that induces dephosphorylation of HeLa cytoplasmic/membrane proteins and precedes a cell cytotoxicity response. These changes were linked to bacterial overgrowth - associated media acidification - which could be avoided by changing the media every 3hrs or decreasing the infection time (to 6hr; not shown but see below).

By contrast, EPEC and *espZ*₍₈₁₎ strains delivered Tir, as evidenced by detecting T'' form in the soluble fractions (Figure 64C), but whereas the T'' form was evident in the soluble fractions of EPEC infected cells at all, but not the 9hr time point, it was absent from most of the corresponding samples from *espZ*₍₈₁₎-infected cells linked to increasing T' and/or unmodified Tir forms (Figure 64C). This change in Tir signal was associated with tubulin/actin signal loss linking it to cell cytotoxicity (Figure 64C); latter supported by absence of most cells (~70%) at final (9h) infection time point. Interestingly, no corresponding loss of EspB and EspF signals was evident from the soluble fractions suggesting that they are mainly membrane inserted/associated and/or

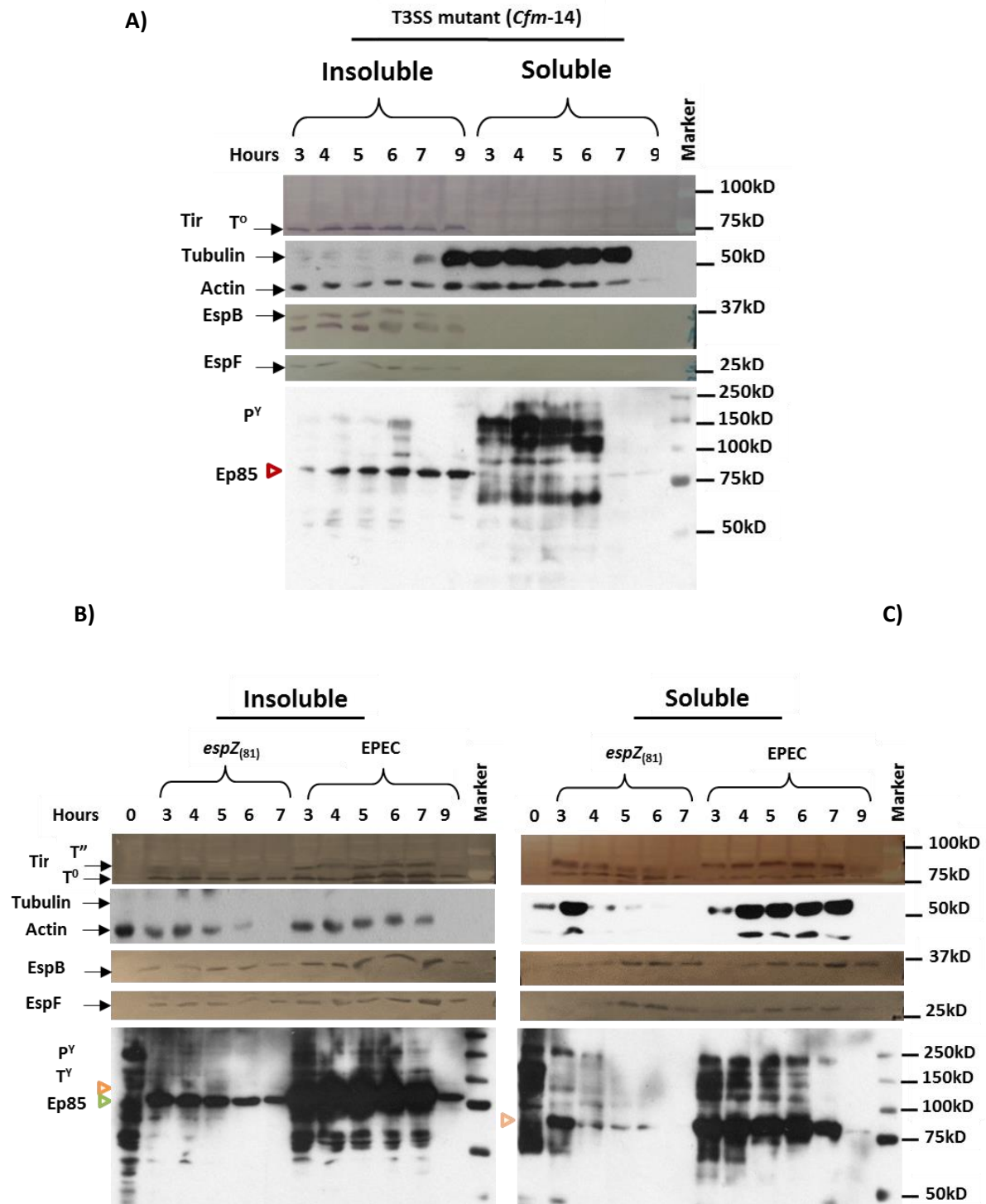


Figure 64: T3SS-independent and -dependent loss of Tir T'', phosphotyrosine proteins and tubulin/actin signals in *espZ*₍₈₁₎-infected cells. HeLa cells were left uninfected or infected with indicated strains, for indicated times, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T[°]) and host kinase modified Tir (T'' and P^Y [T^Y]) forms are indicated as are Ep85 (EPEC tyrosine-phosphorylated cytoplasmic protein), EspF, EspB, tubulin, actin and protein markers bands. Strains used were EPEC, T3SS-deficient (T3SS/*cfm-14*) and EspZ-deficient (*espZ*₍₈₁₎). Note, data is representative of that from two or more independent experiments.

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within organelles, for example, EspF accumulates within mitochondria (Nagai *et al.*, 2005). As expected, the phosphotyrosine antibodies detected Tir and host proteins in the soluble fractions from *espZ* and EPEC-infected cells but with a rapid dramatic loss in these signals, over time, from *espZ₍₈₁₎*-infected cells (Figure 64C). Interestingly, while the Tir T^Y modified form migrated (due to binding Intimin) into the insoluble fraction of EPEC-infected cells at each time point, there was little evidence of this for *espZ*-infected cells suggesting that Tir is no longer available to, or cannot stably, interact with Intimin (Figure 64B). It should be noted that strain binding levels are reflected by Ep85, EspB, EspF and unmodified Tir (within bacteria) signals in insoluble fractions, with Ep85 in samples from EPEC-infected cells obscured by strong phosphotyrosine Tir band (Figure 64B). This work links the T3SS-dependent loss of i) T^Y form, ii) tyrosine dephosphorylated Tir and host proteins and iii) cytoplasmic tubulin/actin proteins to the failure of EPEC to deliver EspZ in cells leading to a cytotoxic response.

5.3.2 Critical role for Intimin, Map and EspF proteins in the *espZ*₍₈₁₎-associated phenotypes

As our previous work suggested that the, above described, *espZ*₍₈₁₎-associated phenotypes could be prevented by inactivating the Intimin gene, with most Intimin subversive activities relating to it binding Tir (Kenny & Finlay, 1997, Kenny *et al.*, 1997b, Kenny *et al.*, 2002b, Rosenshine *et al.*, 1996), studies examined the impact on infecting cells with EspZ-deficient strains unable to express other effectors and/or Intimin. These studies were restricted to a 6 h period; which is sufficient to reveal the T3SS-dependent cytotoxicity-associated phenotypes linked to *espZ*₍₈₁₎ infection (Figure 64). The screened strains and resulting western blot data (representative blots shown in Figure 65A-D) are summarised in Table 9. HeLa cells were infected with control strains, EPEC and Intimin-deficient (*eae*) mutant, for only 3 and/or 6 hr with 3, 4, 5 and 6hr infections for strains lacking EspZ alone (*espZ*₍₈₁₎) or with other effectors (Tir, Map, EspH, EspF, EspG/Orf3) and/or Intimin proteins.

Strain	Figure #	3h	4h	5h	6h	Notes
EPEC	64, 65A/C/D	E	E	E	E	T'' is stable, No pTyr nor tub/actin loss
<i>espZ</i> ₍₈₁₎	64, 65A/C	E	Δ*●○	Δ*●○	Δ*●○	
<i>fz:km</i>	65A	E	Z	Z	Z	Slower T'' and tub/actin loss
<i>mz:km</i>	65A	E	Z	Z	Z	Faster pTyr proteins loss; slower tub/actin loss
<i>go3z:km</i>	65A	E	Z	Z	Z	Faster pTyr proteins and tub/actin loss
<i>hz:km</i>	65A	E	Z	Z	Z	Faster pTyr proteins and tub/actin loss
<i>ez:km</i>	65B	E	E	E	E	Loss pTyr proteins at 5/6h
<i>m fz:km</i>	65B	E	E	Z	Z	Loss pTyr proteins & Tir T'' at 5/6h & tub/act 6h
<i>m fz:kmgo3</i>	65C	E	E	Z	Z	Loss tub/actin 5/6h and pTyr proteins 5/6h
<i>m ez:km</i>	65D	E	E	E	E	Loss of pTyr proteins & some Tir T'' at 6h
<i>fz:km^e</i>	69	E	E	E	E	Loss of pTyr proteins at 6h
<i>m fz:km</i>	65B/D	E	E	E	E	
<i>m fz:kmgo3^e</i>	65C	E	E	E	E	

Table 9: Summary of differences between cells infected with EspZ-deficient and EPEC strains.

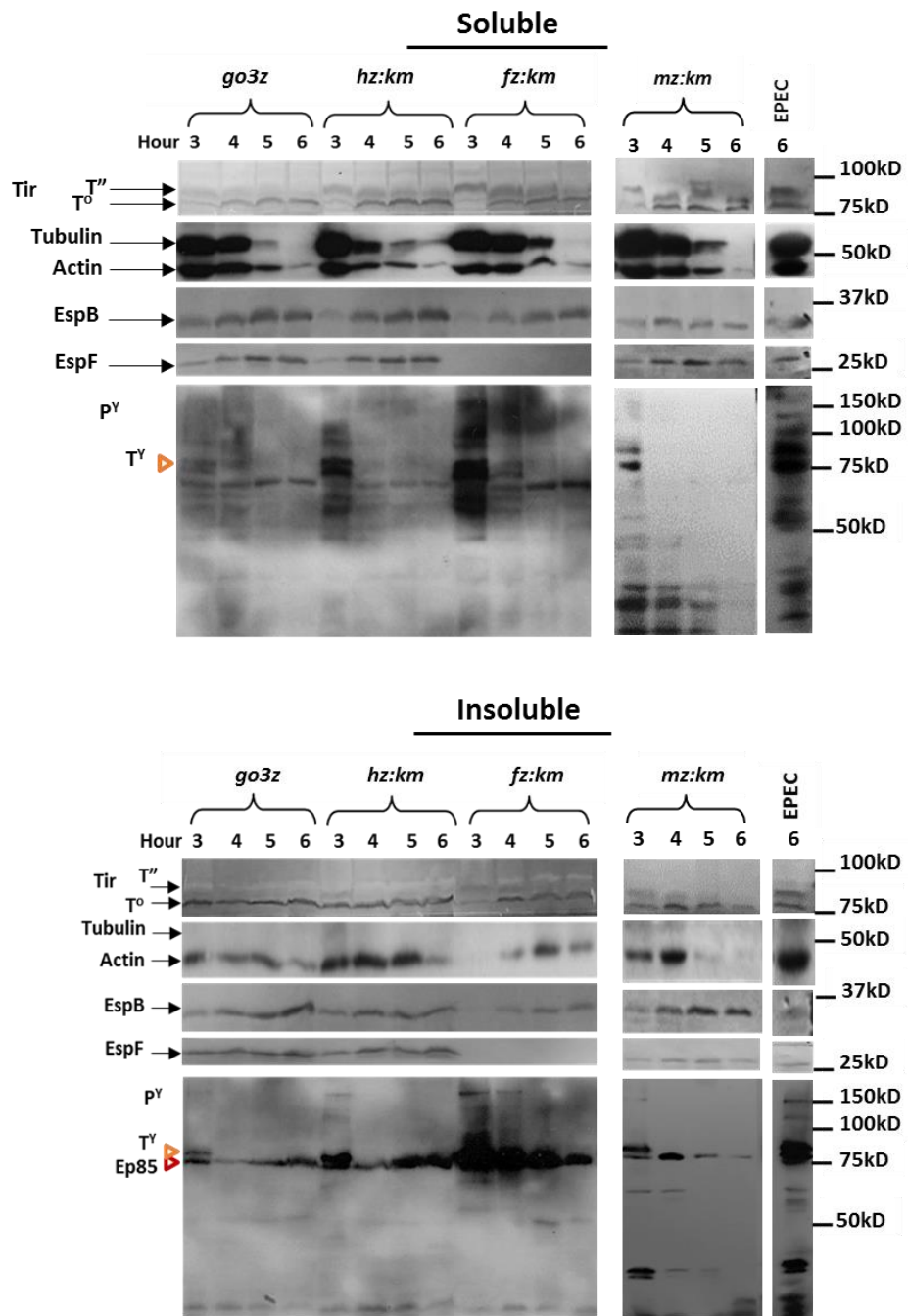
Summary of western blot data from HeLa cells infected with indicated strains after 3, 4, 5 and 6h. Strains used were EPEC or those unable to express EspZ alone (*espZ*₍₈₁₎) or EspZ plus one or more of indicated effectors – EspF (*f*), Map (*m*), EspH (*h*), EspG/EspG2 (*go3*) – and/or Intimin (*e*) proteins. E indicates normal T3SS dependent changes at indicated time point, while Z indicates data similar to that obtained with the *espZ*₍₈₁₎-infected samples at that time point with, when present, some differences in probed signals (Δ reveals dephosphorylation of Tir and host tyrosine phosphoproteins; * loss to Tir T'' band, ● loss of tubulin (tub) signal, and ○ loss of actin signal). Orange highlights *espZ* multi-mutants associated with minor phenotypic changes at later time points. Yellow highlights *espZ* multi-mutants that behave like EPEC. The *espZ* gene was inactivated by deleting 81 (of 98) residues or introducing kanamycin-encoding gene indicated by ₍₈₁₎ and :*km* respectively.

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Interestingly, most *espZ* multiple mutants behaved like the *espZ*₍₈₁₎ single mutant (Table 9; Figure 65) but there were some notable, subtle and dramatic differences. The former include an apparent delay in Tir T'' and tubulin/actin signal loss by the *fz* and, to a lesser extent, *hz* mutants suggestive of EspF and EspH roles in promoting these changes. By contrast, the *mz* and *go3z* mutants were linked to a more rapid loss of the Tir T'' form and slower tubulin/actin loss suggestive of roles for Map and the EspG homologues in hindering and promoting these events respectively (Table 9; Figure 65A). A more dramatic difference was evident with the remaining strains (*ez*, *mfz*, *mez*, *fze mfez*, *mfzgo3* and *mfzgo3e* multi-mutants) which behaved like EPEC though three (*ez*, *fze* and *mez*) were associated with one or more minor phenotypes at later time points (Table 9; Figure 65B-D). The EPEC-like profile for the *ez* double mutant is suggestive of a central role for Intimin functionality in all the phenotype, except tyrosine protein dephosphorylation where it appears to play a suppressive role. The latter is presumably mediated by Map and EspF activities as no tyrosine protein dephosphorylation is apparent with the *mfez* mutant in contrast to some for triple mutant-infected cells *mez* and *fze* (Table 9; Figure 65BD and 69). Interestingly, the *mfz* triple mutant is only associated with little loss of the Tir T'' form linked to some loss in tyrosine phosphorylated protein and tubulin/actin signals (5/6h time point). The latter suggests that Map and EspF promote loss of the Tir T'' form with roles for other effectors, presumably the EspG homologues as the *mfzgo3* mutant does not induce Tir T'' loss (Table 9; Figure 65C). Crucially, two *espZ*-deficient strains (*mfez* and *mfzgo3e*) failed to display any *espZ* mutant-associated phenotypes revealing key cooperative roles for Intimin, Map and EspF activities.

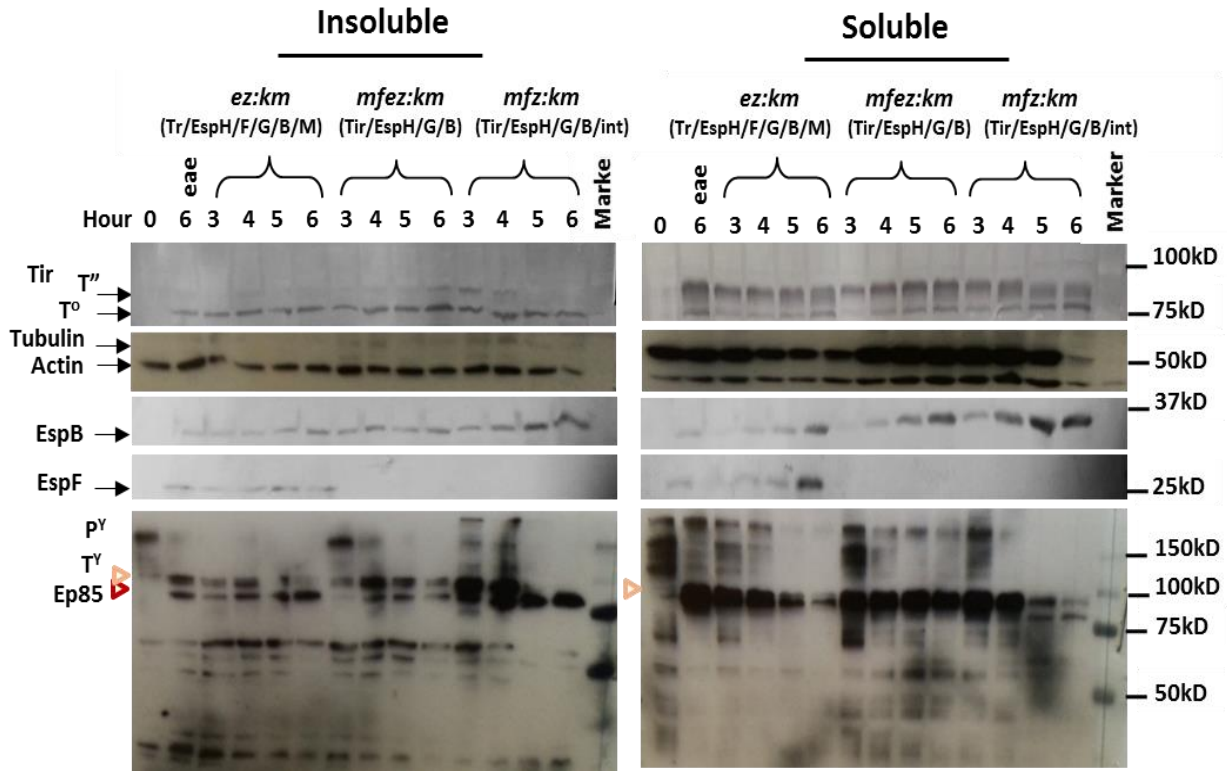
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A)

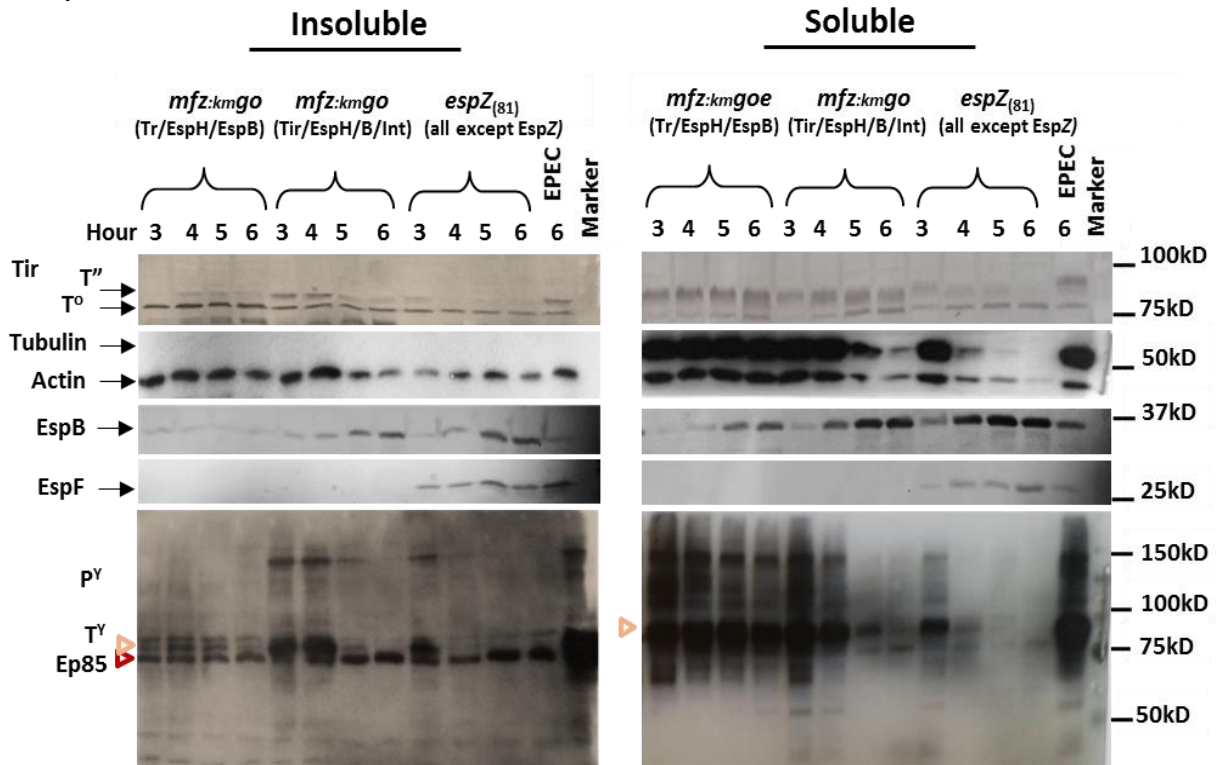


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B)



C)



D)

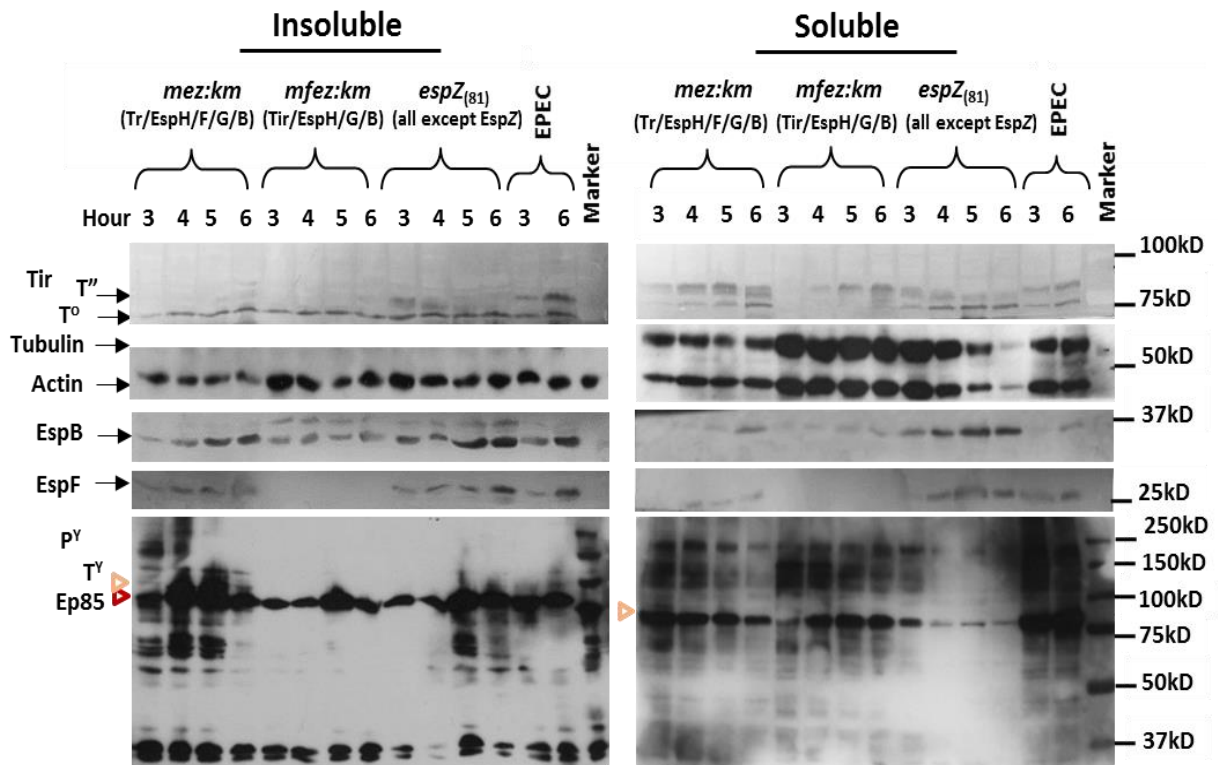


Figure 65: Assessment of *espZ* multi-mutants. HeLa cells were left uninfected or infected with indicated strains, for indicated times, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified Tir (T^{''} and P^Y [T^Y]) forms are indicated as are Ep85 (EPEC tyrosine-phosphorylated cytoplasmic protein), EspF, EspB, tubulin, actin and protein markers. Strains used were EPEC strains lacking EspZ (*espZ₍₈₁₎* or *z*) alone and one or more of the indicated proteins: Map (*m*); EspF (*f*), Tir (*t*), EspH (*h*), EspG/EspG2 (*go3*) or Intimin (*e*). Below the strain name (note indicates order genes were inactivated) is the identity of proteins of interest (Tir, Map [M], EspH, EspZ, EspF [F], EspG/EspG2 [G], EspB [B] effector and/or Intimin [Int] proteins) expressed by the strain. The *espZ* gene was inactivated by deleting 81 (of 98) residues or introducing kanamycin-encoding gene indicated by ₍₈₁₎ and :km respectively. Note, data is representative of that from two or more independent experiments.

5.3.3 Restoring *espZ* associated-phenotypes of *mfez:km* mutant by complementing with Map, EspF or Intimin-expressing plasmids

The previous work suggested possible roles for EspF, Map and/or Intimin proteins in the *espZ*₍₈₁₎-associated phenotypes. To support this premise, plasmids carrying genes for Map, EspF or Intimin were, individually, introduced into the *mfez:km* (lacks Map, EspF, Intimin and EspZ proteins) mutant (see Chapter 2; Section 2.2.2 & Table 5) to determine if ‘rescued’ the defect to a level found for the respective triple mutants (Table 9). PCR analyses supported *mfez:km* mutant genotype and plasmid introduction of the appropriate gene (Figure 66).

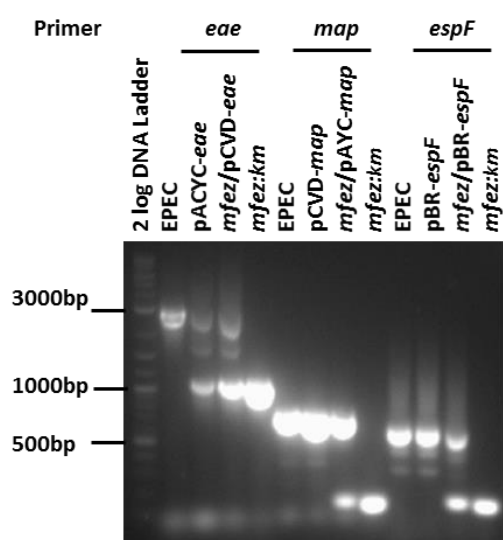


Figure 66: Absence of *eae*, *espF* and *map* genes from the *mfez:km* mutant and plasmid reintroduction of individual genes. Primers specific for the *eae*, *map* and *espF* genes were used to amplify DNA from the indicated strains or, as a control, purified plasmids (used to transforming strains) encoding Intimin (pCVD-*eae*), Map (pACYC-*map*) or EspF (pBR-*espF*). Strains examined were EPEC, the *mfez:km* mutant (missing *map*, *espF*, *eae* and *espZ* genes; Km indicated antibiotic cassette within *espZ* gene). PCR products were run on 1% Gel with molecular weight makers (2 log DNA ladder) visualized using Red Nucleic Acid Stain.

Western blot analysis of Triton X-100 soluble and insoluble fractions from HeLa cells infected with the Intimin complemented strain (*mfez/p-eae*) confirmed an EPEC-like phenotype for the *mfez:km* mutant (though some loss of host/Tir phosphotyrosine protein signal was evident at 6hr time point in this experiment) with plasmid expression of Intimin - supported by Western blot analysis – linked to a much more prominent impact on the T^Y form, host/Tir phosphotyrosine protein and tubulin/actin profiles (4-6hr; Figure 67). Tir (T^Y or T^Y^{pY} forms) were not evident, due to the absence of Intimin, in the insoluble fractions (Figure 67). This data supports a key role for Intimin in the *espZ*₍₈₁₎-associated phenotypes.

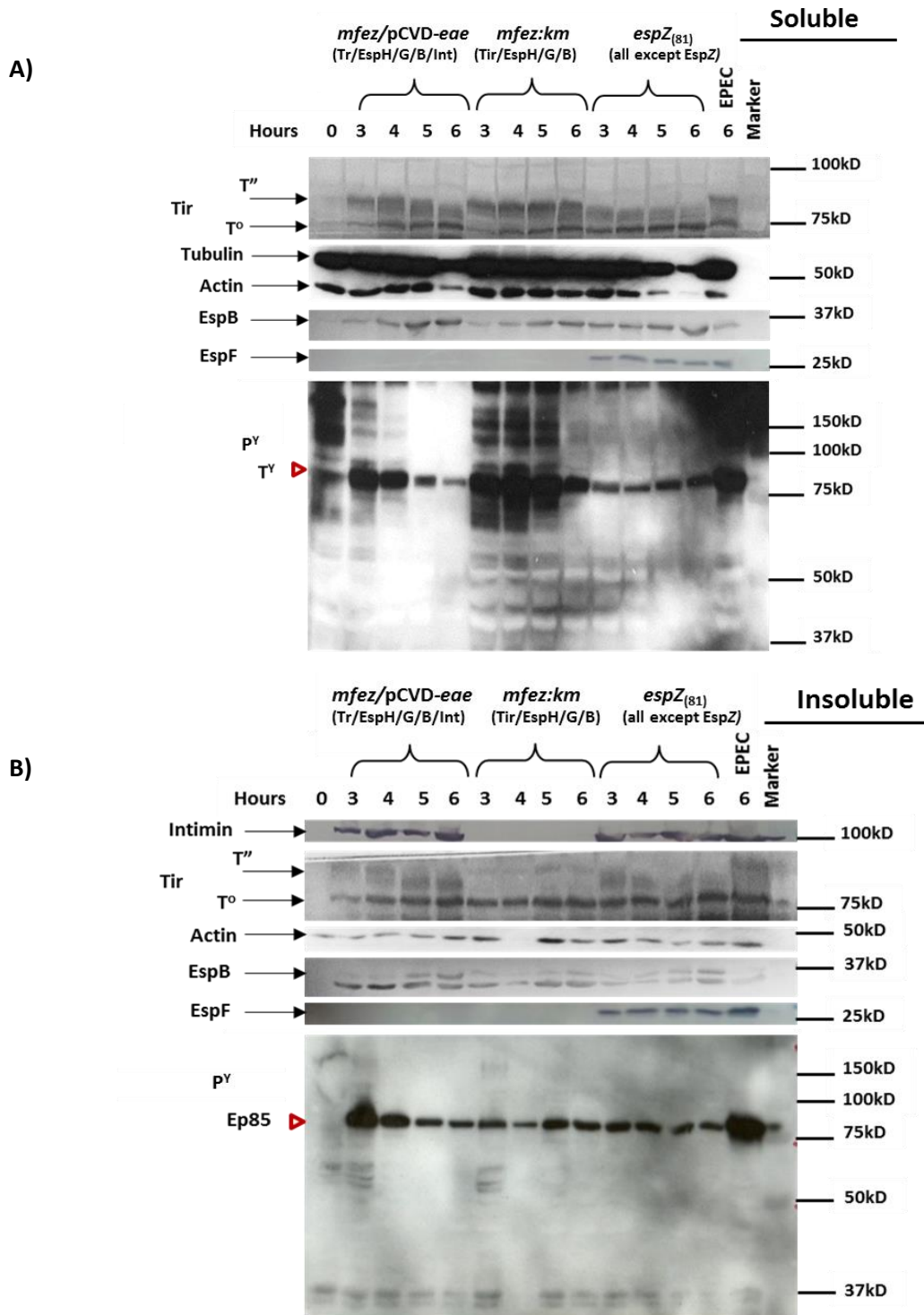


Figure 67: Critical role for Intimin to trigger Tir T^o to T^o' shift and Tir tyrosine dephosphorylation by EspZ-deficient strains. HeLa cells were left uninfected or infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PA gels) and probed for phosphotyrosine proteins (P^Y), Intimin, Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified (T^o' and T^o'^{P^Y}) Tir forms are indicated as are the positions of Ep85 (EPEC tyrosine-phosphorylated protein), Intimin, EspB, EspF, tubulin, actin and protein markers. Strains used were EPEC, *espZ*₍₈₁₎ and strains lacking 3 or 4 of the genes encoding Map (*m*), EspF (*f*), EspZ (*z*) and Intimin (*e*) with, when appropriate, strains carrying an Intimin-expressing plasmid (*pCVD-eae*). Note tyrosine dephosphorylation with *mfez:km* mutant at 6hr is unusual (see Figure 68 and 69).

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Studies with the EspF complemented strains confirmed an EPEC-like phenotype for the *mfez:km* mutant with the *mfez/p-F* equivalent triple mutant (*mez:km*) linked to loss of host and Tir phosphotyrosine protein signals at 6h time point (Figure 68). Importantly, the complemented (*mfez/pF*) strain mimicked, as predicted, the *mez:km* mutant supporting a role for EspF in promoting host and Tir phosphoprotein signal loss. Strain genotype was supported by EspF absence in samples from only the *mfez:km* infected cells (Figure 68). It should be noted that the timing and, perhaps, level of EspB and (when expressed) EspF signals was different between *mfez:km* (evident by 3hr in soluble fraction), *mez:km* (evident by 4hr) and *mfez/pF* (evident by 5hr; Figure 68).

EspF and Map proteins target the mitochondria, via mitochondrial targeting sequences [MTS], leading to loss of mitochondrial dysfunction hence host cell death (Nagai *et al.*, 2005, Kenny & Jepson, 2000, Papatheodorou *et al.*, 2006) and EspZ is proposed to inhibit the impact of these effectors on mitochondrial function (Shames *et al.*, 2011, Roxas *et al.*, 2012). In an attempted to examine whether EspF and Map associated phenotypes (in the absence of EspZ) are linked to their import to the mitochondria, the *mfez:km* mutant was complemented with plasmids encoding EspF variants unable to target mitochondria, due a single substitution; L16E (Nagai *et al.*, 2005) or replacing the N-terminal 101 residues - contains mitochondrial targeting sequence - with N-terminal 101 Tir residues (Quitard *et al.*, 2006). However, no defects were evident (data not shown) implicating dependence on other features.

Consistent with our previous findings, introducing the Map-encoding plasmid into the *mfez:km* mutant, generating an equivalent triple mutant (*fz:kmE*), was linked to loss of host and Tir phosphotyrosine proteins at later (4-6hr) time points (Figure 69). Strain genotype was supported by absence of EspF and Intimin protein signals (antibodies against EPEC Map were not available). Interestingly, the EspB levels in samples from cells infected with the complemented strain were greater than that from control *mfez:km*-infected cells (Figure 69), but similar to that in the *fz:kmE* infected cells. Complementation studies were also undertaken with Map variants known to have subversive defects i.e. don't target mitochondria as N-terminal 41 residues - contains mitochondrial targeting sequence - replaced by N-terminal 101 Tir residues (*mapΔMTS*), can't activate Cdc42 (*mapE78A*) and can't sustain Map-triggered Cdc42 activity (*mapΔTLR* or *mapHA*); latter also enables antibody detection of Map (Kenny &

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Jepson, 2000, Papatheodorou *et al.*, 2006, Wong *et al.*, 2012a, Dean *et al.*, 2013). These studies revealed a significant defect for one variant, *map* Δ *MTS*, implicating a key role for Map targeting mitochondria or another N-terminal feature, though it is possible that the Tir domain prevents the responsible Map activity (Figure 70). Interestingly, expressing the other variants were linked to a faster and greater level of phosphotyrosine protein loss than the native, plasmid encoded, Map (Figure 70) suggesting that these features may hinder Map activity that promotes loss of phosphotyrosine protein signal. Probing for HA and Tir epitopes supported expression and delivery of MapHA and Tir_ Δ MTSMap proteins, respectively (Figure 71).

Collectively, these data support a major role for Intimin in the cytotoxicity-associated phenotypes of EspZ-deficient strains with Map and EspF proteins promoting dephosphorylation of host/Tir phosphotyrosine proteins dependent on the N-terminal, MTS containing, domain of Map but not EspF effector protein.

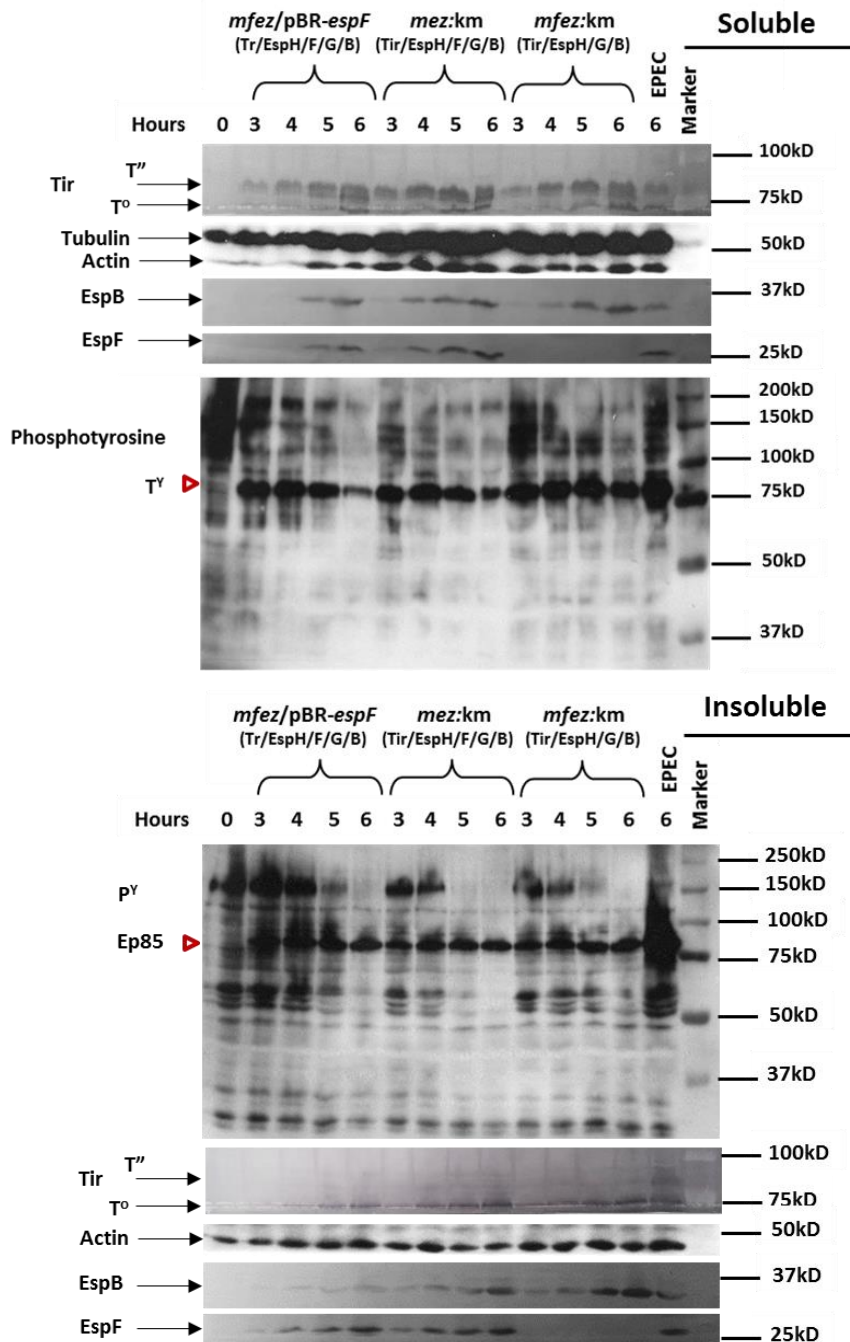


Figure 68: Minor role for EspF in the dephosphorylation of host and Tir tyrosine phosphorylated proteins following infection by EspZ-deficient strains. HeLa cells were left uninfected or infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PA gels) and probed for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified (T^Y and T^Y [T^Y]) Tir forms are indicated as are the positions of Ep85 (EPEC tyrosine-phosphorylated protein), EspB, EspF, tubulin, actin and protein markers. Strains used were EPEC and strains lacking 3 or 4 of the genes encoding Map (*m*), EspF (*f*), EspZ (*z*) and Intimin (*e*) with, when appropriate, strains carrying a plasmid encoding for EspF (pBR-*espF*).

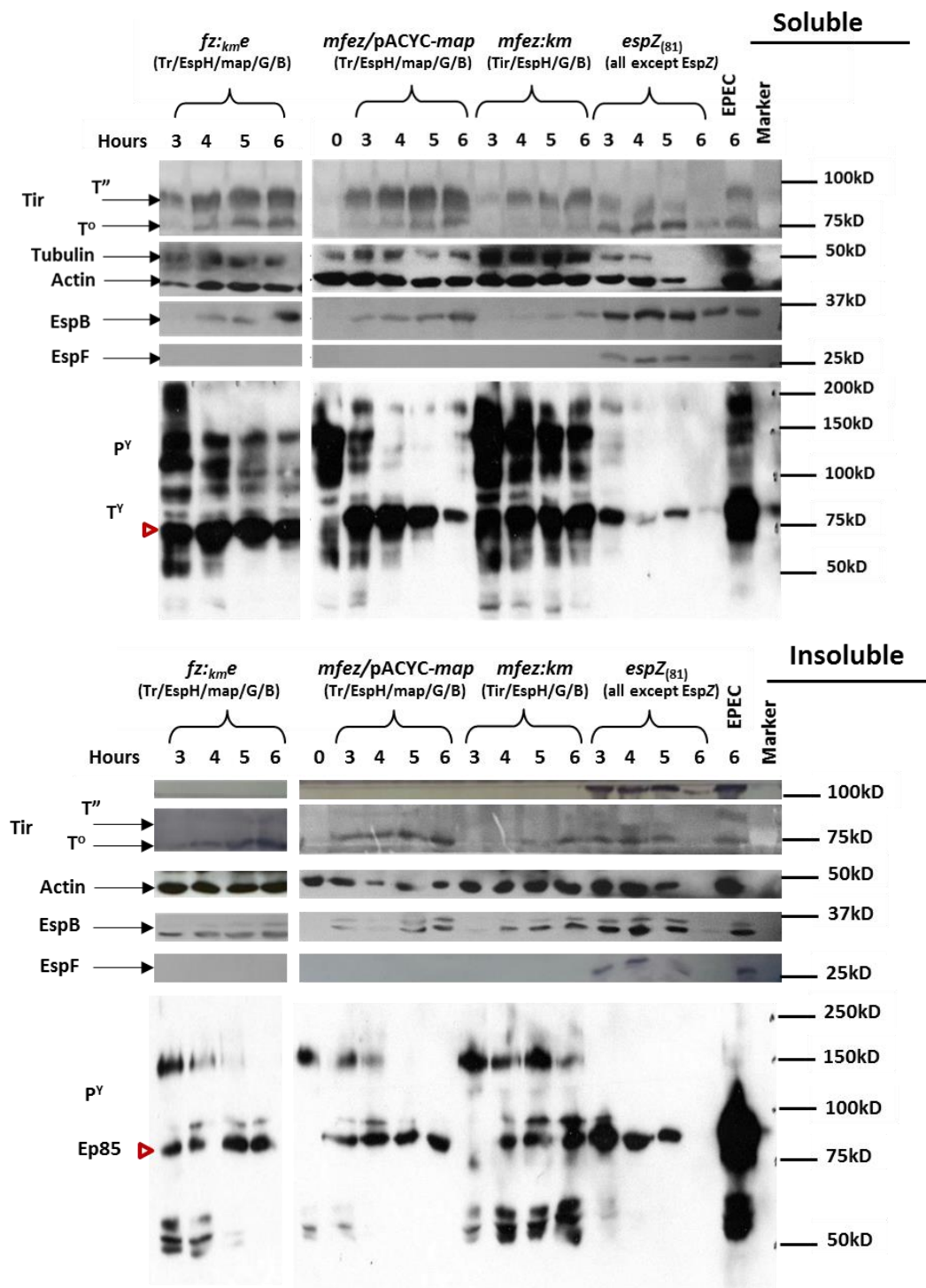


Figure 69: Prominent role for Map in triggering tyrosine dephosphorylation of host proteins of EspZ/Intimin-deficient strains. HeLa cells were left uninfected or infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PA gels) and probed for phosphotyrosine proteins (P^Y), Intimin, Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified (T'' and T''^{pY} [T^Y]) Tir forms are indicated as are the positions of Ep85 (EPEC tyrosine-phosphorylated protein), Intimin, EspB, EspF, tubulin, actin and protein markers. Strains used were EPEC, *espZ₍₈₁₎* mutant and strains lacking 3 or 4 of the genes encoding Map (*m*), EspF (*f*), EspZ (*z*) and Intimin (*e*) with, when appropriate, strains carrying a plasmid encoding for Map (*pACYC-map*)

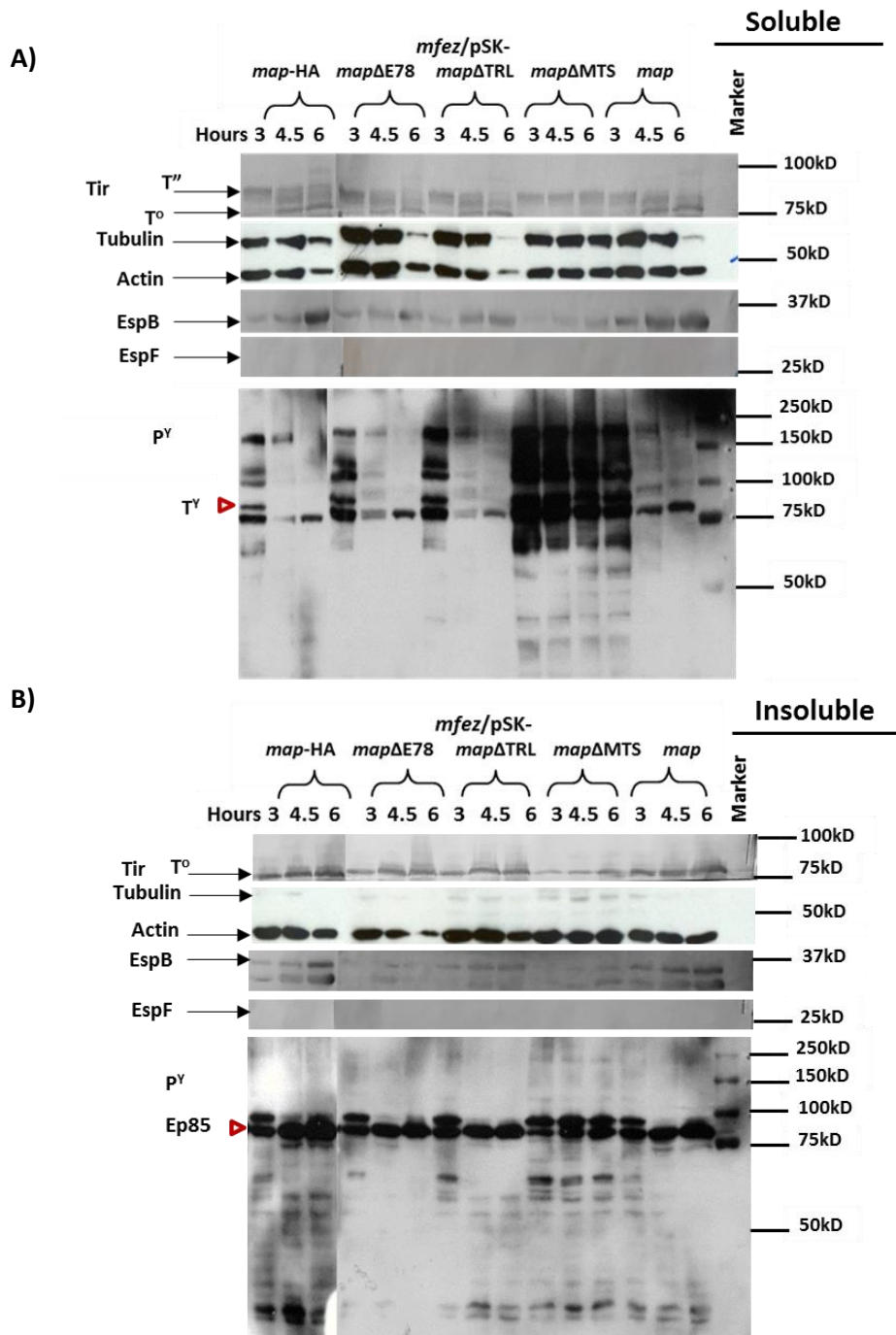


Figure 70: Map triggered *espZ* associated Tir/host protein tyrosine dephosphorylation phenotypes is N-terminal dependent. HeLa cells were infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PA gels) and probed for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified (T^o and T^oP^Y [T^Y]) Tir forms are indicated as are the positions of Ep85 (EPEC tyrosine-phosphorylated protein), EspB, EspF, tubulin, actin and protein markers. Strains used were *espZ* quadruple (*mfez*) mutant (lacking genes encoding Map (*m*), EspF (*f*), EspZ (*z*) and Intimin (*e*) carrying a plasmid encoding for Map (pSK-*map*, pSK-*map*-HA) or Map variant that is missing mitochondrial targeting sequence (*map*ΔMTS), as N-terminal 41 residues - contains mitochondrial targeting sequence - replaced by N-terminal 101 Tir residues, (*map*ΔE78A) can't activate Cdc42 and (*map*ΔTRL) can't sustain Map-triggered Cdc42 activity. The immunoblots (where appropriate) cropped and moved for presentation purposes. Note, data is representative of that from two or more independent experiments.

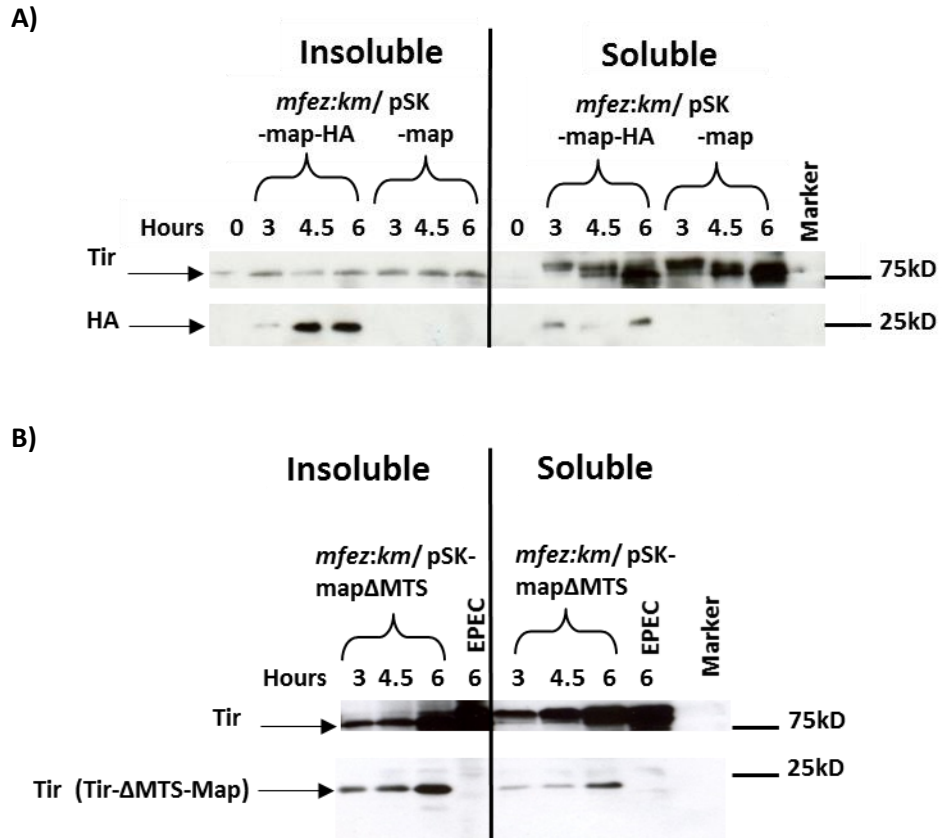


Figure 71: Expression and delivery of MapHA and Tir- Δ MTS-Map fusion proteins. Sample from previous experiment were processed for Western blot analysis (10% SDS-PA gels) and probed for **A)** HA-tagged or **B)** Tir protein. The position of Tir, HA-tag or Tir- Δ MTS-Map (Map-MTS is swapped with N-terminal domain of Tir effector protein) are indicated as are the position of the protein markers. Strains used were as described in Figure 69.

5.3.4 Critical role for Tir in triggering *espZ*₍₈₁₎ associated phenotypes

Intimin subverts host cellular process through binding mammalian receptors, such as beta Integrin and nucleolin, or Tir (Frankel *et al.*, 1996, Sinclair *et al.*, 2006, Kenny & Finlay, 1997, Kenny *et al.*, 1997b). Thus, we decided to examine whether Tir has a possible role in triggering or suppressing *espZ*₍₈₁₎ phenotypes. Therefore, a *z*₍₈₁₎*t* double mutant strain was generated (see Chapter 2; Section 2.2.7), PCR confirmed (data not shown) and HeLa cells were then infected with *espZ*₍₈₁₎ and *z*₍₈₁₎*t* mutant strains for indicated time course with Triton X-100 soluble and insoluble fractions isolated for analyses. It should be noted that, since all *espZ*₍₈₁₎ phenotypes have been detected between 3 and 6hrs post infection with no real differences observed between 4 and 5hr, thus, this strain was examined at only 3, 4.5 and 6hrs post-infection time points.

Western blot analysis showed, as expected, no evidence for Tir expression nor delivery for cells infected with the *z*₍₈₁₎*t* mutant strain (Figure 72), supporting strain genotype. Interestingly, probing with anti-tubulin and actin showed no change in the amount of proteins in cells infected with *z*₍₈₁₎*t* mutant at all-time points. Similarly, probing with anti-phosphotyrosine revealed no tyrosine dephosphorylation event (noting Tir is missing in this strain) was evident in *z*₍₈₁₎*t* mutant infected cells (3hr) with >3hr, a slight host proteins dephosphorylated was observed, confirming a possible role for other effector proteins, Map and/or EspF, to induce this phenotype (Figure 72).

Collectively, these data suggested that Tir seems to have an important role in triggering *espZ*₍₈₁₎ phenotypes, via/or with not Intimin, with cooperative roles for other EPEC effector proteins (presumably Map and EspF).

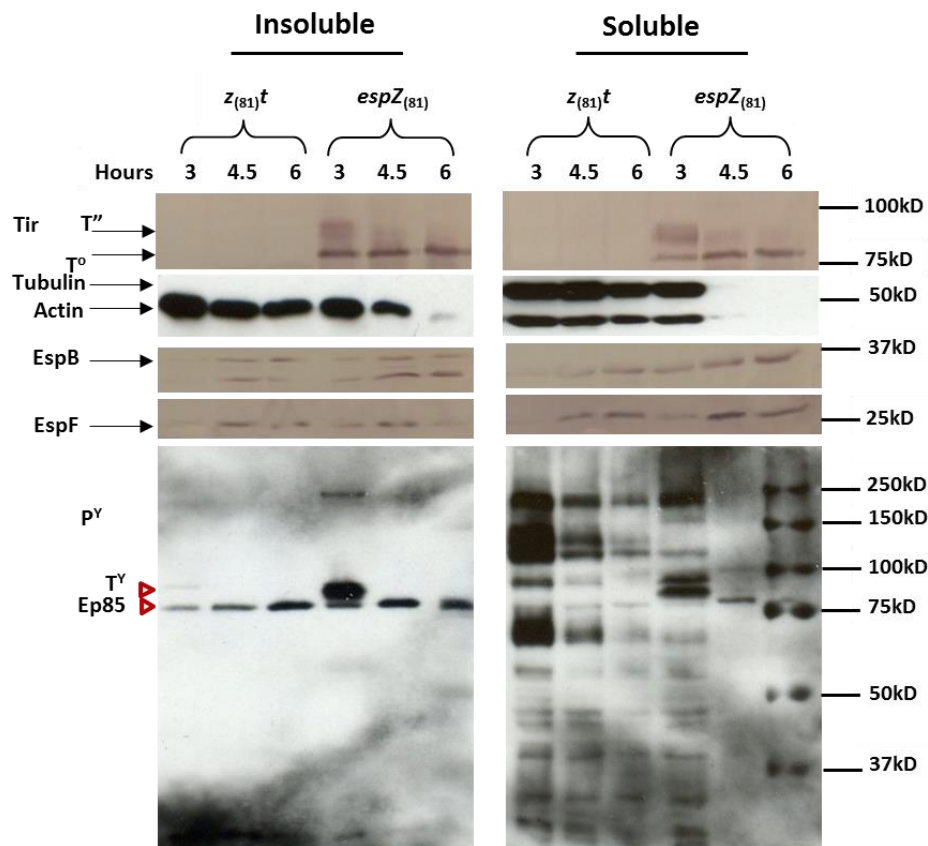


Figure 72: Tir is critical for *espZ*₍₈₁₎ associated phenotypes. HeLa cells were left uninfected or infected with indicated strains, for indicated times, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified Tir (T^{''} and P^Y [T^Y]) forms are indicated as are Ep85 (EPEC tyrosine-phosphorylated protein), EspF, EspB, tubulin, actin and protein markers bands. Strains used were EspZ-deficient (*espZ*₍₈₁₎) mutants and Tir/EspZ-deficient strain (*z*₍₈₁₎*t*). The *espZ* gene was inactivated by deleting 81 (of 98) residues and indicated by ₍₈₁₎, while *tir* was inactivated by deleting the whole gene. Note, data is representative of that from two or more independent experiments.

5.3.5 Supporting roles for Map and EspF in $z_{(81)}t$ mutant associated phenotypes

To test the hypothesis that dephosphorylation events detected at latest time point infection with the $z_{(81)}t$ mutant (Figure 72) are linked to other EPEC effectors, presumably Map and/or EspF, we examined $z_{(81)}t$ multiple mutant strains missing one or more EPEC LEE effector proteins. The screened strains and resulting western blot data (representative blots shown in Figure 72&73) are summarised in Table 10.

Strain	Figure #	3h	4h	5h	6h	Notes
EPEC	64, 65A/C/D	E	E	E	E	T ^{''} is stable, No pTyr nor tub/actin loss
$espZ_{(81)}$	72	E	Δ*•○	Δ*•○	Δ*•○	
$z_{(81)}t$	72	iE	iE	iE	Z	Slower pTyr proteins loss at 5/6h
$etz:km$	73B	iE	iE	iE	Z	Slower pTyr loss at 5/6h
$mtz:km$	73A	iE	iE	iE	Z	Slower pTyr and tub loss at 5/6h
$emtz:km$	73A	iE	iE	iE	Z	Slower pTyr loss at 5/6h
$etfz:km$	73A	iE	iE	iE	Z	Slower pTyr loss at 6h
$tfz:km$	73A	iE	iE	iE	iE	
$tmfz:km$	73B	iE	iE	iE	iE	
$mfz_{(81)}go3t$	74	iE	iE	iE	iE	

Table 10: Summary of differences between cells infected with EspZ-deficient and Tir/EspZ negative mutants strains. Summary of western blot data from HeLa cells infected with indicated strains after 3, 4, 5 and 6h. Strains used were $espZ_{(81)}$ mutant or those unable to express EspZ alone ($espZ_{(81)}$ mutant) or EspZ plus Tir with one or more of indicated effectors – EspF (*f*), Map (*m*), EspG/EspG2 (*go3*) – and/or Intimin (*e*) proteins. E indicates normal T3SS dependent changes at indicated time point. While **iE** indicates normal T3SS dependent changes, except Tir data, as these mutants lack Tir, **Z** indicates data similar to that obtained with the $espZ_{(81)}$ -infected samples at that time point with, when present, some differences in probed signals (Δ reveals dephosphorylation of Tir and host tyrosine phosphoproteins; * loss to Tir T^{''} band, • loss of tubulin (tub) signal, and ○ loss of actin signal). Orange highlights $espZ$ multi-mutants associated with minor phenotypic changes at later time points. Yellow highlights $espZ$ multi-mutants that behave like EPEC. The $espZ$ gene was inactivated by deleting 81 (of 98) residues or introducing kanamycin-encoding gene indicated by $_{(81)}$ and $:km$ respectively.

As expected, Western blot analysis showed no evidence for Tir expression in the soluble/insoluble fractions of all infected strains, as these strains are missing Tir (Figure 73). Interestingly, while no evidence for tubulin and actin proteins loss in the soluble/insoluble fractions of all infected strains, >4h infection with $mtz:km$ led to some tubulin loss (Figure 73AB). Probing with anti-phosphotyrosine revealed that infection for >4hr with $etz:km$, $emtz:km$ or $mtz:km$ mutant strains (lacking Tir and EspZ with Map or Intimin or both) led to host proteins being dephosphorylated at later time points (Figure 73A). Interestingly, deleting EspF from *tz* mutant ($tfz:km$, $etfz:km$ and $tmfz:km$) showed little if any host protein dephosphorylation (Figure 73B). The latter suggest that EspF promote loss of the host protein phosphorylation with possible role for other effectors, presumably the EspG homologues, as the $mfz_{(81)}go3t$ mutant does not induce this phenotype (Table 10; Figure 74). While a similar EspB profile was observed

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in all soluble and insoluble fractions, EspF was only detected in cells infected with EspF positive strains (Figure 73AB).

Collectively, these data (with our previous work findings) support a critical role for Tir and/or Intimin (via or not Tir-Intimin interactions) in triggering *espZ*₍₈₁₎ phenotypes and suggested a cooperative role for EspF and Map in the absence of Intimin and/or Tir, to induce *espZ*₍₈₁₎ associated dephosphorylation event.

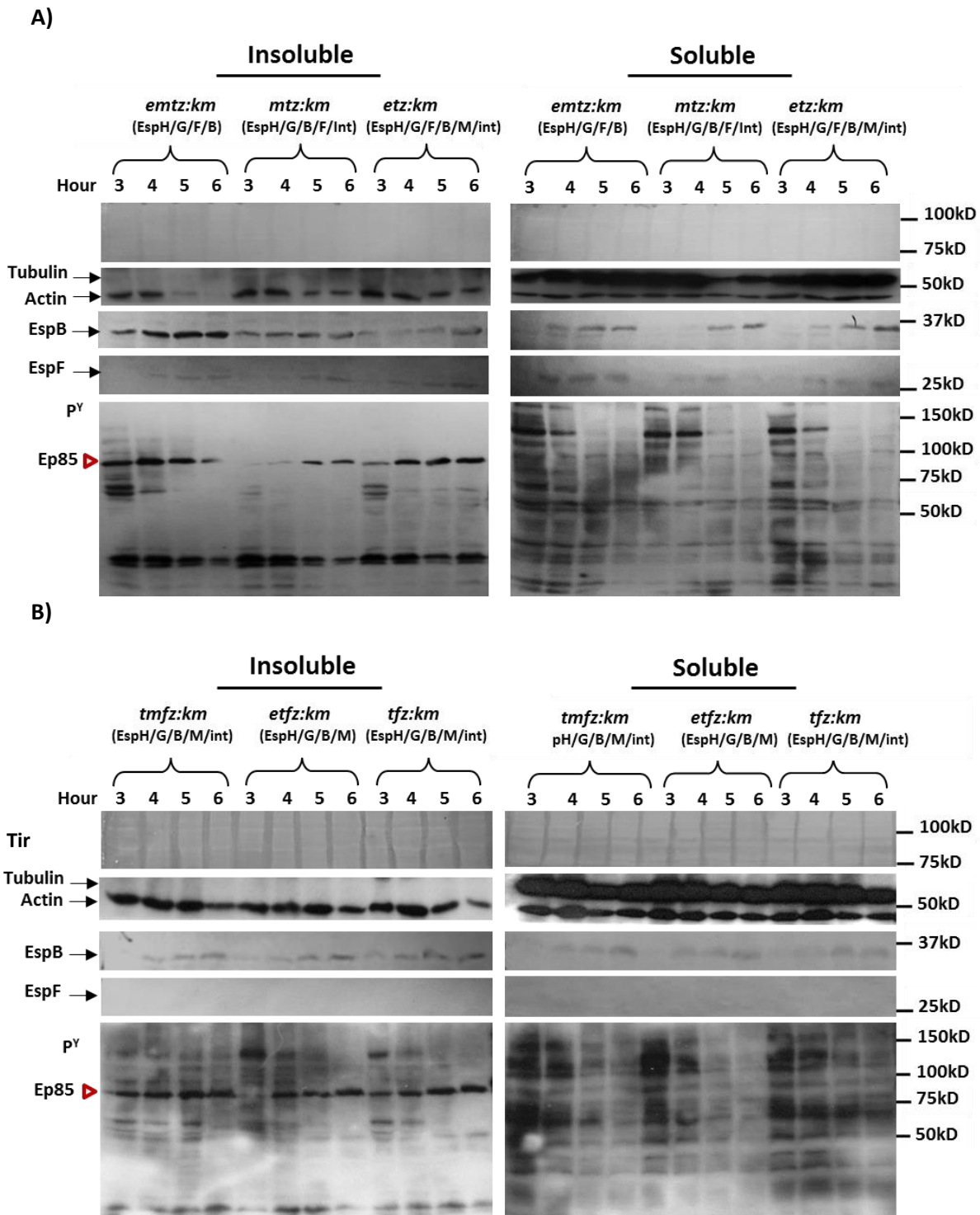


Figure 73: Assessment of Tir/EspZ-deficient mutant strains. HeLa cells were infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PA gels) and probed for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of Ep85 (EPEC tyrosine-phosphorylated protein), EspB, EspF, tubulin, actin and protein markers are indicated. Strains used were EPEC strains lacking EspZ (*espZ*₍₈₁₎ or *z*) and Tir with one or more of the indicated proteins: Map (*m*); EspF (*f*) or Intimin (*e*). Below the strain is the identity of proteins of interest (Map [M], EspH, EspF [F], EspG/EspG2 [G], EspB [B] effector and/or Intimin [Int] proteins) expressed by the strain. The *espZ* gene was inactivated by introducing kanamycin-encoding gene indicated by :km.

5.3.6 *espZ*₍₈₁₎ cytotoxicity-associated phenotypes depend on Tir with critical role for kinase modified residues

To examine the role of Tir features in the *espZ*₍₈₁₎ associated phenotypes, complementation studies were undertaken with the *mfz*₍₈₁₎*go3t* mutant which showed no *espZ*₍₈₁₎ associated phenotypes, in contrast to the *mfz*₍₈₁₎*go3* mutant (doesn't express EspZ, Map, EspF, EspG or EspG2 effectors) (Table 9; Figure 65).

Western blot analysis showed that *mfz*₍₈₁₎*go3* mutant infection was linked to loss of tubulin (didn't assess actin) and phosphotyrosine protein signals (at late time points; Figure 74) In contrast, the Tir-deficient variant (*mfz*₍₈₁₎*go3t*) appeared not to induce these alterations indicating a critical role for Tir for these events. Strain genotype was supported by absence of Tir bands in samples from *mfz*₍₈₁₎*go3t*, unlike *mfz*₍₈₁₎*go3*, mutant-infected cells (Figure 74). To gain insight on how Tir may be contributing to this process, complementation studies were undertaken with variants that were i) unable to interact with Intimin (TirHA_{into}; p3*gt*HA_{into}) as HA epitope tag within Intimin binding domain, ii) carrying a HA tag C-terminus with no known impact on Tir functionality or iii) kinase substrate residues serine (434 and 463) and tyrosine (454 and 474) substituted to alanine/phenylalanine (Tir Δ SY; p3*gt* Δ SY). Interestingly, only the TirHA variant rescued the *mfz*₍₈₁₎*go3t* mutant defect with complementation supported by Western detecting Tir (increased molecular mass when HA epitope tagged) and failure to detect Tir Δ SY variant with anti-phosphotyrosine antibodies (Figure 74). This data is suggestive of a key role for Tir-Intimin interaction and one or more of the examined Tir kinase phosphorylation substrates in the ability of EspZ-deficient strains to induce tubulin (and presumably actin) loss i.e. cytotoxicity and loss of tyrosine phosphorylated proteins.

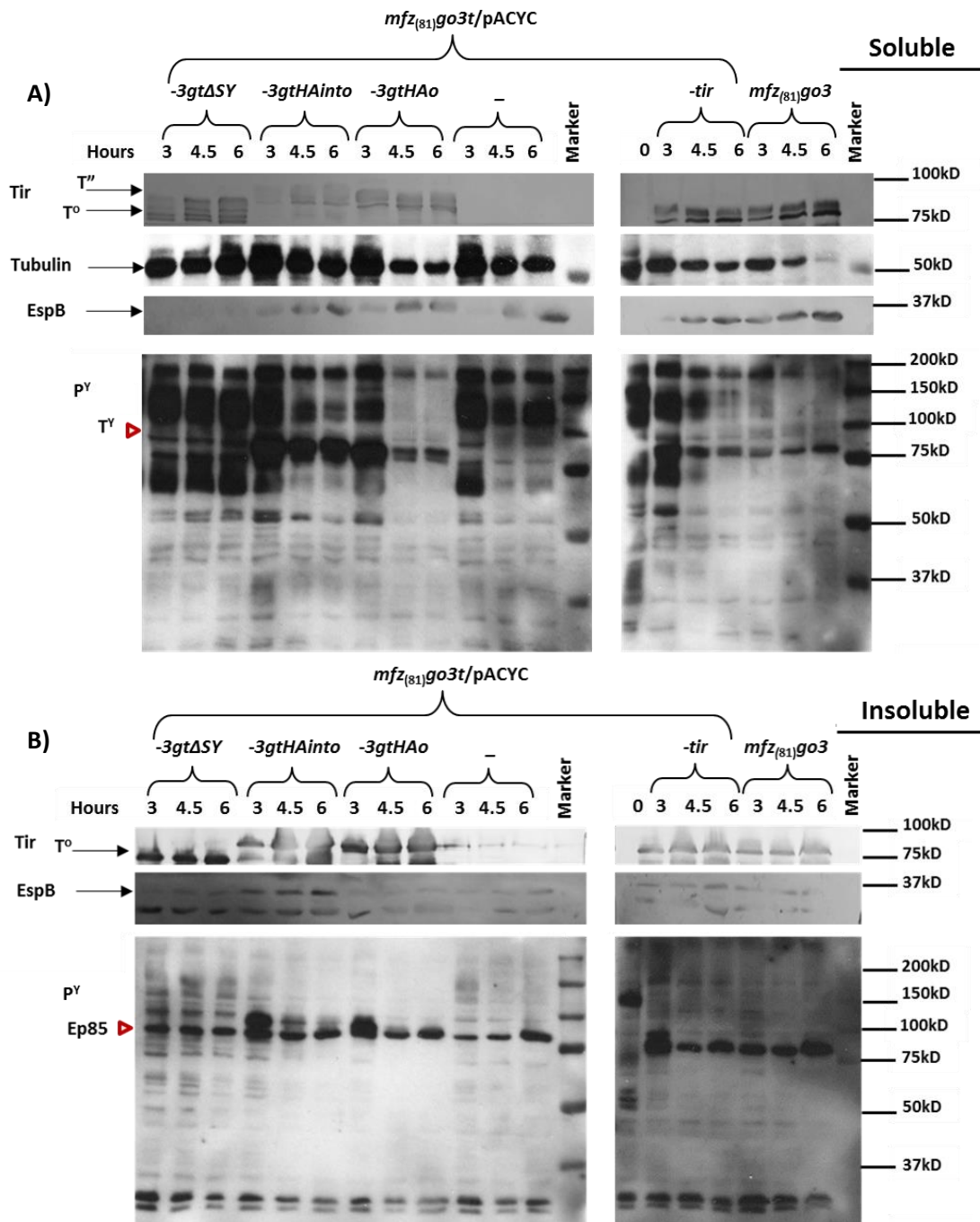


Figure 74: Intimin induced *espZ*₍₈₁₎ phenotypes is Tir dependent with critical role for serine and/or tyrosine residues. HeLa cells were infected with indicated strains (for indicated times) prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions. The samples were processed for Western blot analysis (10% SDS-PAGEs) and probed for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T⁰) and host kinase modified (T^{''} and T^{'''} [T^Y]) Tir forms are indicated as are the positions of Ep85 (EPEC tyrosine-phosphorylated protein), EspB, EspF, tubulin and protein markers. Strains used were *mfz*₍₈₁₎*go3* lacking EspZ (*espZ*₍₈₁₎ or *z*) with other indicated proteins: Map (*m*); EspF (*f*) and EspG/EspG2 (*go3*), *mfz*₍₈₁₎*go3t* as *mfz*₍₈₁₎*go3* but lacks Tir, and *mfz*₍₈₁₎*go3t* complemented with pACYC- *tir* (expressing Tir), pACYC-*3gtHAinto*, (expressing Tir with HA-taq inserted in the Intimin binding area (IBA) to prevent Tir-Intimin interaction), pACYC-*3gtHAo* (expressing Tir with HA-taq inserted in the C-terminal of Tir), or *tir*Δ*SY* (kinase phosphorylated serine (434 and 463) and tyrosine (454 and 474) residues substituted to alanine/phenylalanine). Note, data is representative of that from two or more independent experiments.

5.3.7 Additional complexity in the *espZ*₍₈₁₎ mutant phenotypes

Although our study with the *mfz*₍₈₁₎*go3e* and *mfz*₍₈₁₎*go3t* mutant strains (Figure 65 & 74) implicated a key role for Tir via Intimin in triggering *espZ*₍₈₁₎ associated phenotypes with contribution from EspF, Map and EspG, additional complexity was evident by an earlier observation that the *espZ* multiple mutant; *mfz*₍₈₁₎*go3eh:km* (As *mfz*₍₈₁₎*go3e* but lacks EspH [*h*]) behaved like the *espZ*₍₈₁₎ (Figure 75).

While Western blot analysis showed no evidence of the *espZ*₍₈₁₎ associated phenotypes was observed in cells infected with either EPEC or *mfz*₍₈₁₎*go3e* mutant (Figure 75), the *mfz*₍₈₁₎*go3eh:km* infection linked to a slower loss of Tir T'' form and tubulin/actin were detected at latter time point of infection (Figure 75). Furthermore, while EspB was detected in all loaded samples (Figure 75), EspF was absent from soluble and insoluble fractions of cells infected with either *mfz*₍₈₁₎*go3eh:km* or *mfz*₍₈₁₎*go3e* mutant, contrasting to an EPEC strain, which support strains' genotype (Figure 75).

This data suggestive of a possible suppressive role for EspH against other EPEC protein(s), presumably Tir or a non-LEE effector protein(s).

5.3.8 LDH release depends on *espZ* mutants expressing Tir or Intimin

Our previous results (Figure 65 & 72) revealed Intimin and Tir proteins are essential for triggering the *espZ*₍₈₁₎ associated phenotypes. To support this proposition, we quantified cellular cytotoxicity mediated by different *espZ*₍₈₁₎ strains (express or not Tir and/or Intimin proteins) using the Lactate dehydrogenase (LDH) assay. Thus, HeLa cells were infected with EPEC, T3SS mutant (*cfm-14*), *espZ* single or multiple mutants (lacking Tir and/or Intimin with or without other EPEC LEE effector proteins) before cell culture supernatants were isolated to quantify the level of released LDH. The findings are summarised in Table 11 and Figure 76.

As reported (Shames *et al.*, 2010), the T3SS mutant strain induced minimal host cell cytotoxicity, with ~10% of LDH enzyme released into the cell culture media by 6hr post infection (Figure 76). In contrast, cells infected with the EPEC strain was linked to more LDH release with ~18% of LDH enzyme released by 6hr post-infection (Table 11; Figure 76). In contrast, *espZ*₍₈₁₎ infection led to the greatest release of LDH enzyme with 20, 65 and ~90% of LDH enzyme being released after 3, 4.5 and 6hr infection periods, respectively (Table 11; Figure 76). Similar results were obtained with only one strain, *mfz*₍₈₁₎*go3* mutant (lacks four EPEC LEE effector proteins and EspG2, but has both Tir and Intimin) (Table 11; Figure 76). Interestingly, deleting either Tir or Intimin from all examined *EspZ* deficient, including *mfz*₍₈₁₎*go3* led to an EPEC-like profile (Table 11; Figure 76). This finding illustrates a critical role for Tir and Intimin proteins to induce *espZ*₍₈₁₎ associated cytotoxicity.

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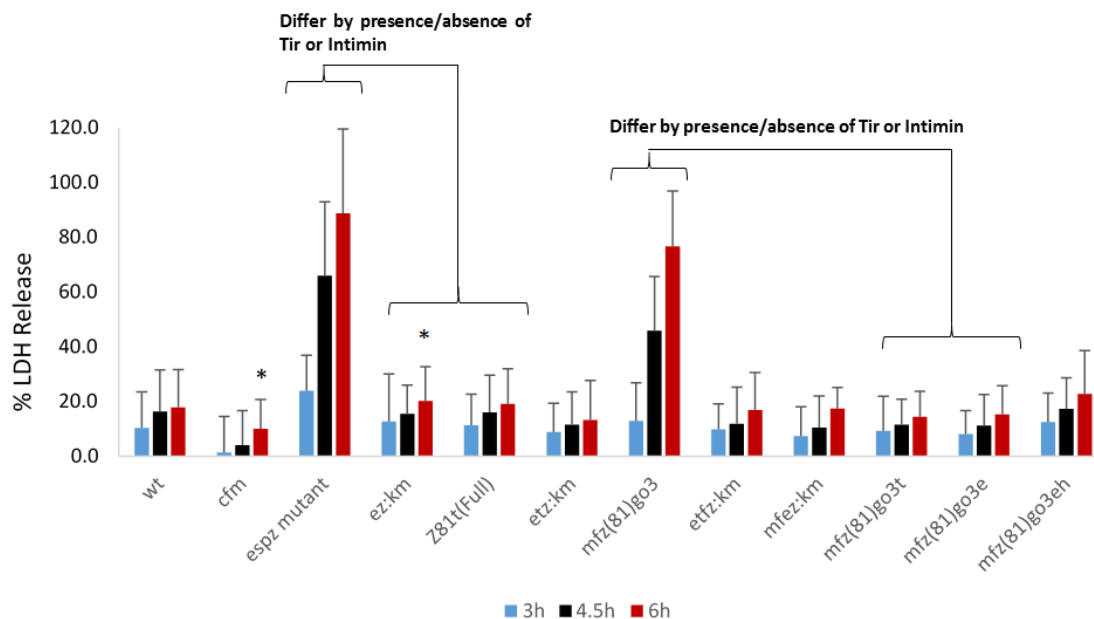


Figure 76: LDH release depends on *espZ* mutant expressing Tir or Intimin proteins. HeLa cells were infected with indicated strains (for indicated times) prior to processing extracellular media for LDH release as a measure for cell cytotoxicity. Strains used were EPEC, *cfm*-14 (T3SS mutant) or *espZ* single or multiple mutants (lacking Tir (*t*) and/or Intimin (*e*) with or without one or more of indicated LEE effectors – EspF (*f*), Map (*m*), EspG/EspG2 (*go3*) and EspH (*h*). LDH releases was measured and data expressed as means \pm S.D. from four independent experiments with asterisk indicating when only involved three independent experiments.

Strain	Figure #	3h	4h	5h	6h	Notes	Mean value of %LDH released (6h)
EPEC	64&65	E	E	E	E		17.9
<i>cfm</i> (T3SS)	64	E	E	E	E		10
<i>espZ</i> ₍₈₁₎	64&65A/C	E	$\Delta^* \bullet \circ$	$\Delta^* \bullet \circ$	$\Delta^* \bullet \circ$		88.8
<i>ez:km</i>	65B	E	E	E	E	Loss pTyr proteins at 5/6h	20.2
<i>Z</i> ₍₈₁₎ <i>t</i>	72	tE	tE	tE	Z	Slower pTyr proteins loss at 6h	19.2
<i>etz:km</i>	73B	tE	tE	tE	Z	Loss pTyr loss at 5/6h	13.3
<i>mfz:kmgo3</i>	65C	E	E	Z	Z	Loss Tub/actin 6h and pTyr proteins 5/6h	76.5
<i>etfz:km</i>	73A	tE	tE	tE	Z	Slower pTyr proteins loss at 6h	16.9
<i>mfez:km</i>	65B/D	E	E	E	E		17.5
<i>mfz</i> ₍₈₁₎ <i>go3t</i>	74	tE	tE	tE	tE		14.5
<i>mfz:kmgo3e</i>	65C	E	E	E	E		15.3
<i>mfz</i> ₍₈₁₎ <i>go3eh</i>	75	E	E	Z	Z	Loss pTyr proteins/Tir T'' at 4.5/6h & tub/act 6h	22.8

Table 11: Summary of differences between cells infected with *EspZ*-deficient and EPEC strains. Summary of western blot and LDH data (6h only) from HeLa cells infected with indicated strains after 3, 4, 5 and 6h. Strains used were EPEC, *cfm* (T3SS mutant) or those unable to express *EspZ* alone (*espZ*₍₈₁₎ mutant) or *EspZ* plus one or more of indicated effectors – Tir (*t*) EspF (*f*), Map (*m*), EspH (*h*), EspG/EspG2 (*go3*) – and/or Intimin (*e*) proteins. E indicates normal T3SS dependent changes at indicated time point. While tE indicates normal T3SS dependent changes, except Tir data, as these mutants lack Tir, Z indicates data similar to that obtained with the *espZ*₍₈₁₎-infected samples at that time point with, when present, some differences in probed signals (Δ reveals dephosphorylation of Tir and host tyrosine phosphoproteins; * loss to Tir T'' band, \bullet loss of tubulin (tub) signal, and \circ loss of actin signal). Yellow highlighting indicate *espZ* multi-mutants that release the higher percentage of LDH enzyme. The *espZ* gene was inactivated by deleting 81 (of 98) residues or introducing kanamycin-encoding gene indicated by ₍₈₁₎ and :*km* respectively.

5.3.9 Ruling out any possible role for non-LEE effector proteins in *espZ*₍₈₁₎ phenotypes

Finding that, deleting the *espH* gene from *mfz*₍₈₁₎*go3e* mutant revealed its ability to trigger *espZ*₍₈₁₎ associated phenotypes (Figure 75; *mfz*₍₈₁₎*go3eh:km*), implicated additional complexity in which EspH may suppress cytotoxicity inducing activity of, presumably, non-LEE encoded effector protein(s). In EPEC (strain E2348/69), 24 non-LEE putative effectors have been identified (Dean & Kenny, 2009, Iguchi *et al.*, 2009, Deng *et al.*, 2004b). Thus, we decided to examine the putative role for these effector proteins in the *espZ*₍₈₁₎ phenotypes by generating a strain lacking the *espZ* gene and as many LEE and non-LEE-encoded effectors as possible with the prediction that it would have little if any *espZ*₍₈₁₎ phenotypes. Thus, I took an advantage of an available mutant TOE-A7 (Yen *et al.*, 2010) that lacks 14 non-LEE effectors and EspG, from which *espZ*, *espF*, *map*, *espH*, *eae*, *tir* and/or *espB* genes were deleted using available suicide vectors and developed strategies (see Chapter 2; Section 2.2.7). This led to the generation of ten strains (Table 12) with PCR analysis confirming disruption of *espZ*, *espF*, *map*, *espH*, *eae*, *tir* and/or *espB* genes (data not shown). The screened strains and resulting Western blot data (shown in Figure 77A-D) are summarised in Table 12.

Western blot analysis of isolated fractions revealed that TOE-A7, as expected, behaved like EPEC with no evidence for any *espZ*₍₈₁₎ associated phenotypes (Table 12; Figure 77A). In contrast, TOE-A7 mutants, Δfz , Δfzm and $\Delta fzmh$ (lacking EspZ with one or more of the LEE effectors EspF, Map and EspH) behaved like *espZ*₍₈₁₎ (Table 12; Figure 77AB). Interestingly, while $\Delta fzmh$ (lacks all classical LEE effectors [but not EspB] and the 14 non-LEE effectors) was associated with one or more minor phenotypes at later time points (Table 12; Figure 77D), $\Delta fzmthe$ (as $\Delta fzmh$ but lacks Intimin) was linked to a more rapid loss of the proteins tyrosine phosphorylation and tubulin/actin proteins, with, of course, no information about Tir modification as strains are *tir*-negative strains (Table 12; Figure 77C). Similarly, TOE-A7 Δ core (lacks the 14 non-LEE effectors and loss of LEE region genes encoding the EspH, CesF, Map, Tir, CesT and Intimin) also behaved like *espZ*₍₈₁₎, suggestive of a role for Tir and Intimin in promoting and a suppressing these events, respectively (Table 12; Figure 77D). Noticeably, the cytoplasmic tubulin protein, was detected in the insoluble fractions of most EspZ deficient TOE-A7 mutants, suggesting a putative role for EspZ with other 14 non-LEE effectors to protect against that. In addition, EspB was detected in all

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infected cells (soluble and insoluble fractions) with a gradual increase in HeLa cells infected over 3hr with *espZ*₍₈₁₎ deficient strains, suggested that EspB might be regulated by EspZ or other missing effectors. In contrast, EspF was only detected in EspF positive strain (EPEC, TOE-A7 and *espZ*₍₈₁₎) which is a further support for strains genotype.

These findings suggested that Tir, but not Intimin, is responsible for triggering the *espZ*₍₈₁₎ associated phenotypes in the TOE-A7 genetic background.

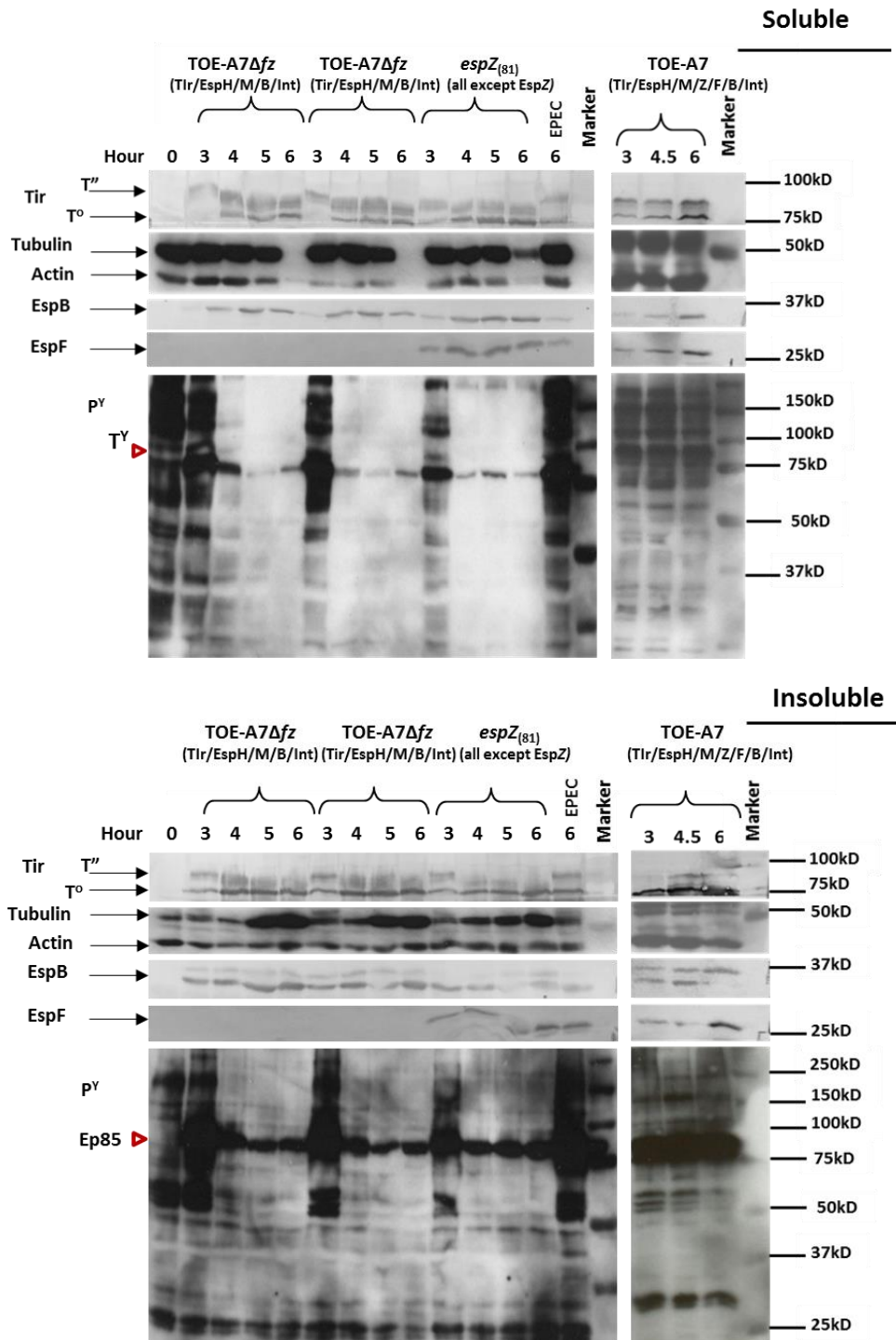
Strain	Figure	3h	4h	5h	6h	Notes	Mean value of %LDH released (6h)
EPEC	64/77	E	E	E	E	T ^{''} is stable, No pTyr nor tub/actin loss	17.9
<i>espZ</i> ₍₈₁₎	77A/B	E	Δ*●○	Δ*●○	Δ*●○	Slower tub/actin 6h	88.8
TOE-A7	77A/C	E	E	E	E	As EPEC	12.5
TOE-A7 <i>b</i>	N/A	–	–	–	–		11.1
TOE-A7Δ <i>fz</i>	77A	E	Z	Z	Z	As <i>espZ</i> ₍₈₁₎	28.2
TOE-A7Δ <i>fzm</i>	77B	E	Z	Z	Z	As <i>espZ</i> ₍₈₁₎	36.0
TOE-A7Δ <i>fz</i> <i>mh</i>	77B	E	Z	Z	Z	As <i>espZ</i> ₍₈₁₎	38.6
TOE-A7Δ <i>fz</i> <i>me</i>	N/A	–	–	–	–		29.2
TOE-A7Δ <i>fz</i> <i>meh</i>	77C	E	Z (4.5h)		Z	Slower T ^{''} and pTyr loss; Faster tub/actin 4h	37.4
TOE-A7Δ <i>fz</i> <i>mt</i>	N/A	–	–	–	–		9.7
TOE-A7Δ <i>fz</i> <i>mth</i>	77C	tE	tE (4.5h)		Z	Slower loss pTyr proteins at 6h & tub/act 6h	11.4
TOE-A7Δ <i>fz</i> <i>mthe</i>	77C	tE	Z (4.5h)		Z	Faster pTyr and tub/actin loss 4.5h	9.8
TOE-A7Δ <i>core</i>	77C	tE	tE (4.5h)		Z	Slower loss pTyr proteins at 6h & tub 6h	16.3
TOE-A7Δ <i>fz</i> <i>mehb</i>	N/A	–	–	–	–		6.0

Table 12: Summary of differences between cells infected with EspZ-deficient TOE-A7 strains.

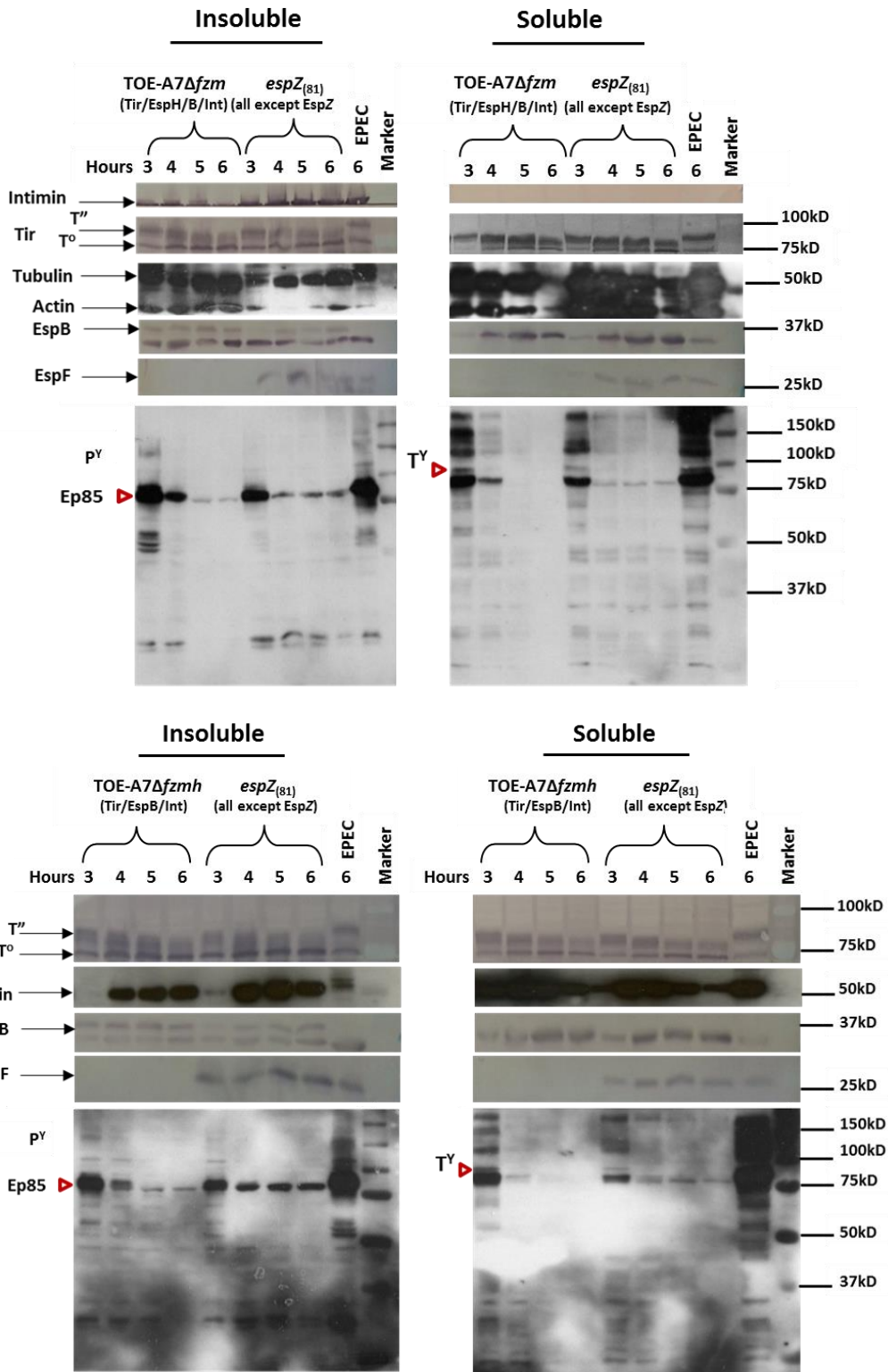
Summary of western blot and LDH (only 6hr) data from HeLa cells infected with indicated strains and time. Strains used were TOE-A7 (lacks 14 non-LEE effectors and one LEE effector, EspG) or those (TOE-A7 mutants) unable to express EspZ plus one or more of indicated LEE effectors – EspF (*f*), Map (*m*), EspH (*h*), Tir (*t*) – and/or Intimin (*e*) proteins and core region (loss of LEE region genes encoding the EspH, CesF, Map, Tir, CesT and Intimin). E indicates normal T3SS dependent changes at indicated time point. While tE indicates normal T3SS dependent changes, except Tir data, as these mutants lack Tir, Z indicates data similar to that obtained with the *espZ*₍₈₁₎-infected samples at that time point with, when present, some differences in probed signals (Δ reveals dephosphorylation of Tir and host tyrosine phosphoproteins; * loss to Tir T^{''} band, ● loss of tubulin (tub) signal, and ○ loss of actin signal). N/A indicate that this strain was not assisted. Yellow highlighting indicates *espZ* multi-mutants that release the higher percentage of LDH enzyme.

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A)



B)



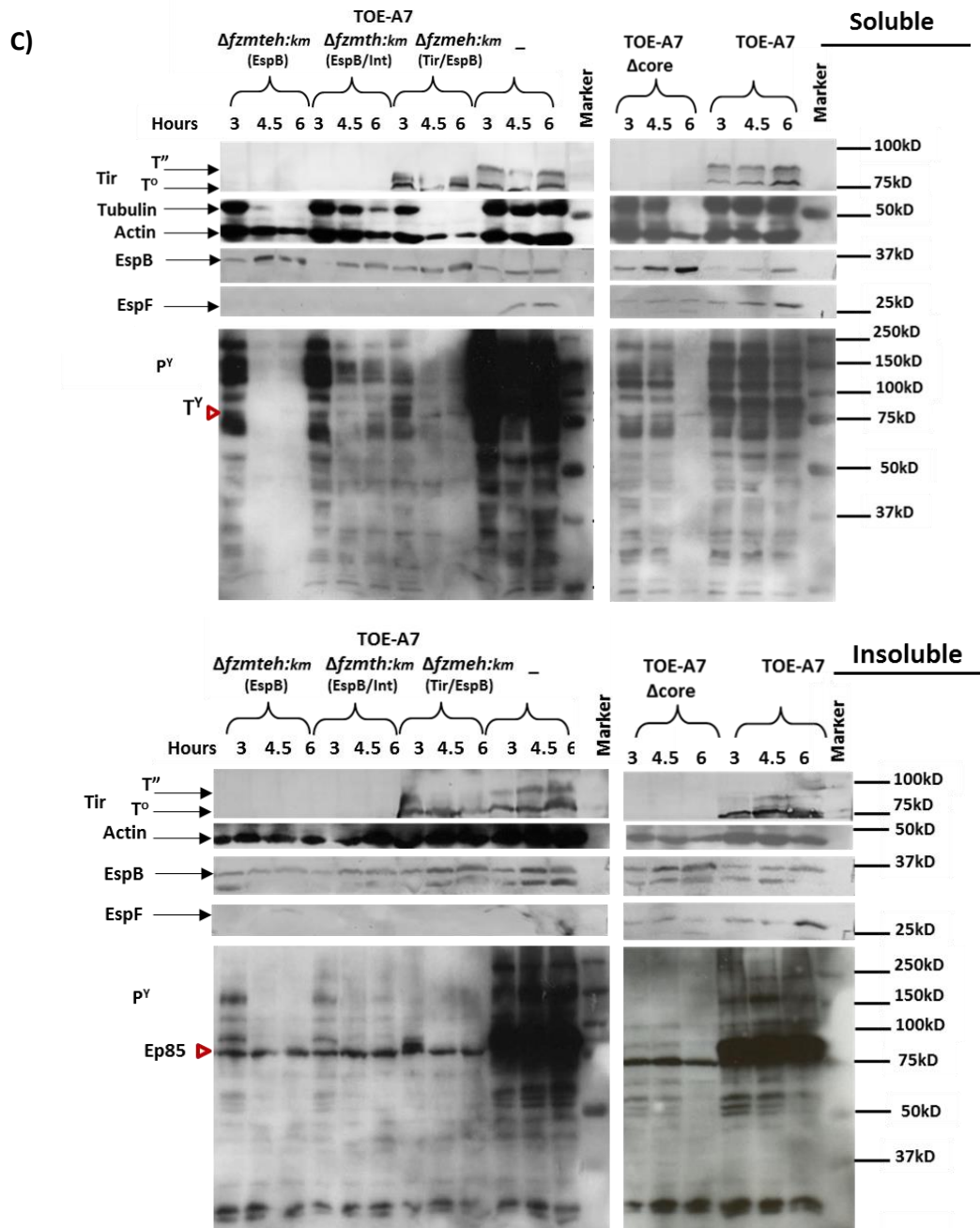


Figure 77: Assessment of EspZ deficient TOE-A7 multi-mutants. HeLa cells were left uninfected or infected with indicated strains, for indicated times, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) for Western blot analysis probing for phosphotyrosine proteins (P^Y), Intimin, Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified Tir (T'' and P^Y [T^Y]) forms are indicated as are Ep85 (EPEC tyrosine-phosphorylated protein), Intimin, EspF, EspB, tubulin, actin and protein markers. Strains used were EPEC, TOE-A7 strains lacking EspZ ($espZ_{(81)}$ or z) with one or more of the indicated proteins: Map (m); EspF (f), Tir (t), EspH (h) or Intimin (e), or core region (loss of LEE region genes encoding the EspH, CesF, Map, Tir, CesT, EspG and Intimin protein). Below the strain name (note indicates order genes were inactivated) is the identity of proteins of interest (Tir, Map [M], EspH, EspZ, EspF [F], EspB [B] effector and/or Intimin [Int] proteins) expressed by the strain. The $espZ$ gene was inactivated by deleting 81 (of 98) residues while $espH$ by introducing kanamycin-encoding gene indicated by (81) and $:km$ respectively. The immunoblots (where appropriate) cropped and moved for presentation purposes. Note, data is representative of that from two or more independent experiments.

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The cellular cytotoxicity mediated by EspZ deficient TOE-A7 mutants (express or not Tir and/or Intimin proteins) was also quantified using the Lactate dehydrogenase (LDH) assay and data are summarised in Table 12. As before, the *espZ*₍₈₁₎ infection lead to the greatest release of LDH enzyme with 20, 65 and ~90% of LDH enzyme being released after 3, 4.5 and 6hr infection periods, respectively (Figure 78). In contrast, cells infected with the TOE-A7 strain was linked to ~18% LDH released by 6hr post-infection with less in cells infected with T3SS mutant strain (TOE-A7 Δ *espB*; ~11%) (Figure 78). Interestingly, TOE-A7 mutants, Δ *fz*, Δ *fzm* and Δ *fz mh* (lacking EspZ with one or more LEE effectors EspF, Map and EspH) were linked to more LDH enzyme released ~30% compared to TOE-A7 (Figure 78). Importantly, deleting Tir, but not Intimin, (Δ *fz mt* and Δ *fz mth*) linked to a great reduction in the amount of LDH enzyme release (~10%) (Figure 78).

Collectively, these data suggested that the LDH release, in the TOE-A7 genetic background, is Tir but not Intimin dependent.

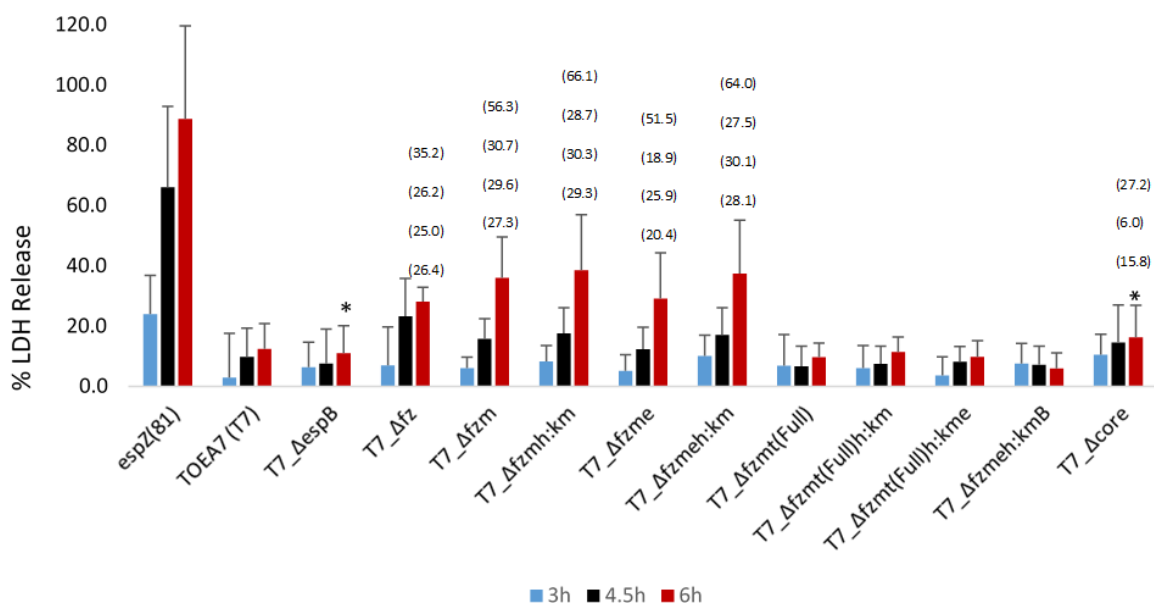


Figure 78: LDH release depends on EspZ deficient TOE-A7 mutants expressing Tir but not Intimin. HeLa cells were infected with indicated strains (for indicated times) prior to processing extracellular media for LDH release as a measure for cell cytotoxicity. Strains used were the *espZ*₍₈₁₎, TOE-A7, TOE-A7 Δ *b* (T3SS mutant), EspZ deficient TOE-A7 multiple-mutant strains (lacking EspZ plus one or more of indicated effectors EspF (*f*), Map (*m*), EspH (*h*) and/or –Tir (*t*) /Intimin (*e*) proteins) or lacking the core region (loss of LEE region genes encoding the EspH, CesF, Map, Tir, CesT and Intimin), LDH releases was measured and data expressed as means \pm S.D. from four independent experiments with asterisk indicating when only involved three independent experiments.

5.3.10 K12 'mini-LEE' strain behaves like *espZ*₍₈₁₎ mutant

In an effort to avoid the above-mentioned complexities, studies explore the possibility that a recently published non-pathogenic *E. coli* strain (SIEC-eLEE5; genes introduced to express EPEC's effector delivery system, Intimin and only one classical LEE effector, Tir) would be cytotoxic to HeLa cells as lacks the *espZ* gene product. The EPEC T3SS-associated genes in are under the control of an IPTG-inducible promoter (Ruano-Gallego *et al.*, 2015). Initial HeLa cell infection studies revealed that, as expected, SIEC-eLEE5 could deliver Tir into cells where modified to the T^{''} form and interacts with Intimin (Figure 79); latter evidenced by presence in insoluble fraction. By contrast, no Tir or EspB bands were evident in cells infected with the negative control strain SIECΔp1-eLEE5 which cannot express a T3SS as lacks promoter to drive expression of T3SS components (Ruano-Gallego *et al.*, 2015). It should be noted that there appears to be 'leaky' expression of EspB and Tir, but not the T3SS delivery system, as Tir is not modified to T^{''} form (Figure 79) with no evidence of cell cytotoxicity. Thus, the infection time was extended and carried out at two MOI's.

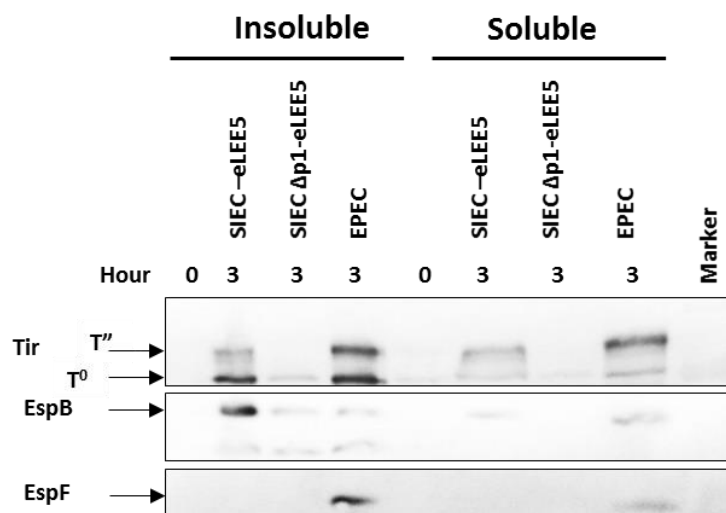


Figure 79: SIEC-eLEE5 delivers Tir where modified to T^{''}-like form and interacts with Intimin.

HeLa cells were left uninfected or infected with indicated strains for 3hr, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) for Western blot analysis probing for Tir, EspB and EspF. The position of unmodified (T[°]) and host kinase modified Tir (T^{''}) forms are indicated as are EspF and EspB. Strains used were EPEC, SIEC-eLEE5 (expresses T3SS/Esp translocons, effector delivery system, Intimin, Tir and its chaperone CesT) or SIECΔp1-eLEE5 (same as SIEC-eLEE5 but lacks promoter to drive expression of T3SS components needed for the effector-delivery process).

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Interestingly, the Western blot profile obtained with the SIEC-eLEE5 strain, at both MOI's (Figure 80), was similar to that with extended *espZ*₍₈₁₎ mutant infections (Chapter 5; Figure 64) i.e. loss of Tir T'' form over the extended infection period linked to decreasing levels of host and Tir phosphotyrosine proteins as well as tubulin and actin proteins. Crucially, these phenotypes were not evident with the negative control, SIEC Δ p1-eLEE5, strain linked to very poor expression of effectors (Tir and EspB) with absence of functional T3SS as no Tir modification (Figure 80). It should be noted that probing for tyrosine phosphorylated proteins did not reveal Ep85 (insoluble fraction) as this protein is not expressed by K12 *E. coli* (Ilan et al., 1999).

Collectively, this work illustrated that SIEC-eLEE5 strain behaves like the *espZ*₍₈₁₎ despite lacking all known EPEC effectors except Tir and EspB providing a simple model to examining the hypothesis that EspZ protects infected cells from a cytotoxic outcome in response to Tir-Intimin interactions.

5.3.11 The SIEC-eLEE5 associated phenotypes are CesT dependent

To examine the predicted key roles for Tir and Intimin in the cytotoxic-associated phenotype of the SIEC-eLEE5 strain, we wanted to inactivate the Tir and Intimin genes. However, this was not possible with available suicide vectors due to how the *tir*, *cesT* *eae* (encodes Intimin) operon was inserted into the K12 genome as this provided little homology (<200bp) for crossover events. Indeed, numerous attempts to disrupt the genes, via, the small degree of homology, were unsuccessful. Therefore, it was decided to disrupt the gene encoding the Tir chaperone, CesT, as it is critical for Tir delivery (Thomas *et al.*, 2005) using an available suicide vector and developed strategies (see Chapter 2; Section 2.2.7). PCR analysis confirmed disruption of the *cesT* gene (Figure 81A) as evidenced by a larger fragment reflecting insertion of Kanamycin resistance encoding gene in the 5' end of *cesT*.

Next, HeLa cells were infected with the new strain to determine its ability to induce cytotoxicity-associated alterations after an extended (9h) infection period. However, data interpretation was hindered by apparent inability of the SIEC-eLEE5 Δ *cesT* mutant to bind to the infected cells suggested by absence of EspB-related bands detected in the soluble or insoluble fractions in contrast to cells infected with the SIEC-eLEE5 or SIEC Δ p1-eLEE5 strains (Figure 81B).

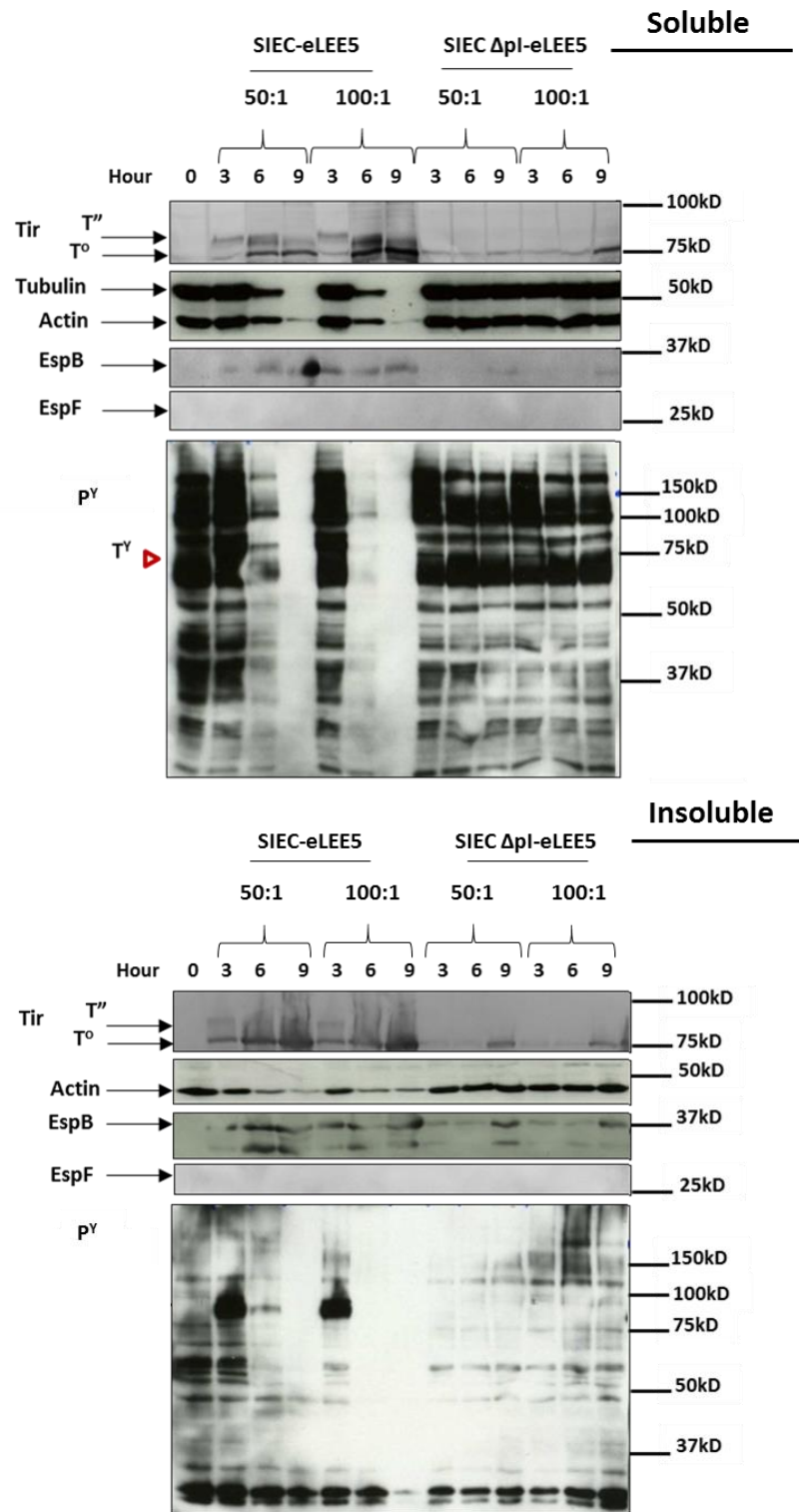


Figure 80: Cell cytotoxic responses following infection with SIEC-eLEE5. HeLa cells were left uninfected or infected with indicated strains, for indicated times and MOI, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for phosphotyrosine proteins (P^Y), Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified Tir (T^o' and P^Y [T^o']) forms are indicated as are EspF, EspB, tubulin, actin and protein markers. Strains used were EPEC, SIEC-eLEE5 (expresses T3SS/Esp translocons, effector delivery system, Intimin, Tir and its chaperone CesT) or SIECΔp1-eLEE5 (same as SIEC-eLEE5 but lacks promoter to drive expression of T3SS components needed for the effector-delivery process).

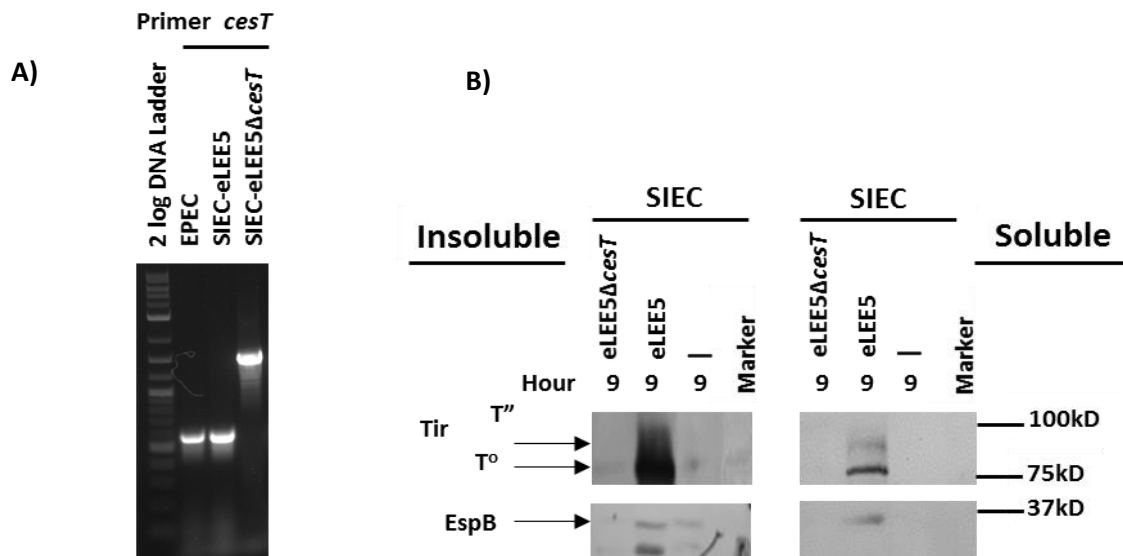


Figure 81: The SIEC-eLEE5Δ*cesT* strain does not deliver EspB. **A)** Confirmation of the disruption of the *cesT* gene in the SIEC-eLEE5 strain. Primers designed to amplify *cesT* gene was used to interrogate the absence or presence of *cesT* gene in the SIEC-eLEE5Δ*cesT* with EPEC, SIEC-eLEE5 were used as a positive control. PCR products and 2 Log DNA ladder marker (100ng-BioLabs) were run on a 1% agarose gel containing Gel Red Nucleic Acid Stain. **B)** HeLa cells were left uninfected or infected with indicated strains, for indicated times, prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for, Tir and EspB. The position of unmodified (T[°]) and host kinase modified Tir (T^{''}) forms are indicated as are EspB and protein markers. Strains used were SIEC (expresses T3SS/Esp translocons, effector delivery system but not Intimin, Tir or CesT), SIEC-eLEE5 (as SIEC but also expresses Intimin, Tir and CesT) and SIEC-eLEE5Δ*cesT* (as SIEC-eLEE5 but lacks functional *cesT* gene)

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Therefore, we attempted to promote strain binding by introducing a plasmid, pIL14, which encoding an afimbrial adhesin (Labigne-Roussel *et al.*, 1984) in each of the SIEC-related strains. Indeed, a similar (9h) infection with the three strains resulted in similar levels of EspB proteins in the Triton X-100 soluble and insoluble fractions (Figure 82) support the idea that disruption of CesT hinders SIEC-eLEE5 interaction with HeLa cells. As expected, only the SIEC-eLEE5 strain delivered Tir into cells as evidenced by T'' form in both fractions (Figure 82).

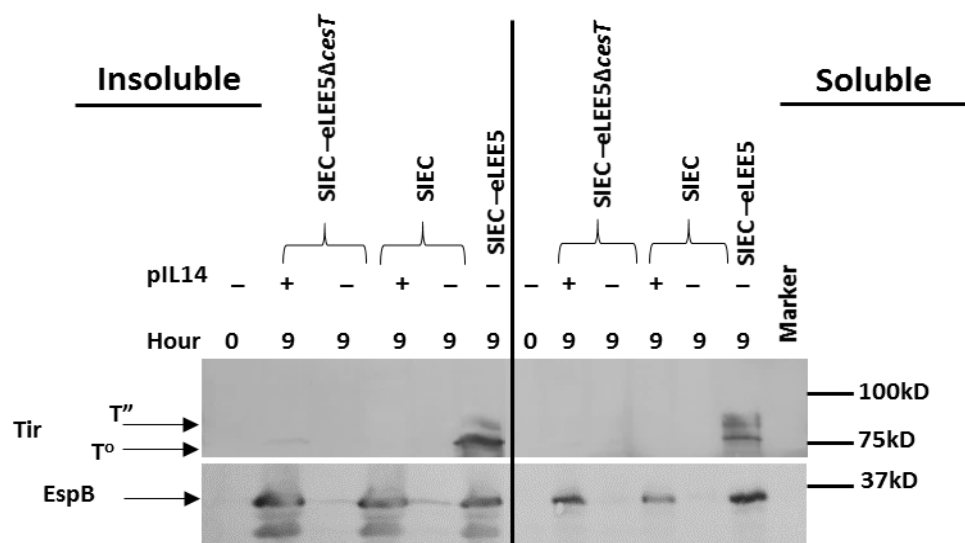


Figure 82: EspB delivery defect of the CesT-deficient SIEC-eLEE5 strain is rescued by plasmid expressing an afimbrial adhesin. HeLa cells were left uninfected (0) or infected for 9 h with indicated strains prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for Tir and EspB. The position of unmodified (T°) and host kinase modified Tir (T'') forms are indicated as are EspB and protein markers. Strains used were SIEC (expresses T3SS/Esp translocons, effector delivery system but not Intimin, Tir or CesT), SIEC-eLEE5 (as SIEC but also expresses Intimin, Tir and CesT) and SIEC-eLEE5 Δ cesT (as SIEC-eLEE5 but lacks functional *cesT* gene). Each strain either carried no additional plasmid (-) or the pIL14 plasmid (+) that encodes an afimbrial adhesin.

Crucially, a time course infection with these afimbrial adhesin-expressing strains revealed that only the SIEC-eLEE5 strain induced *espZ* mutant-associated phenotypes despite all three strains displaying similar EspB signals in the soluble and insoluble fractions (Figure 83). This finding demonstrates a critical role for CesT, again presumably via Tir, in driving cytotoxicity-associated events that are normally prevented by the T3SS-dependent delivery of EspZ.

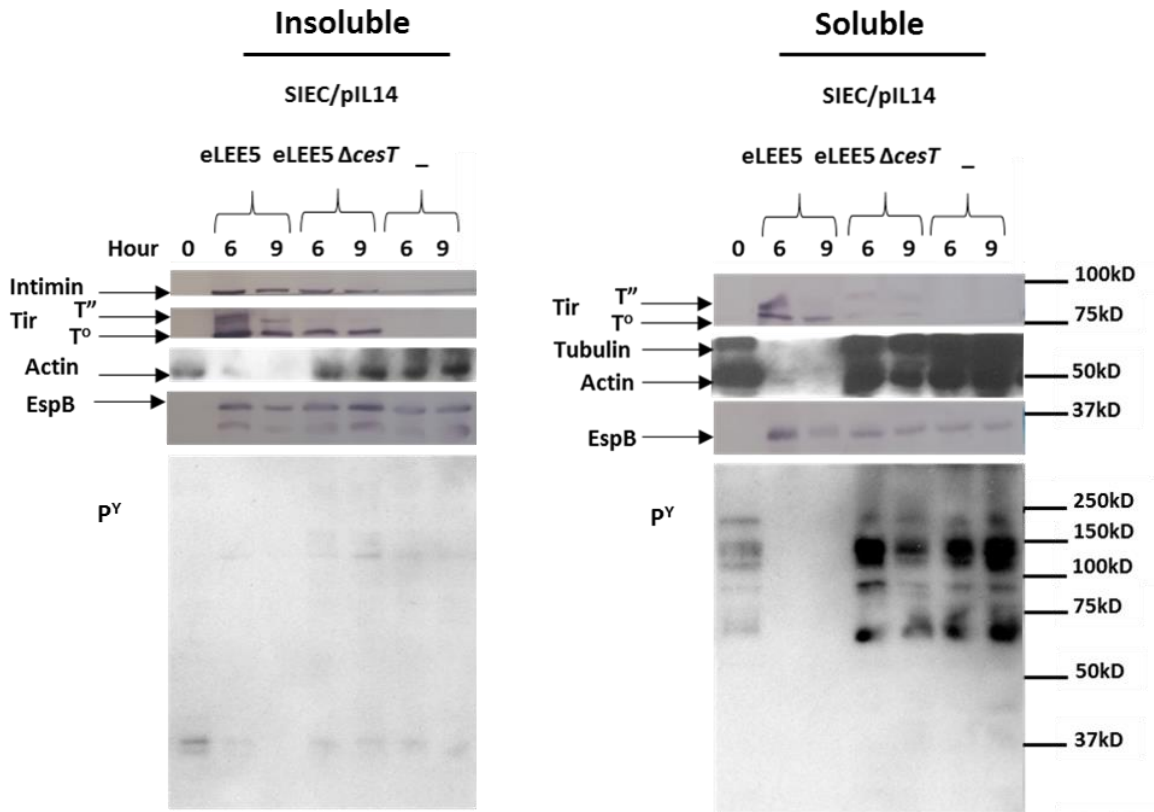


Figure 83: *CesT* activity is critical for SIEC-eLEE5 to induce *espZ* mutant-associated phenotypes. HeLa cells were left uninfected (0) or infected for 6 or 9 h with indicated strains prior to isolating Triton X-100 soluble (contains host cytoplasm and membrane proteins plus delivered effector proteins) and insoluble (contains host nuclei and cytoskeletal proteins plus adherent bacterial proteins) fractions for Western blot analysis probing for phosphotyrosine proteins (P^Y), Intimin, Tir, EspB, EspF, tubulin and actin. The position of unmodified (T^o) and host kinase modified Tir (T^{''} and P^Y [T^Y]) forms are indicated as are Intimin, EspB, tubulin, actin and protein markers. Strains used were SIEC (expresses T3SS/Esp translocons, effector delivery system but not Intimin, Tir or *CesT*), SIEC-eLEE5 (as SIEC but also expresses Intimin, Tir and *CesT*) and SIEC-eLEE5 $\Delta cesT$ (as SIEC-eLEE5 but lacks functional *cesT* gene). Each strain carried the pIL14 plasmid which encodes an afimbrial adhesin. It should be noted that phosphotyrosine protein signal is unusually weak (eg compare to Figure 80) in the soluble fraction of uninfected cells.

5.4 Discussion

The aim of the described work was to explore the idea, based on data from Chapter 3, that the delivered EspZ effector protects cells from Intimin-dependent activities that trigger a cytotoxicity response. Indeed, studies with available and newly generated EspZ multi-mutants supported a key role not only for Intimin but also its receptor Tir with contributory, minor, roles for other examined effectors. However, additional complexity was evidenced by unexpected results with some multi-mutants but crucially a simple model supports the idea of a new protective mechanism, whereby EspZ prevents cells undergoing a cytotoxic response initiated as a consequence of Tir-Intimin interaction.

Studies on the role of LEE effectors in the Tir modification and insertion process led to data questioning EspZ's proposed role in protecting cells from cytotoxicity by different mechanisms (Shames *et al.*, 2011, Berger *et al.*, 2012, Roxas *et al.*, 2012). However, our data argues that EspZ protects against the cell cytotoxicity response stemming from Tir-Intimin interaction, to perhaps explaining why EspZ, Tir and intimin, unlike other classical effectors, are critical for the virulence of EPEC (Wilbur *et al.*, 2015, Marchès *et al.*, 2000). The key roles for Tir and Intimin were suggested by a dramatic reduction in *espZ*₍₈₁₎ associated phenotypes when either *tir* or *eae* gene was inactivated (*z*₍₈₁₎*t* or *ez:km*, respectively). However, there are residential minor phenotypes at later time points which can be prevented through the deletion of other effectors (Map, espF and EspG), suggesting a cooperative role for these effectors in triggering the *espZ*₍₈₁₎ associated phenotypes. Unexpectedly, multiple mutant strain revealed an additional complexity. For example, inactivation of the *esph* gene from the *mfz*₍₈₁₎*go3e* mutant (which behaves like EPEC) led to the generation of a *mfz*₍₈₁₎*go3eh:km* mutant and the restoration of *espZ*₍₈₁₎ activities. This implies a possible suppressive role for EspH against, (presumably) Tir or other non-LEE effectors. In addition, inactivation of the *eae* gene from a TOE-A7 Δ *fz**mh* mutant (lacking 14 non-LEE effectors and LEE Map, EspF, EspZ, EspH & EspG2/Orf3) did not prevent the appearance of *espZ*₍₈₁₎ associated phenotypes, suggests that these phenotypes are intimin independent in the TOE-A7 genetic background. However, the ability of TOE-A7 Δ *fz**mhe* (lacking 14 non-LEE effectors and LEE Map, EspF, EspZ, EspH, EspG2/Orf3 and Intimin) mutant to trigger these phenotypes might be attributed to the remaining protein(s), particularly Tir or other non-LEE effectors, EspC, LifA and EspJ (Deng *et al.*, 2012, Vidal & Navarro-Garcia, 2008). The EspJ is localized to mitochondria (Kurushima *et al.*, 2010)

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and may cause mitochondrial dysfunction in the absence of the EspZ. The EspC, however, regulates the secretion levels of EspD and EspA with an *espC* mutant showing increased levels of cell-associated EspA and EspD, as well as increased pore formation activity associated with cytotoxicity (Guignot *et al.*, 2015).

Thus, to avoid the previous complexity and to support the key role for Tir and/or Intimin in triggering the *espZ*₍₈₁₎ associated phenotypes, we have used a simple model, *E. coli* K-12 (SIEC-eLEE5) strain (Ruano-Gallego *et al.*, 2015). This strain encodes Intimin and the effector delivery system, but lacks all EPEC LEE effectors, except Tir and EspB (Ruano-Gallego *et al.*, 2015). Conclusively, the ability of SIEC-eLEE5 strain to behave like *espZ*₍₈₁₎ discounted any possible role for all LEE and non-LEE effectors and implicated a crucial role for Tir, its chaperone CesT, and/or Intimin, in triggering the *espZ*₍₈₁₎ phenotypes, though it is possible that these phenotypes may be linked to the translocators (EspA/D/B). However, the inability of SIEC (which encodes a functional T3SS) and eLEE5 Δ *cesT*/pIL14 (as SIEC-eLEE5 but lacks CesT, Tir chaperone) to trigger the *espZ*₍₈₁₎ phenotypes discounted a possible role for the translocator proteins and implicated a key role for the CesT dependent effector, Tir, with or without Intimin interaction. It is possible that CesT has an unknown function that helps to bring about these phenotypes, but that is unlikely as we have implicated a key role for Tir in triggering these phenotypes, and CesT is required for Tir delivery (Thomas *et al.*, 2005). Taken together, these findings provide further arguments against the balancing hypothesis, in which EspZ is thought to regulate the translocation of EPEC effectors into host cells (Berger *et al.*, 2012) and instead support a key role for Tir/Intimin in triggering the *espZ*₍₈₁₎ associated phenotypes. Further work is required to inactivate *tir* and/or the *eae* gene from SIEC to further confirm these results.

Intimin interacts with Tir to i) mediate an intimate attachment to the host cells, ii) promote pedestal formation, iii) stimulate host phospholipase C- γ 1 phosphorylation and iv) trigger actin rearrangements (Kenny & Finlay, 1997, Kenny *et al.*, 1997b, Kenny *et al.*, 2002b, Rosenshine *et al.*, 1996). Thus, the inability of the *ez:km* or *z*₍₈₁₎*t* mutant to trigger the *espZ*₍₈₁₎ phenotypes might be due to a weaker interaction between the mutant strain bacteria and host cells. However, the binding capacity of *ez:km* and *z*₍₈₁₎*t* mutants are reflected by Ep85, EspB or unmodified Tir (within bacteria) signals in insoluble fractions which argues against any binding issue. Thus, it is more likely that Intimin interacts with Tir or other receptors such as β integrin and nucleolin (Strong *et*

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al., 2011), and subverts a signal leading to the *espZ*₍₈₁₎ phenotypes, while EspZ protects against that. It was reported that Td92, a surface protein of the periodontal pathogen *Treponema denticola*, interacts with the cell membrane integrin $\alpha 5\beta 1$ and activates NOD-leucine-rich repeat protein (NLRP3) mediated cytokine and caspase-1 processing, leading to cell death (Jun *et al.*, 2012, Eldridge & Shenoy, 2015), and so Tir/Intimin might function via a similar pathway while EspZ protect against that.

Interestingly, the key role filled by Tir and Intimin was further supported by re-introducing a gene encoding Intimin or Tir in the *espZ* multiple mutant (*mfez:km* or *mfez*₍₈₁₎*go3t* respectively) which restored their capacity to trigger the *espZ*₍₈₁₎ phenotypes. Importantly, preventing the Tir-Intimin interaction led to a dramatic reduction in the ability of complemented strain (*p3gtHAinto-mfez*₍₈₁₎*go3t*) to trigger these phenotypes, in contrast to (*ptir-mfez*₍₈₁₎*go3t*), suggesting that the Tir-Intimin interaction is critical in triggering these events. Tir serine (S363/S343) and tyrosine (Y454/Y474) residues are linked to EPEC Tir's subversive activities (Kenny, 1999, Kenny & Warawa, 2001, Allen-Vercoe *et al.*, 2006, Yan *et al.*, 2013). Crucially, the inability of Tir Δ SY (Y45/Y474 and S363/S343 residues were substituted to alanine/phenylalanine, respectively), to complement *mfez*₍₈₁₎*go3t* and restore the *espZ*₍₈₁₎ phenotypes, supported a key role for these residues in triggering the *espZ*₍₈₁₎ phenotype. Further studies are needed to investigate which residue is necessary for triggering the *espZ*₍₈₁₎ phenotype and how these residues contribute to the *espZ*₍₈₁₎ phenotype.

Although many studies reported the cytotoxicity of the *espZ* mutant (Shames *et al.*, 2011, Berger *et al.*, 2012, Roxas *et al.*, 2012), here we have demonstrated other *espZ* mutant associated phenotypes: the modification of Tir T'' to the T' and T^o-like forms, Tir/host protein tyrosine dephosphorylation, and tubulin/actin loss. Perhaps it is not surprising that the cytoplasmic host proteins tubulin/actin were lost, as *espZ*₍₈₁₎ infection led to rapid membrane permeabilization (Berger *et al.*, 2012). This was not evident for Tir (T^o), EspF or EspB, as they are membrane proteins or within organelles and thus were not lost following the plasma membrane permeabilization event. Noticeably, the distinguishable level of EspB, which is linked to the *espZ*₍₈₁₎ infection, but not the EPEC infected cells, could implicate a possible role for EspZ in the regulation of EspB and pore formation. Crucially, the *espZ*₍₈₁₎ phenotypes are promoted or suppressed by one or more EPEC proteins. While modification of Tir T'' to the T' and T^o-like form is an Intimin dependent event and Tir/host protein tyrosine

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dephosphorylation appears to be attributed to the Map and EspF effectors, the tubulin/actin loss is a Tir/Intimin dependent event. It should be noted that, the tubulin/actin loss is unlikely to be linked to cytotoxicity as the TOE-A7 Δ *fzmthe* and TOE-A7 Δ *core* mutants showed approximately the same levels of released LDH as TOE-A7, though these strains were correlated with tubulin/actin loss.

The contribution role of Map, at least in the *mfez.km* background, can be attributed to its import into the mitochondria, as a *map*- Δ MTS complemented strain was not able to trigger *espZ*₍₈₁₎ phenotypes compared to Map or other Map variants. This is consistent with the idea that Map and EspF target mitochondria and induce cytotoxic signals suppressed by EspZ (Nagai *et al.*, 2005, Papatheodorou *et al.*, 2006, Shames *et al.*, 2011). However, in our model, EspF's contribution role could not be prevented by blocking targeting to mitochondria (L16E-EspF and Δ MTS-EspF; latter equivalent to Δ MTS-Map as MTS region was swapped for the 1st 101 AA of Tir). Indeed, the Map's role is to remain unaltered when known subversive activities were abolished (*mapE78A* can't activate Cdc42 and *map* Δ *TLR* can't sustain Map-triggered Cdc42 activity), implied there is no role for these motifs in triggering the *espZ*₍₈₁₎ associated phenotypes. However, it should be noted that the high degree of Tir/host protein dephosphorylation observed with the *map*-complemented strain is likely to be attributed to the overexpression of Map by the *map*-complemented strain. This allows large amounts of Map to translocate to the mitochondria, resulting in mitochondrial dysfunction, release of cytochrome *c* and hence host cell death.

In summary, while EPEC showed a key role for Tir via Intimin, TOE-A7 revealed these phenotypes are Tir, but not Intimin dependent, suggesting a complexity behind different effectors activities and *espZ*₍₈₁₎ phenotypes that can be triggered by different pathways. However, this study provided a simple model, the SIEC strain, to investigate EspZ associated cytotoxicity and our results confirmed, using SIEC strain, a key role for Tir/Intimin in triggering the *espZ*₍₈₁₎ phenotypes. Importantly, this work argued against the suggested alternate mechanisms by which EspZ protects against cell death and discounted any putative role for all known LEE/non-LEE effectors and the translocators. Crucially, this data also confirmed that the Tir-Intimin interaction is critical in triggering the *espZ*₍₈₁₎ phenotypes. Thus, we speculated that Intimin interacts with a functional Tir and EspZ to trigger a signal leading to the *espZ*₍₈₁₎ associated phenotypes, while EspZ protects against this process. Therefore, immunoprecipitation

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experiments are required to examine whether Intimin-Tir-EspZ interact with each other and further studies are needed to investigate how the Tir-Intimin interaction can lead to cell cytotoxicity while EspZ provides a protection against it.

Chapter 6: Final Discussion

Final Discussion

The pathogenesis of enteropathogenic *Escherichia coli* (EPEC) depends on a functional T3SS to deliver two virulence proteins Tir and EspZ into the host cells (Wilbur *et al.*, 2015, Marchès *et al.*, 2000, Coburn *et al.*, 2007). Upon delivery into the host cytosol, Tir acts as a substrate for the host kinase that phosphorylate Tir at two different serine residues, leading to an increased apparent Tir molecular mass by a total of 7kD (to T[~]-like form). The T[~]-like form Tir then inserts into the host cell's membrane and binds Intimin. Although the LEE region was shown to be sufficient to aid Tir modification to T[~] form, this study discounted a role for the classical LEE encoded protein effectors (EspG, Map, EspF, EspH and EspZ) and Intimin protein in the modification of Tir to the T[~] form and instead implicated roles for other T3SS-dependent substrates - i.e translocators or a non- delivered effector, SepL. The N-terminus of SepL carries the secretion signal and binds to the CesL chaperone, while the carboxy terminus binds to the Tir effector (Wang *et al.*, 2008, Younis *et al.*, 2010).

Evaluation of translocators (EspA, EspD and EspB) and SepL protein in effector functionality is not possible using a gene deletion strategy, as these proteins are essential for the functionality of T3SS. However, an EspB variant (EspB-mid) lacks 60 residues (residues 159-218) and cannot bind host myosin proteins to inhibit phagocytosis (Iizumi *et al.*, 2007), and *E. tarda* EspB (37.8% identity to EPEC one) restored the functionality of T3SS and revealed no role for missing and homologous motifs, respectively, in the Tir modification and insertion mechanisms. However, the possible role for other, non-homologous EspB motifs cannot be discounted. It is well recognised how toxins and translocators are inserted directly into the host cell's membrane (Dal Peraro & van der Goot, 2016), but conceptually, it is still very difficult to understand how proteins like Tir and EspZ are delivered with two transmembrane domains and inserted in the hairpin like structure into the host cells membrane.

Importantly, the *E. tarda* LEE-like region is missing all known EPEC LEE effector homologues, except Tir and EspZ (Nakamura *et al.*, 2013), and *E. tarda* Tir lacks all EPEC Tir residues that are linked to EPEC Tir's subversive activities (Kenny, 1999, Kenny & Warawa, 2001, Allen-Vercoe *et al.*, 2006, Yan *et al.*, 2013). Thus, we speculated that *E. tarda* Tir is no longer required to manipulate any effector function and would be inserted directly into the host cells membrane to act as a receptor for *E. tarda* Intimin (Tir-Intimin interaction) (Kenny *et al.*, 1997b). Further studies are needed to investigate the direct or indirect insertion mechanism of Tir. This can be investigated

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via making an MTS-Tir fusion protein where Tir can only be imported to the mitochondria if it is present in the host cytoplasm. In addition, expressing CesT (Tir chaperone) or EspB (cytoplasmic and membrane forms that are potential candidates factor aiding Tir insertion) within the host cells to see if they can interact and inhibit the Tir insertion into host cells membrane thus accumulation in the host cells cytoplasm. These strategies will support a cytoplasmic intermediate form of Tir.

Interestingly, our work revealed an unexpected role for EspA and EspD, not in aiding Tir modification to T⁺ form, but actually in facilitating the Tir T⁺-Intimin interaction. This suggested that these translocators (EspA/D) might interact with Intimin in order to stabilise its interaction with the Tir T⁺ form. Further work needs to be done to i) examine whether these proteins interact with each other (immunoprecipitation) and ii) examine whether the detected T⁺-like form is inserted in the host cells membrane (looking the topology of Tir by detecting the inserted tag in the cytoplasmic or extracellular domain). In addition, by swapping either the N- or C- terminal domains, or individual amino acids, of the EPEC and *E. tarda* strains, acritical features linked to the Tir-Intimin interaction and insertion into the host cells membrane might be revealed. A previous study (Luo & Donnenberg, 2011) suggested that EspA interacts with EspD while EspD interacts with EspB. Our study confirmed that *E. tarda* EspA and EspD proteins are only functional interchangeable when co-expressed, suggesting species-specific protein-protein interactions. This observation also provides a possible explanation for the lack of interchangeability of some *E. tarda* proteins (SepL, EscL and EscK; components of a sorting platform) as these proteins might be expressed and functional but lack the specific motif(s) required to mediate interact with their partner homologue. Thus, the expression of these non-interchangeable *E. tarda* proteins needs to be examined by, for example, generating a tagged fusion protein to enable their detection. These proteins (for example components of a sorting platform), if expressed, should be co-expressed to interrogate their functionality. Indeed, this work provides a good model, *E. tarda* T3SS, for investigating different T3SS components as these components are highly different to the EPEC one. Thus, further work needs to be done to investigate different T3SS components motifs that might be responsible for interaction and functionality of these proteins.

Interestingly, the detection of Intimin, Tir and EspZ, but not other LEE classical proteins, in the LEE like region of a fish infecting bacteria strain (*E. tarda*), supports the idea of a key role for these proteins in the virulence of pathogenic strain. EspZ is

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anti-toxic protein that is protecting against cell death by different suggested mechanisms (Shames *et al.*, 2011, Berger *et al.*, 2012, Roxas *et al.*, 2012). However, our study argued against all the suggested mechanisms (Shames *et al.*, 2011, Berger *et al.*, 2012, Roxas *et al.*, 2012) and clearly illustrated that EspZ protects against the Tir-Intimin interaction that leads to cell death. Tir interacts with Intimin to form a pore in the host cell's membrane (unpublished data). Thus, we speculate that EspZ is likely to interact with a translocator, in this case Tir and/or Intimin. This interaction aids EspZ in blocking these pores, which could be examined via immunoprecipitating these proteins. Crucially, the key Intimin-Tir role can also be linked to the hypothesis that this interaction might lead to unauthorized actin polymerization and activation of inflammasome-mediated cell death (MacPherson *et al.*, 2017) which might be prevented by EspZ. This process is further supported by the initial data (data not shown) revealed that the Cytochalasin D (Cyto D), an actin polymerization inhibitor, prevented *espZ*₍₈₁₎ associated phenotypes. Further work will need to be done to examine EspZ, Tir and intimin interactions by pulling down all these proteins together with a translocator, presumably EspB or EspD, in order to fully investigate this hypothesis.

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