Defining the effector(s) and mechanism(s) by which enteropathogenic *E. coli* (EPEC) inhibits Akt signalling upstream of PI3 kinase

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Abstract

Defining the effector(s) and mechanism(s) by which enteropathogenic *E. coli* (EPEC) inhibits Akt signalling upstream of PI3 kinase.

Disease associated with enteropathogenic *Escherichia* coli (EPEC) depends on a Type 3 Secretion System (T3SS) that delivers 'effector' proteins into infected cells. The EPEC E2348/69 strain has approximately 6Mb of horizontally acquired DNA provided, mostly, by 21 prophage (PP) and integrative element (IEs). The T3SS, six effectors and two chaperones - latter aid the export of T3SS substrates - are encoded on an IE named LEE (Locus of Enterocyte Effacement). The remaining effectors are encoded on 7 other IE/PPs. EPEC inhibits the activity of a host kinase, Akt, that regulates important cellular processes, including cell survival, but the responsible effectors and inhibitory mechanism remain undefined. Previous work discounted roles for 17 of 21 known effectors but linked the inactivation process to Akt dephosphorylation in a manner requiring the LEE chaperone, CesT. Here, additional screening strategies undertaken to identify the responsible effector(s) uncovered key but redundant roles for two, previously unexamined, T3SS substrates: LifA (3223 residue, IE6-encoded) and LifA-like (2624 residue, IE2-encoded) proteins. These proteins have putative glycosyltransferase and protease activities with LifA thought to be delivered into host cells. Other studies support CesT dependence of the inhibitory process and, surprisingly, revealed LifA's inhibitory activity requires IE2-encoded factor(s). Screening IE2-related fragments implicated factor(s) on a cloned 1.7kB region but further studies are needed to confirm the results and provide mechanistic insights. Studies on LifA's inhibitory mechanism revealed its T3SS- and CesT-dependent accumulation with host membrane proteins with dephosphorylation of Akt not requiring motifs needed for its known glycosyltransferase and protease activities. By contrast, immunoprecipitation studies linked the inhibitory mechanism to O-glycosylation of Akt and revealed infection-induced O-GlcNAc of an Akt-sized band that gradually disappeared in a T3SS-dependent manner. Studies described in this thesis not only identify the EPEC effectors responsible for inhibiting Akt activity, but also provide important insights into the inhibitory mechanism.

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List of Abbreviations

A:-

A/E lesion: attaching and effacing lesion

Ab: antibody

AD: activation domain

AIEC: adherent invasive *Escherichia coli*

Arf: ADP-ribosylation factor

ARP2/3: actin related protein 2/3

B:-

BD: binding domain

BFP: bundle forming pilus

BSA: bovine serum albumin

B171: EPEC O111: NM B171

C:-

Cif: cell cycle inhibiting factor

Carb: Carbenicillin

Cmp: Chloramphenicol

CR: Citobacter rodentium

C-terminal: Carboxyl-terminal

D:-

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

DEC: diarrhoeagenic *Escherichia coli*

DH/PH: Dbl-homology/ pleckstrin homology

DMEM: Dulbecco's minimal Eagle's medium

DMSO: dimethylsulphoxide

E:-

EAF: EPEC adherence factor

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

EHEC: Enterohemorrhagic *Escherichia coli*

EIEC: Enteroinvasive *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

Esc: EPEC secretion

Esp: EPEC secreted/signalling protein

ESCRT: endosomal sorting complexes required for transport

F:-

FADD: Fas-associated death domain

FAK: focal adhesion kinase

FCS: Foetal calf serum

G:-

GAP: GTPase-activating protein

GEF: guanine nucleotide exchange factor

GFP: green fluorescent protein

GI: genomic island

Glc: Glucose

H:-

HRP- horseradish peroxidase

I:-

IE: integrative element

IEC: intestinal epithelial cell

IKK: IκB kinase

IMS: inner membrane space

IRTKS: insulin receptor tyrosine kinase substrate

IκB: inhibitor of kappa B

ITIM: Immune receptor tyrosine based inhibition motifs

J:-

JAM: Junction adhesion molecule

JNK: c-Jun N-terminal kinase

K:-

Km: Kanamycin

L:-

LEE: locus of enterocyte effacement

LB: Luria broth

Ler: LEE-encoded regulator

LPS: lipopolysaccharide

LRR: leucine-rich repeat

M:-

Map: Mitochondrial associated protein

MAPK: mitogen activated protein kinase

MOI: Multiplicity of infection

MS: mass spectrometry

MSA: multiple sequence alignment

MTS: mitochondrial targeting sequence

N:-

NF-κB: nuclear factor kappa-B

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

Nle: Non-LEE encoded

N-WASP: Neural Wiskott-Aldrich syndrome protein

O:-

OD600: Optical density 600 nm

P:-

PAK: p21-activated kinase
PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

Per: Plasmid encoded regulator

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

PKA: Protein kinase A

PIC: Protein inhibitor cocktail

PMSF: Phenylmethane sulfonyl fluoride

PPR: Polyproline region

PRR: pattern recognition receptor

Q:-

R:-

RDEC/REPEC: rabbit diarrhoeagenic *Escherichia coli*/ rabbit EPEC

RHIM: Rip homotypic interaction motif

RID: Rho GTPase inactivation domain

RIPK: receptor-interacting protein kinase

RTK: Receptor tyrosine kinase

S:-

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

SPATE: serine protease autotransporters of Enterobacteriaceae

STEAEC: Shiga toxin-producing enteroaggregative *Escherichia coli*

Stx: Shiga toxin

T:-

T1SS: Type I secretion system

T2SS: Type II secretion system

T3SS: Type III secretion system

T4SS: Type IV secretion system

T5SS: Type V secretion system

T6SS: Type VI secretion system

T7SS: Type VII secretion system

Tet: Tetracycline

Tir: Translocated intimin receptor

Thr: Threonine

TNFα: Tumour necrosis factor alpha

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

TRAF: TNF receptor associated factor

U:-

UPEC: Uropathogenic *Escherichia coli*

W:-

WT: Wild type

Chapter 1. Introduction

1.1 General introduction

The human gastrointestinal (GI) tract provides one of the largest interfaces (250-400m^2) with the external environment and contains trillions of microorganisms – the so called microbiome (Clements *et al.*, 2012; Thursby and Juge, 2017). The gut microbiota is a complex community of mostly bacteria, with many important functions beneficial to the host including the breakdown of nutrients (not degradable by the host), preventing pathogen colonisation through competition, and promoting the maturation of immune cells (Clements *et al.*, 2012). The GI tract is lined by a single layer of epithelial cells with the majority of nutrient, ion and fluid uptake occurring in the small intestine where most cells (>80%) are absorptive epithelia (enterocytes in small intestine; colonocytes in large intestine) with other cells including goblet (secrete mucin), Paneth (secrete antimicrobial peptides), enteroendocrine (release hormones) and antigen-presenting microfold (M)-cells (Snoeck *et al.*, 2005). This monolayer is covered in glycocalyx and mucus layers which provide effective barriers to bacteria accessing the epithelial surface (Liao *et al.*, 2009). Absorptive epithelia have a distinct gut lumen-exposed (apical) surface, often referred to as the brush border (Figure 1) as they are covered in microvilli (actin-rich, finger-like projections) that increase the cells' absorptive surface area and contain transporters for uptake of ions, nutrients and fluids (Snoeck *et al.*, 2005; Vereecke *et al.*, 2011) Loss of transporter function and/or microvilli has diarrhoeagenic consequences. Key to enterocyte development are tight junctions (TJ) between adjacent epithelia as they prevent the movement of molecules between the epithelial cells and also between apical and baso-lateral membrane (Figure 1) allowing the accumulation of molecules such as transporters in specific regions (Snoeck *et al.*, 2005; Zihni *et al.*, 2016). TJs prevent the unregulated movement of ions, nutrients, and foreign antigens across the epithelial layer. The basolateral membrane has high levels of receptors for detecting specific foreign antigen so TJ disruption would not only promote a diarrhoeal outcome but would also trigger antigen receptor driven signalling that induces anti-microbial and inflammatory responses (Corr *et al.*, 2008).

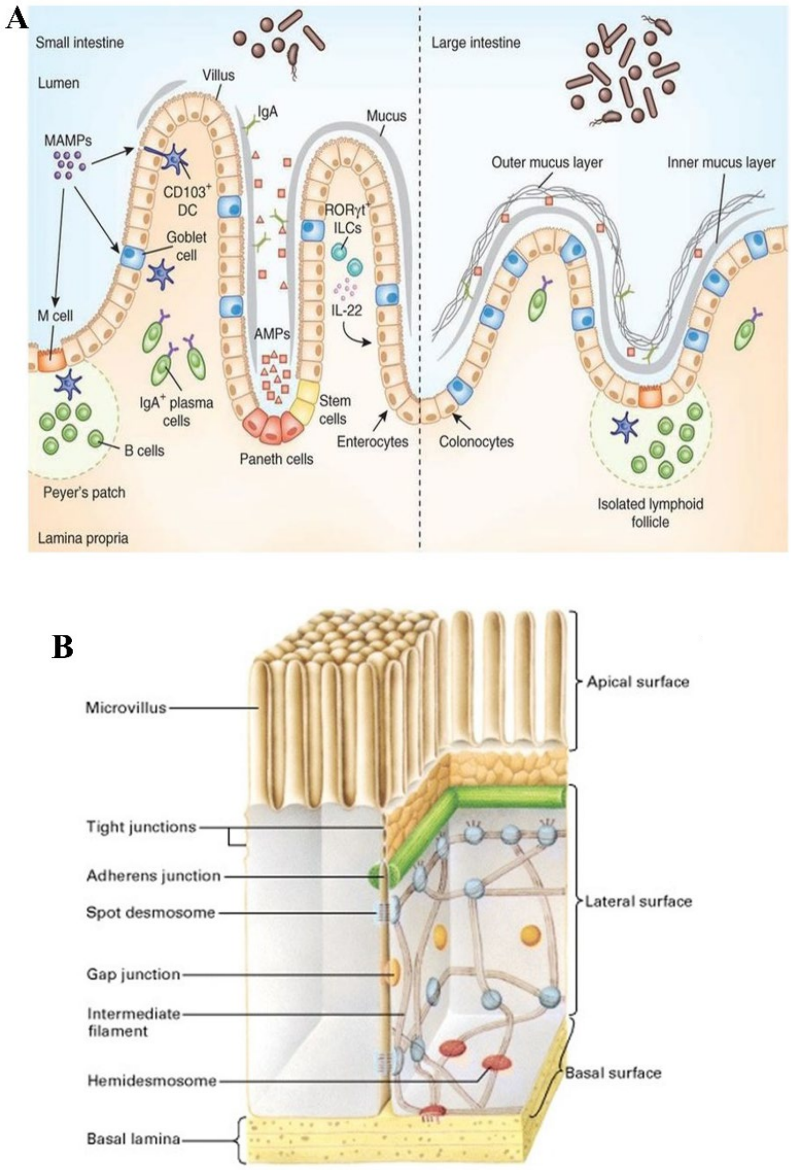


Figure 1 Schematic of epithelial cells lining the intestine and of the enterocyte.

Panel A- Components of the intestinal epithelial barrier. The intestinal epithelium is made up of a single layer of enterocytes (in the small intestine) or colonocytes (in the large intestine), with specialised cells scattered between them. Paneth cells abundant in the bases of small intestinal crypts generate antimicrobial peptides, whereas Goblet cells create mucins that form a dense inner mucus layer and a loose outer mucus layer (AMP). Microfold (M) cells and dendritic cells (DC) that extend their dendrites into the gut lumen facilitate antigen sampling. Abbreviations IgA, ILC, IL-22 and MAMP refer to immunoglobulin A, innate lymphoid cell, interleukin-22 (a cytokine) and microbe-associated molecular pattern (recognised by antigen receptors) respectively. Taken from Bossche *et al*., 2017 (Van den Bossche, 2017) Panel B- Schematic of an enterocyte: the basal surface rests on the basal lamina, a fibrous network of collagen and proteoglycans that protects the epithelial cell layer with the apical (brush border) surface facing the lumen of the intestine. Tight junctions located just below the microvilli inhibit the diffusion of substances between apical and basolateral surfaces also, between adjacent cells. Image taken from Lodish *et al* (Lodish *et al*., 2000).

1.2 Overview of the gut microbiota

The human gastrointestinal tract (GIT) contains a large number of bacteria, archaea and eukarya which co-evolved to form an intricate mainly mutualistic relationship with the human host (Bäckhed *et al.*, 2005; Neish, 2009). The bacterial components of this '**gut microbiota**' consists of more than 10^{14} microorganisms from 12 different phyla and >2000 species (Neish, 2009; Hugon *et al.*, 2015). The number of bacteria in the GIT is ~10 times more than human cells (Bäckhed *et al.*, 2005) though a recent revised estimate is a 1:1 ratio (Sender *et al.*, 2016). The bacterial community is composed more of anaerobes (60%-90%) than aerobes and can vary largely among different individuals as influenced by factors such as age, gender, diet, health treatments, host immune system, genetics, heath conditions, demographic, and socio-economic factors (Ardissone *et al.*, 2014). The gut microbiota benefits the host in many ways such as enhancing the gut integrity (tight-tight junction interactions), modulating the activity of the epithelia cells (Natividad and Verdu, 2013), regulating host immunity (Gensollen *et al.*, 2016), providing essential vitamins (Thursby and Juge, 2017) and protecting against pathogens (Bäumler and Sperandio, 2016).

An example of how gut bacteria are beneficial to the host comes from species that reside in the colon producing carbohydrate-active enzymes that act on indigestible polysaccharides. such as dietary fibre, generating short chain fatty acids (SCFAs) that can impact on host physiology (Musso *et al.*, 2010). Three SCFAs - propionate, butyrate and acetate - are linked to regulation of various cellular processes such as cell differentiation, proliferation and apoptosis (Corrêa-Oliveira *et al.*, 2016). Acetate is directly produced by anaerobic gut microbes whereas butyrate and propionate are produced by gut microbes using distinct pathways (Louis and Flint, 2017). Glycolysis and acetoacetyl-CoA produce butyrate whereas succinate or propanediol pathways forms propionate respectively (Louis and Flint, 2017). SCFAs play a crucial role in regulating inflammatory and immune responses (Morrison and Preston, 2016) with, for example, SCFAs stimulating interleukin 18 (IL-18) production involved in repairing and maintaining epithelial cell integrity (Corrêa-Oliveira *et al.*, 2016). Butyrate and propionate can also regulate gene

expression as they acts as histone deacetylase inhibitors (Morrison and Preston, 2016; Amin, 2017).

In addition, GIT microbiota are responsible for the *de novo* synthesis of essential vitamins such as B12 produced by lactic acid bacilli (Martens *et al.*, 2002; LeBlanc *et al.*, 2013). Folate, a vitamin critical for DNA synthesis and repair is produced by *Bifidobacteria* (Pompei *et al.*, 2007) with other species synthesising additional vitamins such as thiamine, riboflavin, biotin, nicotinic acid, pyridoxine and panthotenic acid (Hill, 1997). Bile acids which are poorly reabsorbed by the host can also metabolised by these gut microbes (Staley *et al.*, 2017).

However, changes in the microbial composition/diversity - known as microbiota disequilibrium or ***dysbiosis*** **(**Figure 2) can have negative impacts on the host (Thursby and Juge, 2017). For example, an important function of gut microbes is to inhibit the colonization of ingested, potentially pathogenic bacteria in a competitive manner or by providing an environment that hinders colonisation (Walter *et al.*, 2018). The availability of favourable conditions such as nutritional or functional space is crucial for microbial colonization and external factors that cause dysbiosis. Such as antibiotic treatment can enable colonisation by other species including pathogens (Libertucci and Young, 2019). One member of the gut microbiota is *Escherichia coli* (*E. coli*).

1.3 *Escherichia coli* (*E. coli*)

E. coli are gram-negative, facultative anaerobic bacteria that reside in the human gastrointestinal tract where they colonize in the mucous layer of the colon (Nataro and Kaper, 1998). *E. coli* was first identified and described by Theodor Escherich in 1885 (Escherich, 1988). Taxonomically, *E. coli* is categorised in the enteric bacterial family, *enterobacteriaceae* (Nataro and Kaper, 1998). Most *E. coli* strains are commensals, causing disease only in susceptible individuals (Nataro and Kaper, 1998) but several highly adapted *E. coli* strains can cause enteric disease i.e., pathogenic (Kaper *et al.*, 2004). The strains derive their enteropathogenic capacity by acquiring specific factors that enhance their ability to colonize the gastro-intestinal tract (Kaper *et al.*, 2004).

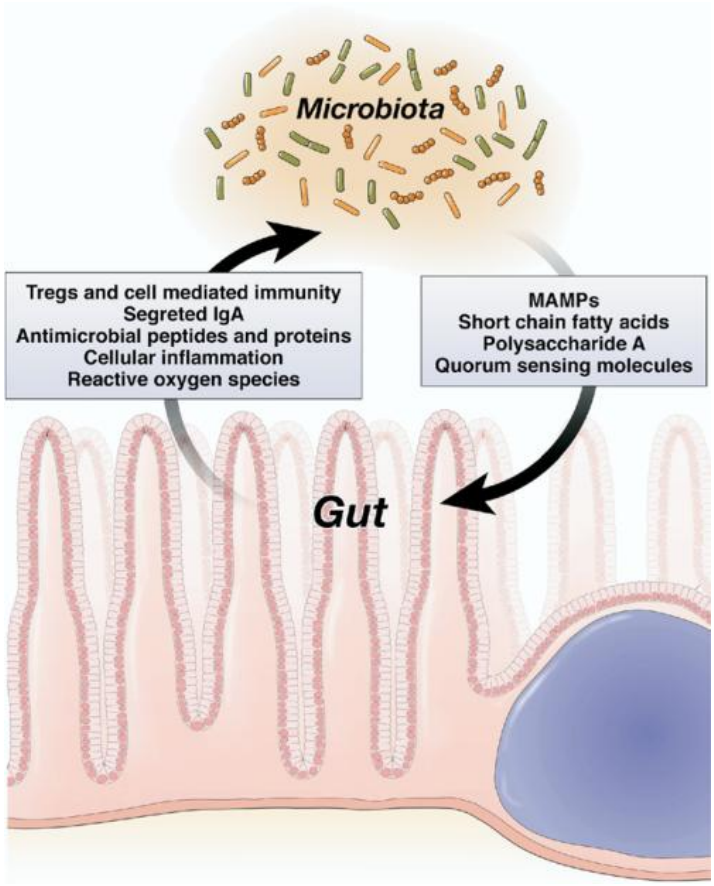


Figure 2 Crosstalk between microbiota and human gut

A balanced equilibrium is necessary for mutualistic relationships between host and gut microbiota. Clinical manifestation results due to a breakdown in host-bacterial crosstalk is called “dysbiosis”. Abbreviations IgA and MAMP refer to immunoglobulin A and microbe-associated molecular pattern (recognised by antigen receptors) respectively. Image taken from Neish *et al* (Neish, 2009).

1.4 Pathogenic *E. coli*

Pathogenic *E. coli* strains have acquired **'*virulence*'** factors that confer important adaptations (such as adherence or cell invasion capacity) facilitating efficient colonization of a particular niche within the GIT (Kaper *et al.*, 2004). Pathogenic *E. coli* are divided into two groups based on the infection site: extra-intestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC) (Rojas-Lopez *et al.*, 2018). ExPEC consists of strains that cause urinary tract infections (UTI) or neonatal meningitis (Kaper *et al.*, 2004). The InPEC strains are subdivided into 8 prominent pathotypes based on their virulence factors, enterocyte interactions, mechanism of infection and tissue tropism. The pathotypes are: (i) enteropathogenic *E. coli* (EPEC), (ii) enterohaemorrhagic *E. coli* (EHEC), (iii) enterotoxigenic *E. coli* (ETEC), (iv) enteroagggregative *E. coli* (EAEC), (v) enteroinvasive *E. coli* (EIEC), (vi) diffusely adherent *E. coli* (DAEC), (vii) adherent-invasive *E. coli* (AIEC) and (viii) Shiga-toxin producing enteroaggregative *E. coli* (STEAEC) (Nataro and Kaper, 1998; Croxen and Finlay, 2010). Key to the pathogenic capacity of bacteria are secretion systems which enable the delivery of proteins to the cell surface (for example to provide adhesins), the extracellular milieu or even into target cells.

1.5 Bacterial secretion systems

The most prevalent secretion mechanisms for transporting proteins across the cytoplasmic membrane are the general secretion (Sec) and twin arginine translocation (Tat) pathways (Natale *et al.*, 2008). The Sec and Tat pathways have been found in all domains of life and are the most highly conserved mechanisms of protein secretion (bacteria, archaea, and eukarya) (Robinson and Bolhuis, 2004; Papanikou *et al.*, 2007).

1.5.1 The Sec Secretion Pathway

The Sec pathway mainly transports proteins that are in an unfolded state and is composed of the SecYEG translocase comprising a protein targeting component, a motor protein and a membrane-integrated conducting channel (Natale *et al.*, 2008)(Figure 3). Sec-dependent secretion leads to proteins being delivered into or

across the cytoplasmic membrane; latter results in their accumulation in the extracellular and periplasmic space of gram-positive and gram-negative bacteria respectively (Korotkov *et al.*, 2012). The Sec pathway relies on a hydrophobic signal sequence at the secreted protein's N-terminus which is generally 20 amino acids long and has three distinct regions: a positively charged amino terminal, a hydrophobic core and a polar carboxyl-terminal region (Natale *et al.*, 2008). Many Sec pathway substrates have a signal sequence recognised by the SecB chaperone which inhibits the pre-secretory protein from folding (Randall and Hardy, 2002). SecB then transfers its substrates to SecA, a multifunctional protein that serves as both a protein guide and an ATPase that supplies energy, for protein translocation (Hartl *et al.*, 1990). A protease (Lep) removes the SecB signal sequence from the secreted protein before transport via the channel, and the released protein is then folded upon delivery to the periplasm (Mogensen and Otzen, 2005)(Figure 3). While SecB-specific signal sequences direct proteins across the cytoplasmic membrane some substrates have signal recognition particle (SRP)-specific signal sequences that directs them for insertion into the inner membrane (Green and Mecsas, 2016) (Figure 3).

1.5.2 The Tat Secretion Pathway

The Tat (twin-arginine translocation) pathway, in contrast to the Sec pathway, largely secretes folded proteins and is made up of three subunits (TatA, TatB, TatC) in gram-negative bacteria (Robinson and Bolhuis, 2004)(Figure 3). In Gram-positive bacteria the system has 2 proteins as TatA and TatB are combined into one multi-functional protein (Sargent *et al.*, 1999; Pop *et al.*, 2002). TatB and TatC bind the signal peptide of Tat-substrate proteins, which then recruits TatA to construct the membrane-spanning channel (Müller, 2005). At the N-terminus of the folded protein, the Tat signal sequence has a pair of "twin" arginine residues in the S-R-R motif (Müller, 2005)(Figure 3). Other protein secretion systems have been identified in gram negative bacteria (Green and Mecsas, 2016). These are shown in Figure 4 and described in sections 1.5.3 – 8.

1.5.3 Type I secretion system (T1SS)

The T1SS was identified in relation to understanding how *E. coli* secreted a protein that could lyse red blood cells and therefore named hemolysin A (HlyA). The secretion system comprises 3 proteins - two in the inner (HlyB, HlyD) and one in the outer (TolC) membrane that transfer HlyA directly from the cytoplasm to the extracellular milieu i.e. bypassing the periplasmic space (Koronakis *et al.*, 2004)(Figure 4).

HlyB is an ABC transporter protein with a cytoplasmic domain that binds HlyA via an uncleaved C-terminal signal peptide. This HlyA C-terminal signal is crucial for secretion and can direct the secretion of other proteins (Kenny *et al.*, 1991; Nakano *et al.*, 1992). The HlyB ATPase forms a complex with the adaptor protein HlyD (membrane fusion protein; MFP) independent of TolC (outer membrane protein; OMP) protein (Thanabalu *et al.*, 1998). However, in the presence of the HlyA substrate HlyD, a trimeric inner membrane protein, interacts with the outer membrane component TolC (Figure 4) which opens the TolC channels in an iris-like mechanism to allow substrate secretion (Koronakis *et al.*, 2004; Dalbey and Kuhn, 2012). T1SS are found in most gram-negative bacteria linked to the release a large variety of virulence proteins.

1.5.4 Type II secretion system (T2SS)

T2SSs are also found in a wide range of pathogenic and non-pathogenic Gram-negative bacteria (Nivaskumar *et al.*, 2014). The T2SS consists of 12-15 components, known as general secretion pathway (Gsp) proteins in enterotoxigenic *Escherichia coli*, Xcp in *P. aeuroginosa* and Eps in *V.cholerae* (Korotkov *et al.*, 2012). The T2SS complex consists of four subassemblies: the OM complex, the IM platform, a periplasmic pseudopilus and a cytoplasmic secretion ATPase (Korotkov *et al.*, 2012). Most studies have been carried out on the *E. coli* T2SS where the OM complex is composed of GspD - also known as the secretin - forming a dodecameric complex GspD (Korotkov *et al.*, 2012).

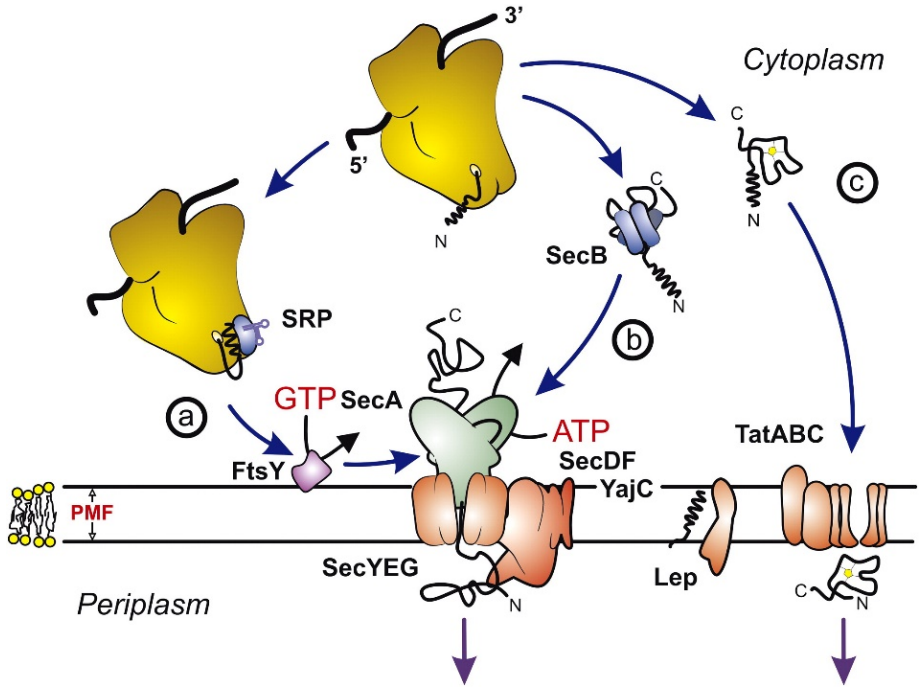


Figure 3 Schematic of the *Escherichia coli* Sec-and Tat protein secretion pathways

Indicated are the protein secretion pathways involving a) co-translational, b) post-translational or c) pre-folded proteins. Image taken from Natale *et al.,* (Natale *et al.*, 2008)

Four membrane proteins (GspC, GspF, GspL, GspM) form the IM platform which connects to the OM complex, by GspC interacting with the periplasmic domain of GspD (Abendroth *et al.*, 2004; Abendroth *et al.*, 2009).The cytoplasmic ATPase, GspF, is a ring-shaped hexamer that is recruited to the IM platform through binding GspL and GspF (Py *et al.*, 2001; Gray *et al.*, 2011; Gao *et al.*, 2013). A pseudopilus within the periplasm (Figure 4) is composed of a major and four minor pseudopilin subunits which are inserted into the IM by the SecYEG translocon with the signal sequence cleaved by the peptidase GspO (Korotkov *et al.*, 2012). The main pseudopilus component, GspG, polymerized into a pseudopilus anchored to the IM by a N terminal helix (Campos *et al.*, 2010). The minor pseudopilins (GspH, GspI, GspJ, GspK) locate at the tip of the GspG helical filament completing the pseudopilus (Cisneros *et al.*, 2012; Korotkov *et al.*, 2012). T2SS substrates are transferred into the periplasm by the Tat or Sec pathways where they can form disulphide bonds or multi-protein complexes before engaging the T2SS pseudopilus (Nivaskumar *et al.*, 2014). The T2SS ATPase is utilised in the formation of the periplasmic pseudopilus and the pushing of substrates (at tip of pilus) through the OM channel. Extension of pseudopilus drive substrates release through the secretin channel in a piston-like manner with disassembly allowing reuse of the system for further rounds of substrate secretion (Nivaskumar *et al.*, 2014).

1.5.5 Type III secretion system (T3SS)

T3SSs - also known as “injectisomes” and “needle and syringe-like appendage” - deliver protein substrates directly into target host cells (Cornelis, 2006; Galán and Wolf-Watz, 2006; Büttner, 2012)(Figure 4). Once transported into the host cell, these effector proteins subvert specific cellular functions to for example promote bacteria invasion and colonization processes (Büttner, 2012). This system is described in more detail in relation to EPEC pathogenesis (see section 1.8. below)

1.5.6 Type IV secretion system (T4SS)

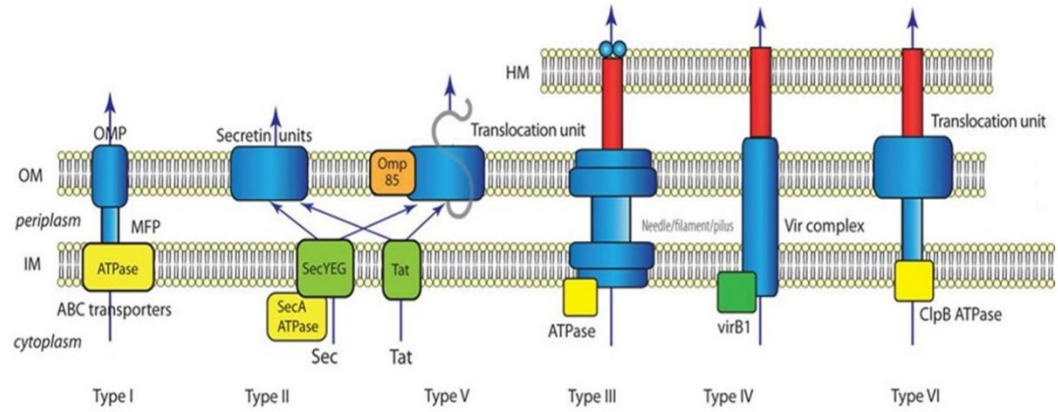
The T4SSs were first identified in relation to transferring DNA between bacteria and have been found in Gram-negative, Gram-positive bacteria and some archaea (Alvarez-Martinez and Christie, 2009). T4SSs can also be dedicated to transferring only effector proteins into target host cells with the prototypic system composed of 12 proteins: VirB1-11 and VirD4 (Figure 4). The VirB3 protein along with B6-B10 form the scaffold and translocation apparatus (Christie *et al.*, 2014), while, the extracellular pilus is formed by VirB2 and VirB5 (Trokter *et al.*, 2014). VirB1 is a periplasmic lytic transglycosylase that remodels the peptidoglycan layer and is crucial for pilus biogenesis (Trokter *et al.*, 2014). VirB4, VirB11 and VirD4 are essential for providing energy for the T4SS system (Trokter *et al.*, 2014) with 14 copies of VirB7, VirB9 and VirB10 making the core-OM complex (Chandran *et al.*, 2009). Substrate secretion is mediated by VirD4 which acts as a molecular **"gate"** beneath the secretion apparatus (Atmakuri *et al.*, 2004) and delivers the substrate to VirB11 for transfer across the periplasm and outer membrane (Chandran *et al.*, 2009) and, for some pathogenic bacteria, transfer into target host cells (Backert and Meyer, 2006).

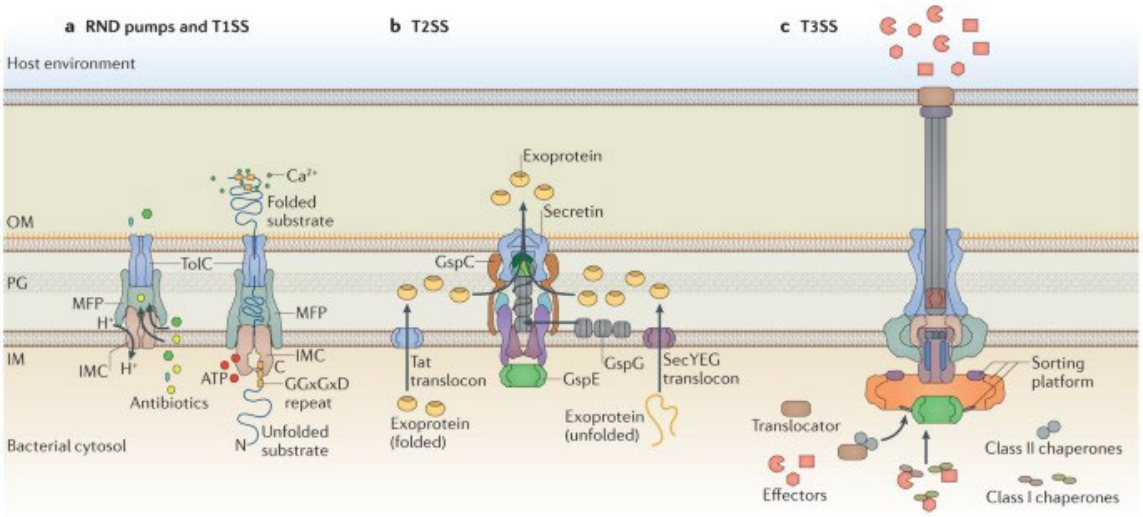
1.5.7 Type V secretion system (T5SS)

The classical T5SS is also known as the autotransporter system as it contains all the information to enable its secretion across the IM, periplasm and OM (Deng *et al.*, 2012; Costa *et al.*, 2015). The SecYEG translocon transfers the T5SS protein, via a cleavable signal sequence, into the periplasm (Leyton *et al.*, 2012)(Figure 4). Once in the periplasm, a β-barrel domain is inserted - by the BAM (β-Barrel Assembly Machinery) system - into the OM to provide a pore by which the 'passenger' domain is transferred across the OM (Leyton *et al.*, 2012). The passenger domain can be either be cleaved to provide a secreted protein e.g. toxin or remain on the cell surface to act, for example, as an adhesin (Junker *et al.*, 2009; Deng *et al.*, 2012).

1.5.8 Type VI secretion system (T6SS)

The Type-6-secretion system (Figure 4) was only described in 2006 but is now known to be widely distributed among Proteobacteria (Pukatzki *et al.*, 2006; Boyer *et al.*, 2009). It is a cell envelope-spanning machine, composed of 14 proteins, that mainly function in bacterial competition - delivers 'toxins' into the periplasm of other bacteria but can also deliver proteins into mammalian cells (Wan *et al.*, 2007; Boyer *et al.*, 2009; Basler *et al.*, 2012; Zoued *et al.*, 2014). The T6SS is composed of two main complexes: a membrane complex consisting of IM proteins homologous to T4SS components and a tail complex with components evolutionary related to those of the bacteriophage contractile tail (Pukatzki *et al.*, 2006; Wan *et al.*, 2007; Leiman *et al.*, 2009). The membrane complex attaches the T6SS tail complex - a long tubular structure that extends deep into the bacterial cell cytoplasm - to the cell envelope (Basler *et al.*, 2012). The membrane complex consists of 3 proteins: TssM an IM protein that binds the OM lipoprotein TssJ to the IM protein TssL (Abendroth *et al.*, 2009; Felisberto-Rodrigues *et al.*, 2011; Zoued *et al.*, 2014). The T6SS tail tube and sheath are assembled on a platform known as baseplate formed by VgrG and TssE - homologs of the T4 phage baseplate proteins (Leiman *et al.*, 2009). In the middle of the baseplate complex, the VgrG trimer forms a spike (Leiman *et al.*, 2009; Basler *et al.*, 2012) which is hypothesised to act as a nucleation platform for the T6SS tail tube's assembly (Brunet *et al.*, 2014) (Figure 4). *In-vitro*, Hcp proteins form hexameric rings with an interior diameter of 40 microns (Mougous *et al.*, 2006; Zoued *et al.*, 2014). In contrast, *in-vivo*, Hcp hexamers form tubular structures by stacking head to tail (Brunet *et al.*, 2014). The tail sheath is predicted to be built by TssB–TssC heterodimers which polymerize around the developing Hcp tube (Basler *et al.*, 2012; Brunet *et al.*, 2014).


HM
OMP
Secretin units
Translocation unit
Translocation unit
OM
Omp 85
periplasm
MFP
Needle/filament/pilus
Vir complex
IM
ATPase
SecYEG
Tat
ABC transporters
SecA ATPase
cytoplasm
Sec
Tat
ATPase
virB1
ClpB ATPase
Type I
Type II
Type V
Type III
Type IV
Type VI


a RND pumps and T1SS
b T2SS
c T3SS
Host environment
Ca2+
Folded substrate
Exoprotein
Secretin
OM
TolC
GspC
PG
MFP
MFP
H+
IM
IMC
H+
IMC
ATP
C
GGxGxD repeat
Tat translocon
GspG
SecYEG translocon
Sorting platform
Antibiotics
GspE
Translocator
Bacterial cytosol
N
Unfolded substrate
Exoprotein (folded)
Exoprotein (unfolded)
Class II chaperones
Effectors
Class I chaperones

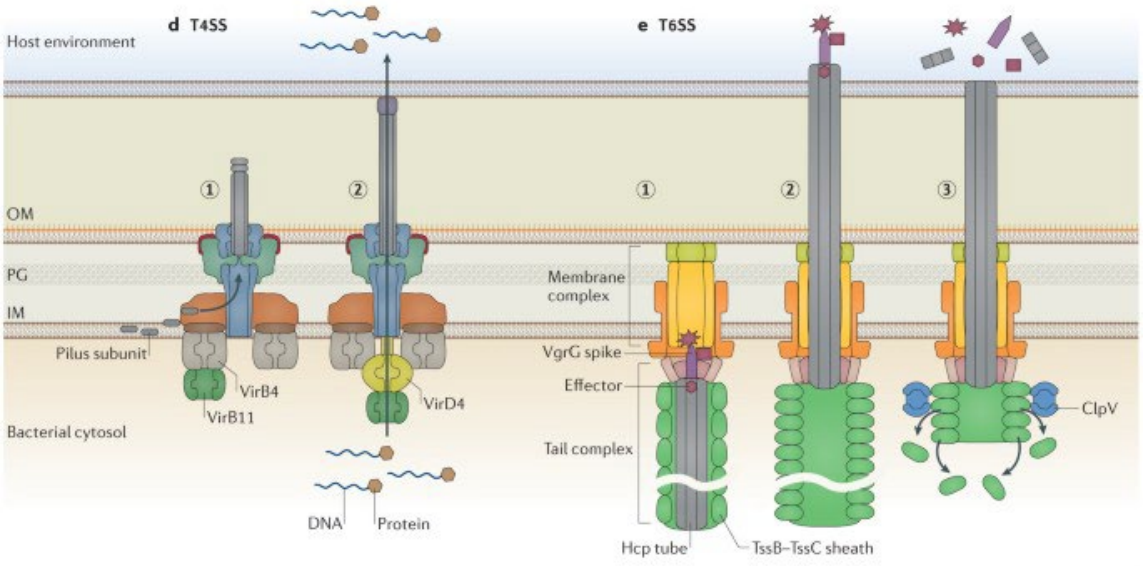

d T4SS
e T6SS
Host environment
1
2
1
2
3
OM
PG
Membrane complex
IM
Pilus subunit
VgrG spike
VirB4
Effector
ClpV
VirB11
VirD4
Tail complex
Bacterial cytosol
DNA
Protein
Hcp tube
TssB–TssC sheath

Figure 4 Models for Gram-negative bacteria's substrate secretion mechanism

Panel A-Schematic of main secretion systems in gram negative bacteria. Abbreviations: HM (host membrane); OMP (outer membrane protein); IM (inner membrane) OMP: MFP (membrane fusion protein). Yellow represents ATPases and chaperones. Adapted from image generated by Tseng and colleagues (Tseng *et al.*, 2009). Panel B- **a-**Antibiotics and small chemicals are translocated to the membrane fusion protein (MFP) by the inner membrane component (IMC) via the IM proton gradient, and they are released into the extracellular compartment by the outer membrane (OM) protein TolC in resistance–nodulation–division (RND) pumps. **b-** The GspC protein recruits the substrate (a folded exoprotein) to the secretin in T2SSs. GspE's ATPase activity is linked to the formation of the periplasmic pseudopilus *via* the IM platform, **c-** T3SS, **d-** The T4SS mechanism for pilus formation and substrate translocation is depicted schematically. VirB11 interacts with VirB4 to increase pilus subunit assembly (step 1), whereas VirB11 interacts with VirD4 to assist substrate translocation (step 2), **e-** On the membrane complex, the T6SS tail complex assembles. Effectors are recruited to the spike–tube complex via the extension domains of VgrG and/or PAAR-repeat proteins (and/or non-covalent binding to them) as well as incorporation into the haemolysin co-regulated protein (Hcp) tube (step 1). Sheath contraction is triggered by an unknown extracellular signal, which causes the spike–tube complex to eject over the target membrane, allowing effector proteins to enter the cell (step 2). ClpV, an ATPase, disassembles the constricted TssB–TssC sheath, allowing a new T6SS complex to be built from the freed subunits (step 3).

1.6 Enteropathogenic *E. coli* (EPEC) pathogenesis

The focus of this thesis is enteropathogenic *E. coli* (EPEC) which was the first *E. coli* pathotype to be linked to human disease (Levine *et al.*, 1978) and remains an important agent of infantile diarrhoea in developing countries (Nataro and Kaper, 1998; Deborah Chen and Frankel, 2005). Diarrhoea is worldwide problem with, in 2016, diarrhoea causing more than 1·6 million deaths and the eighth leading cause of mortality (Moraga and Collaborators, 2017). Children under the age of 5 years are most susceptible and responsible for more than a quarter of the deaths with over 90% recorded in South Asia and Sub-Saharan Africa (Moraga and Collaborators, 2017). An uneven proportion of diarrhoeal morbidity and mortality exists with it being most abundant in low-income countries where there are less resources and poorer health infrastructures compared to high-income countries (Mills, 2014). EPEC remains an important contributor to infantile diarrhoeal cases and death (Liu *et al.*, 2016). Common symptoms reported in EPEC infections are watery diarrhoea, dehydration, vomiting, food intolerance, and low-grade fever (Nataro and Kaper, 1998; Goosney *et al.*, 2001). In addition, EPEC infection may lead to extreme nutrient malabsorption, resulting in persistence diarrhoea and nutritional aggravation (Fagundes-Neto and Scaletsky, 2000).

The main target of EPEC is the enterocyte of the small intestine with disease linked the formation of attaching and effacing (A/E) lesions (Moon *et al.*, 1983). The latter refers to the bacteria penetrating through the microvilli-rich brush border enabling intimate interaction with the host membrane and the formation of pedestal-like structures under the adherent, non-invasive bacteria (Figure 5). Such alterations depend on a horizontally acquired pathogenicity island called **LEE**, which stands for **Locus of Enterocyte Effacement**. LEE encodes a **Type 3 Secretion System (T3SS)** that delivers so-called ‘effector’ proteins into the enterocyte whose alteration of host cellular processes drive the disease (Croxen *et al.*, 2013; Cepeda-Molero *et al.*, 2020).

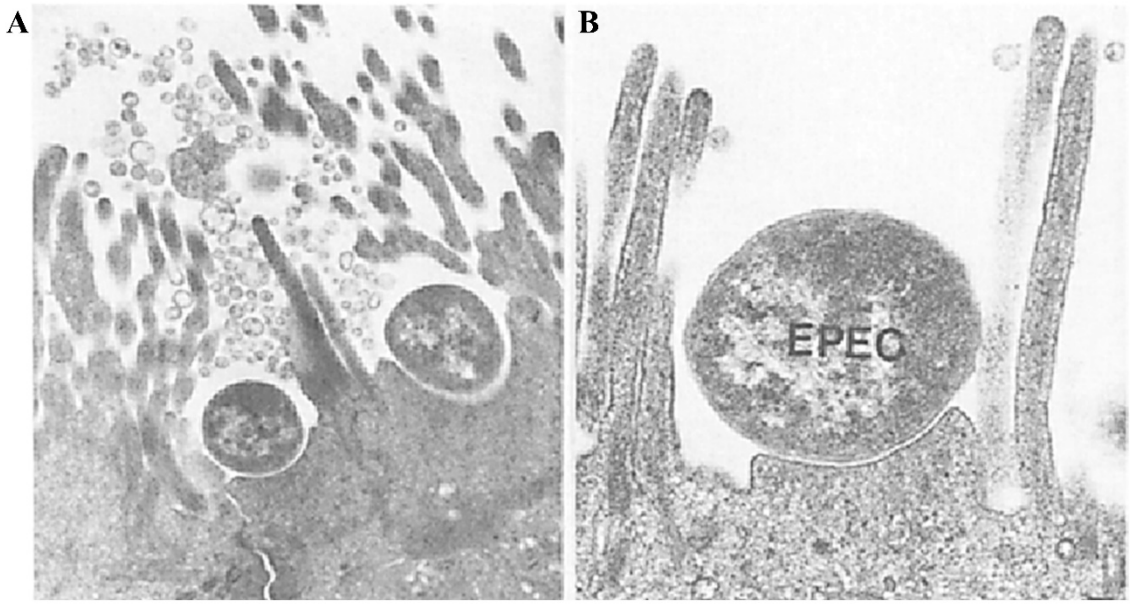


Figure 5 Attaching and effacing (AE) lesions on enterocytes infected by EPEC 2348/69

Panel A) AE lesions: intimately adherent EPEC linked to loss of microvilli and formation of pedestal formation beneath the adhered bacteria. Panel B) higher magnification (×45000) of A/E lesion associated bacteria. Taken from Knutton *et al.,* (Knutton, 1995).

1.7 The Locus of Enterocyte Effacement (LEE)

In 1990, the first chromosomal gene crucial for A/E lesion formation was identified and named *eaeA* (*E. coli* attaching and effacing A) (Jerse *et al.*, 1990). Five years later, the *eaeA* gene was localised to a pathogenicity island (PAI) in EPEC E2348/69 that was named Locus of Enterocyte Effacement, LEE (McDaniel *et al.*, 1995). Transfer of LEE into the non-pathogenic strain *E. coli* K12 provided the ability to form A/E lesions (McDaniel and Kaper, 1997). This LEE region is ~36kb in size and has 41 genes organised into five main operons (LEE1-LEE5; Figure 6) and some smaller transcriptional units (Elliott *et al.*, 2000; Yerushalmi *et al.*, 2014). LEE encodes proteins which form the T3SS, transcriptional regulators, T3SS substrates (several T3SS components including 'translocator' proteins plus effector proteins), chaperones (promote the stability and secretion of T3SS substrates), a protein of unknown function (*rOrf1*) and an outer membrane protein, Intimin - the *eaeA* gene product (Figure 6)(Deng *et al.*, 2004; Barba *et al.*, 2005; Dean and Kenny, 2009).

1.8 The EPEC Type-III-Secretion System (T3SS)

The T3SS apparatus is a syringe-like structure enabling the direct delivery of 'effector' proteins from the bacterial cytosol into the target host cell (Gaytán *et al.*, 2016). The EPEC T3SS is composed of ~20 proteins - most named Esc for *E. coli* secretion apparatus component - which form a basal body, extracellular appendage, and cytoplasm-located components (Coburn *et al.*, 2007) (Figure 7).

The basal body spans the bacterial envelope and consists of three multimeric rings with EscD and EscJ forming the inner membrane-spanning and membrane-tethered rings respectively; EscD interacts with the outer membrane ring formed by membrane-spanning EscC (Creasey *et al.*, 2003; Ogino *et al.*, 2006). The basal body encloses the protein export apparatus (composed of EscR/S/T/U/V), needle like-structure (composed of EscF subunits) that extends into the extracellular space and an adaptor protein (EscI) that connects the export apparatus and EscF needle structures. The entrance to the export channel is provided by the C-terminal

domain of EscV with the order and timing of substrate export controlled by the cytoplasmic membrane-associated C-ring (Diepold *et al.*, 2011).

T3SS substrates include several T3SS components - EscI, EscF and translocator proteins (EspA, EspB, EspD) - regulators (EscP; control the length of the EscF needle and when to secrete different substrates) and effectors. The export process is promoted by an ATPase complex - the ATPase (EscN) plus positive (EscO) and negative (EscL) regulators (Pallen *et al.*, 2005; Biemans-Oldehinkel *et al.*, 2011; Romo-Castillo *et al.*, 2014).

In EPEC, the EscF needle length is regulated by a ruler protein, EscP, interaction with assembling EscF subunits and the export apparatus protein, EscU. When the required length is reached, an interaction occurs that caused a switch in substrate specificity from needle to translocon components, EspA, EspB and EspD (Creasey *et al.*, 2003; Feria *et al.*, 2012; Sal-Man *et al.*, 2012). The export of the EspA protein leads to its polymerisation at the end of EscF to produce a long extension (up to 750nm) which is then tipped by the secreted EspB and EspD translocator proteins (Knutton *et al.*, 1998; Crepin *et al.*, 2005). The EspB and EspD proteins can insert into the host plasma membrane (forming a 3-5 nm pore), thus, completing an effector-delivery conduit between the cytoplasmic compartments of the bacterial and mammalian cells (Ide *et al.*, 2001). The pore formation step induces a regulatory event involving a SepD-SepL-CesL complex, switching export specificity to the effector proteins (O'Connell *et al.*, 2004). It has recently been demonstrated that through its association with SepL, the ruler protein EscP also plays a role in this second switch (Shaulov *et al.*, 2017). The export of most T3SS substrates is aided by chaperones by promoting intracellular stability, preventing unwanted protein-protein interactions, maintaining an unfolded state and delivering the substrate to the T3SS (Mills *et al.*, 2008; Ramu *et al.*, 2013). There are several classes of chaperone: class III, class II and class I that bind secreted needle components, translocators and effector proteins respectively (Gaytán *et al.*, 2016).

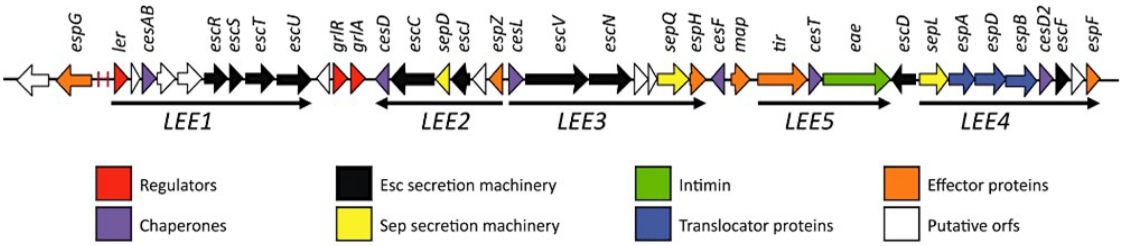


Figure 6 Schematic representing the genetic organization of the locus of enterocyte effacement (LEE) region from EPEC E2348/69

The LEE region of EPEC (E2348/69) includes 41 open reading frames organised in five polycistronic operons indicated LEE1-LEE5. The LEE region encodes proteins which form the T3SS including Esc (Black) and Sep (Yellow), regulatory factors (Ler, GrlA, GrlR, and Mpc; Red), chaperones (Purple), translocator proteins (Blue), effector proteins (Esp; Orange), the bacterial outer membrane protein Intimin (*eae*; Green) and other putative open reading frames (Orf; White). Adapted from Wong *et al.,* (Wong *et al.*, 2011).

Class I chaperones are further divided based on substrate number: class IA bind only one effector while class IB binding multiple effectors. LEE encodes 6 effectors (EspG, EspH, EspF, EspZ, Map, Tir) and one class 1A chaperone, CesF (binds EspF), and one class 1B chaperone, CesT (Elliott *et al.*, 2002; Mills *et al.*, 2013). Originally CesT was classified as a class IA chaperone with one substrate, Tir, (Abe *et al.*, 1999) but was later found to have many substrates (Mills *et al.*, 2013; Gaytán *et al.*, 2016). Some EPEC effectors having no known chaperone (Mills *et al.*, 2013; Gaytán *et al.*, 2016).

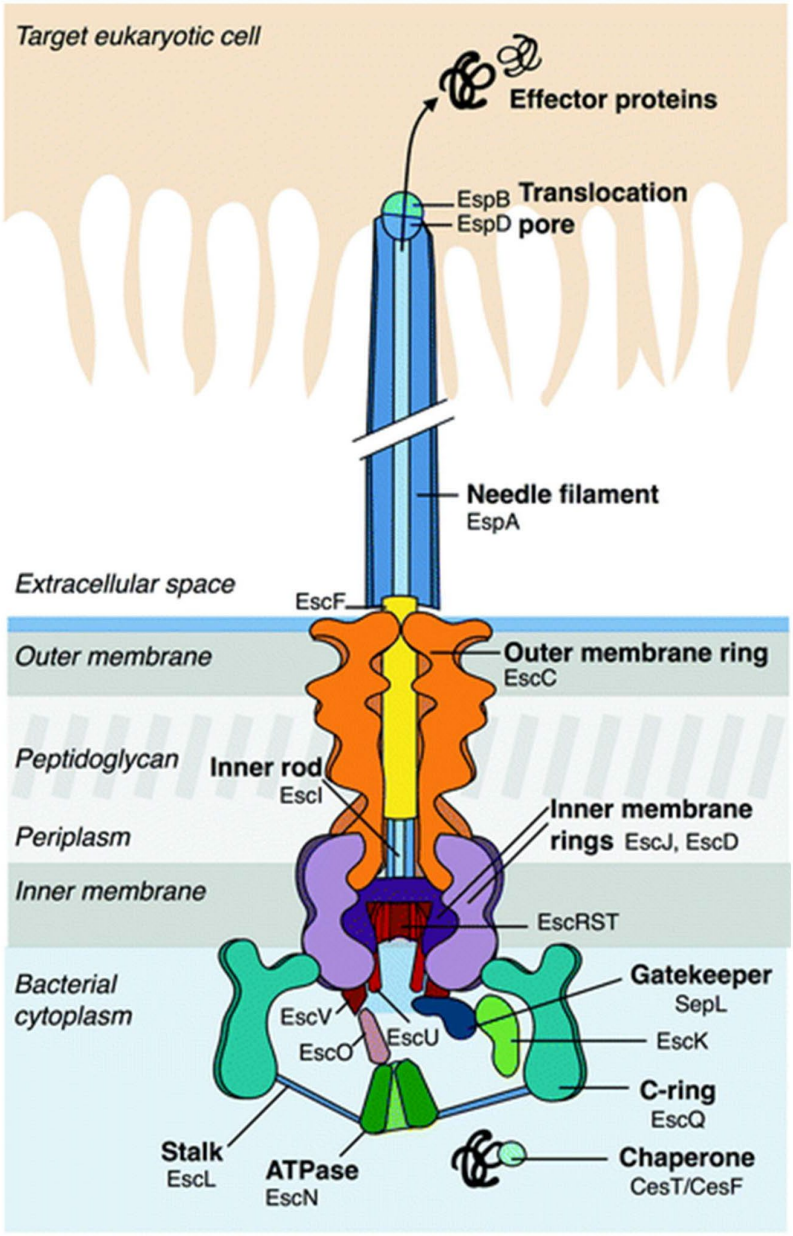


Figure 7 Schematic of the EPEC Type three Secretion System (T3SS)

The T3SS consists of (i) a basal body: three membrane rings connected via a periplasmic inner rod with a needle-like protrusion (ii) extracellular appendage: secreted 'translocator' EspA protein extends the needle and is tipped by secreted, membrane-inserting, EspB and EspD translocator proteins and (iii) cytoplasmic components: chaperones (promote stability/secretion of T3SS substrates), sorting platform proteins (ATPase, gatekeepers) that regulate the timing/order of substrate secretion. Taken from Slater *et al.*, (Slater *et al.*, 2018).

1.9 Other key virulence factors

1.9.1 Bundle Forming Pilus

EPEC targeting of enterocytes depends on adhesins which for typical EPEC strains is mediated by the Bundle forming Pilus (BFP) encoded on a large plasmid named pEAF, for EPEC adherence factor plasmid (Levine *et al.*, 1978; Giron *et al.*, 1991). BFP is produced from the 14 gene *bfp* operon where one gene, *bfpA*, provides the major structural subunit, bundlin (Stone *et al.*, 1996). First described as a bundle of rope like filaments, the BFP (50-500nm wide;14-20μm long) is retractable and is also crucial for bacterium-to-bacterium interaction leading to characteristic microcolonies (Giron *et al.*, 1991; Clarke *et al.*, 2003). BFP expression is regulated by the products of other pEAF genes, the plasmid-encoded regulator genes (*per*A, *per*B, *per*C) produced in response to environmental signals (Puente *et al.*, 1996; Mellies *et al.*, 1999). BFP expression is optimal between 35^0C and 37^0C in the presence of calcium and during exponential growth (Puente *et al.*, 1996; Mellies *et al.*, 1999). BFP is an essential EPEC virulence factor due to its roles in enterocyte colonisation and micro-colony formation for (Giron *et al.*, 1991; Bieber *et al.*, 1998; Knutton *et al.*, 1999). BFP binds to N-acetyllactosamine decorated moieties on the surface of host enterocytes (Hyland *et al.*, 2008) and acts during micro-colony formation in combination with the *E. coli* typical pili (Saldana *et al.*, 2009).

1.9.2 Non-LEE-encoded (Nle) effectors

The existence of T3SS effectors encoded outside the LEE region was first suggested by the identification of an EspG homologue (Orf3/EspG2) encoded on a pathogenicity island encoding another known virulence factor, the secreted EspC autotransporter protein (Elliott *et al.*, 2001). However, most non-LEE-encoded (Nle) effectors were identified from bioinformatics analysis of the EPEC E2348/69 genome sequence (Dean and Kenny, 2009; Iguchi *et al.*, 2009). Firstly, the analysis revealed a 4,965,553bp circular chromosome and two plasmids (Figure 8) with the chromosome sequence remarkably conserved to that of other examined *E. coli* strains - EHEC O157:H7 strain Sakai, ETEC strain E24377A and non-pathogenic

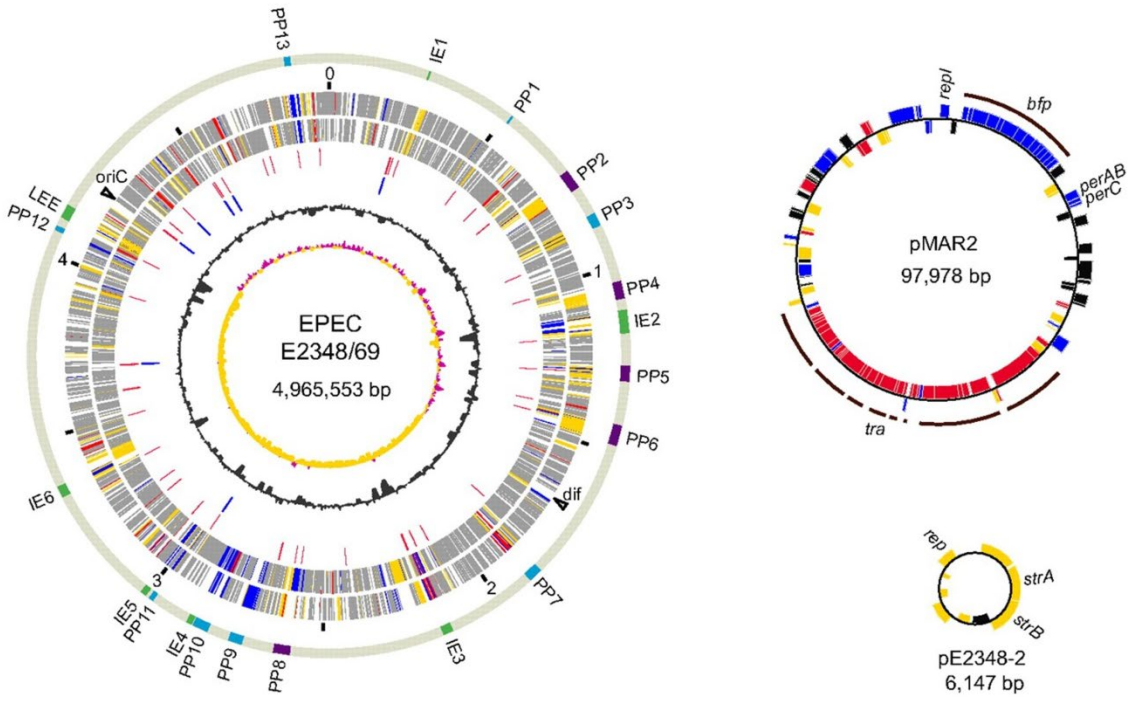


Figure 8 Mobile genetic elements in EPEC E2348/69

Genetic organisation of EPEC E2348/69 PPs and IEs containing gene related to the virulence. The first circle shows the locations of PPs and IEs from the outside (purple, lambda-like PPs; light blue, other PPs; green, IEs and the LEE element), the second circle shows the positions of the nucleotide sequence (in Mbp), the third and fourth circles show clockwise and anticlockwise transcribed CDSs. The fifth circle shows the tRNA (red) genes, the sixth circle shows the rRNA (blue) operons, the seventh circle shows the G+C material, and the GC skew is seen in the eighth circle. B) Plasmids of EPEC strain E2348/69. The boxes represent CDSs transcribed clockwise and anticlockwise, respectively, in the outer and inner circles. Black boxes indicate pseudogenes, and the colours mentioned above for panel A are indicated by other CDSs. Image taken from Iguchi *et al*., (Iguchi *et al.*, 2009).

(laboratory strains) - with approximately 70% of genes (3141 of 4,488) having homologues in the strains (Iguchi *et al.*, 2009). Most EPEC specific genes (349/424) are located on horizontally acquired DNA - 319 on prophages (PPs) and/or integrative elements (IEs) - with the remainder (30 genes) on the plasmids.

There are 13 PPs (PP1 to PP13) and 8 IE's (LEE, IE1a, IE1b, IE2 to IE6) (Figure 8) of which three (PP1, IE1a and IE1b) are very small (<4kb) with the rest between 10-61kb in size (Iguchi *et al.*, 2009). The bioinformatics approach identified 14 putative effectors - most named Nle (non-LEE-encoded) with some retaining the Esp nomenclature – encoded, usually in clusters, on 3 prophages (PP) and 3 Insertion elements (IE) (Dean and Kenny, 2009; Iguchi *et al.*, 2009). Nle effector activity is mainly linked to interfering with signalling cascaded controlling the activity of host transcriptional factors modulating the expression of anti-microbial, cell death and/or inflammatory responses (see below; section 1.11.2) (Hemrajani *et al.*, 2010; Gao *et al.*, 2013; Cepeda-Molero *et al.*, 2020).

Intestinal epithelial cells (IECs) have specific receptors for cytokines and foreign antigens whose activation triggers signalling cascades through the IκB and mitogen-activated protein kinase (MAPK) pathways that control transcriptional factors (e.g., NF-κB, AP-1) directing the expression of proteins involved in anti-microbial, inflammatory and cell death pathways (Takeuchi and Akira, 2010) (Figure 9). Most relevant is the canonical pathway involving the p50/p65 dimer NF-κB isoform which remains in the cytoplasm until the IκB (inhibitory NF-κB) protein is phosphorylated by the IκB kinase complex (IKK) leading to IκB degradation which releases the p50/p65 complex to enter the nucleus to direct the transcription of NF-κB-dependent genes (Misra *et al.*, 2012). Receptor-triggered activation of IKK also leads to the activation of Map kinases (p38, JNK, Erk) which control the activity of other transcriptional factors (Figure 9).

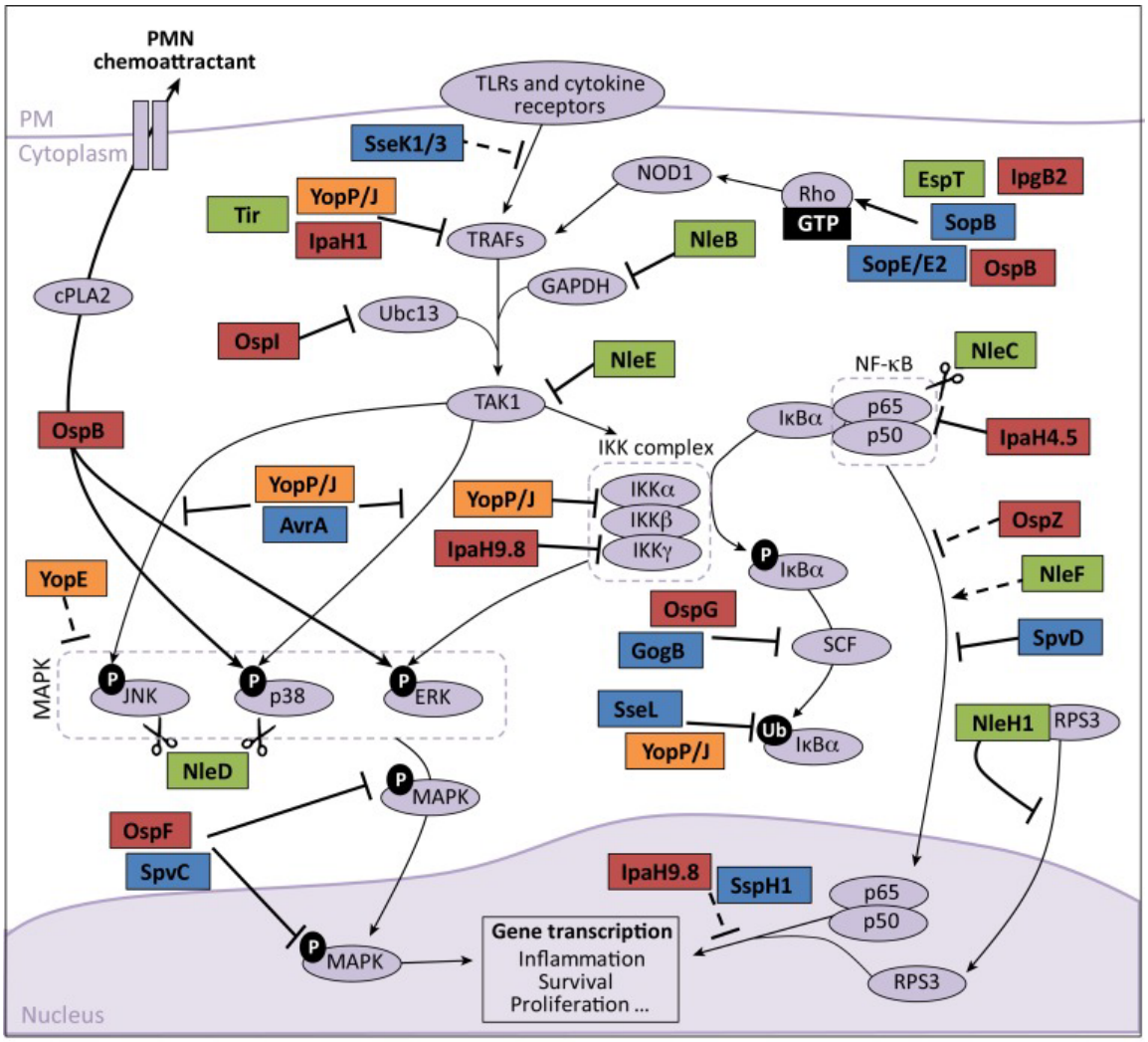


Figure 9 Enteropathogenic *Escherichia coli* (EPEC) targeting of NF-κB and MAPK Signaling Pathways.

EPEC strain activate proinflammatory signaling through sGTPase stimulation, Tir, NleB, and NleE interfere with TRAF signaling. The proteases NleD and NleC cleave activated MAPKs and the p65 subunit of NF-κB, respectively. Despite NleF promoting NF-κB signaling, NleH1 prevents nuclear translocation of RPS3, a component of NF-κB transcription complexes. Broken lines indicate unknown mechanisms. Abbreviations: PM, plasma membrane; TRAF, TNF receptor associated factor; TAK1, TGFβ-activated kinase 1; $cPLA_2$, cytoplasmic phospholipase 2; PMN, polymorphonuclear cell. Image taken from Pinaud *et al.,* (Pinaud *et al.*, 2018).

1.10 Discovery of EPEC T3SS effector proteins

1.10.1 LEE effector proteins

The first EPEC effector was discovered as a receptor for Intimin. Intimin, a 94kDa outer membrane protein identified in 1990 is crucial for intimate EPEC-host cell attachment and is encoded by the *eaeA* gene (Jerse *et al.*, 1990). It was reported that intimin interacts with the tyrosine phosphorylated form of a host receptor with a 90kDa apparent molecular mass, therefore named Hp90, triggering the formation of the pedestal-like structures observed in A/E lesions (Rosenshine *et al.*, 1992; Rosenshine *et al.*, 1996b). However, in 1997, Hp90 was shown not to be a host protein but a LEE-encoded effector that was name Tir for Translocated intimin receptor (Kenny *et al.*, 1997b). This discovery arose from studies examining optimal conditions for T3SS secretion of translocator proteins revealing a novel secreted protein with an apparent molecular mass of ~78kDa (Ep78). The 78kDa protein was isolated in sufficient quantities for N-terminal sequencing and to generate polyclonal antibodies. The former revealed N-terminal residues enabling the design of oligonucleotides to identify the encoding gene (Kenny *et al.*, 1997b). A well-developed strategy for determining effector delivery into host cells is to fractionate infected cells into Triton X100 soluble (contains host cytoplasm and membrane proteins plus T3SS effectors) and insoluble (contains host nuclear and cytoskeletal proteins plus proteins from adherent or remaining bacteria) components and using western blot methodologies to probe the Triton soluble fraction for the presence of the T3SS-dependent effector protein. Indeed, this approach confirmed the T3SS-dependent delivery of Ep78 and revealed that it undergoes phosphorylation within the host cells which increases its apparent molecular mass to 90kDa. Studies to confirm Hp90 is the phosphorylated form of Ep78 involving deleting the encoding gene, plasmid expressing Ep78 or Ep78-epitope tagged versions and illustrating Ep78-Intimin interaction (Kenny *et al.*, 1997b).

The second EPEC effector protein was discovered from studies examining the LEE region revealing a gene encoding a polyproline rich 206 amino acid protein predicted to be an effector (Elliott *et al.*, 1998). Antibodies were raised against the

protein, the encoding gene was deleted and cloned onto an expression vector enabling western blot studies illustrating the 206-residue protein to be T3SS-dependent secreted protein. However, the gene deleted strain behaved like EPEC in examined assays including Tir/Intimin interaction triggering signalling leading to actin nucleation. The authors named this putative effector EspF (Elliott *et al.*, 1998).

The third EPEC effector was identified due to its gene being located immediately upstream of *tir* and thus a potential effector-encoding gene (Kenny and Jepson, 2000). To investigate this possibility the gene (*orf19*) was deleted from EPEC and an epitope-tagged variant was generated to enable Western blot and epi-fluorescent microscopy studies. These approaches illustrated Orf19 to be delivered into host (HeLa) cells in a T3SS-dependent manner where it accumulated in punctate structures. The latter were shown to be mitochondria, leading to Orf19 being remained Map for Mitochondrial-associated protein, with Map-mitochondrial interaction linked to organelle dysfunction (Kenny and Jepson, 2000).

The fourth LEE effector was identified through comparing protein sequences of putative LEE genes to known effectors revealing the *rorf2* gene product had significant sequence homology (21% identity and 40% similarity) to a *Shigella flexneri*/enteroinvasive *E. coli* (EIEC) encoded T3SS effector, VirA (Uchiya *et al.*, 1995). Standard investigative approaches were undertaken (deleting the gene, plasmid re-introducing an epitope tagged variant) enabling Western blot analysis to show T3SS-dependent secretion and delivery into infected cells. Interestingly, rOrf2 - renamed EspG - functionally replaced VirA but the *espG*-deficient strain had no obvious defect in examined *in vitro* cellular models. It was suggested that the latter could be due to functional redundancy as the group had identified a second VirA/EspG homolog - named Orf3/EspG2 - on the pathogenicity island encoding the EspC autotransporter. However, an *espG* double mutant behaved like EPEC with EspG2 also functionally substituting for VirA.

The fifth LEE effector was identified by screening LEE genes for those encoding proteins that could be delivered in host cells in a T3SS-dependent manner (Braunstein *et al.*, 2003). This approach involved plasmid expressing the proteins as Cya (adenylate cyclase) fusion proteins since delivery into host cells would lead to T3SS-dependent increases in cAMP levels. CyaA is a calmodulin-dependent

enzyme and thus only active if transferred into host cytoplasm which contains calmodulin. The work revealed *orf18* to encode a T3SS-dependent effector -named EspH - supported by western blot analysis and epiflourescent microscopy detection of an epitope (Flag) tagged variant. Examining an *espH*-deficient strain for defects in known EPEC T3SS-dependent phenotypes revealed EspH promoted actin nucleation event triggered by Tir/Intimin interaction and inhibited those dependent on Map i.e., formation of actin rich filopodia. It is worth noting that the Cya fusion protein approach was used earlier to demonstrate T3SS-dependent delivery of EspB into host cells, consistent with its role as a membrane inserted translocator protein. However, western blot analyses revealed significant amounts of EspB in the host cytoplasmic fraction (Knutton *et al.*, 1998) suggestive of it being an artefact of the over-expression system or it being a fusion protein or that EspB may have effector functions. Indeed, the latter has been supported by subsequent studies revealing EspB interactions with α-catenin, α_1-antitrypsin and myosin linked to subversive events (Kodama *et al.*, 2002; Iizumi *et al.*, 2007; Hamaguchi *et al.*, 2008).

The sixth, and final, LEE effector was originally thought to regulate T3SS functionality leading to its original Sep designation (Hauser, 2009). However, studies involving the systematic inactivation of all *Citrobacter rodentium* LEE genes found no such defect for the *sepZ*-deficient mutant but revealed an important role for full virulence (Deng *et al.*, 2004). Approaches, which included the use of a CyaA fusion protein, were used to illustrate that EspZ is a T3SS-dependent substrate that is delivered into host cells. Bioinformatics interrogations revealed that, like other effectors, there was a high level of sequence difference between A/E pathogen strains. Moreover, EspZ was predicted to have two transmembrane domains suggesting that, like Tir, it inserts into the host membrane in a hairpin-like structure. Indeed, microscopy studies revealed some EspZ co-localisation with Tir (Kanack *et al.*, 2005).

1.10.2 Non-LEE-encoded effector proteins

Nearly all of the other T3SS effectors (see Figure 10-11) were, as previously mentioned, identified from bioinformatics interrogation of the EPEC E2348/69 genome sequence (Dean and Kenny, 2009; Iguchi *et al.*, 2009). This discovery promoted studies - using the standard approaches - to firstly confirm the gene encoded expressible T3SS-dependent effectors and then to understand effector functions. Notably, some studies used more fluorescent tags - such as β-lactamase (Bla) proteins and GFP – that are useful for screening programs to identify effectors and/or to monitor effector delivery (or location within host cells) in real time (Stebbins and Galan, 2001; Mills *et al.*, 2008; Van Engelenburg and Palmer, 2010).

However, the idea of additional cryptic effectors was illustrated by a proteomics approach revealing 3 novel T3SS substrates - the very large LifA (3223 residues) and LifA-like (2624 residues) proteins and, on a prophage (PP) not previously known to encode effectors, a T3SS translocated protein named NleJ (Deng *et al.*, 2012). The final effector, the EspC autotransporter - a member of the SPATE (serine protease auto-transporters of the Enterobacteriaceae family) and virulence associated factor - is also delivered into host cells in a T3SS-dependent manner (Elisa Drago-Serrano *et al.*, 2006; Vidal and Navarro-García, 2008; Salinger *et al.*, 2009; Serapio-Palacios and Navarro-Garcia, 2016). Subsequent investigations argue that the T3SS can deliver some effectors from the bacterial surface into the colonised host cells though it is not established whether this is through the internal conduit or the extracellular surface of the translocon (Tejeda-Dominguez *et al.*, 2017).

1.11 Effector functionality

Critical for understanding effector functionality is the identification of ‘phenotypes or cellular processes/events altered in a T3SS-dependent manner thereby allowing the contribution of specific effectors to be interrogated. Once an effector or effectors are linked to a T3SS-dependent subversive event then studies can explore the effector(s) contribution. For example, while EPEC is considered a non-

invasive pathogen it was found to induce its uptake into cultured cells enabling screening mutant banks - generated by transposon mutagenesis - for those with invasion defects leading to the discovery of the first virulence gene, *eaeA* (encodes Intimin) and the LEE region (Jerse *et al.*, 1990; McDaniel *et al.*, 1995). Another example relates to the discovery of actin-rich filopodia at the EPEC infection site on HeLa cells with studies revealing a T3SS-dependence requiring the Map effector independent of it targeting mitochondria (Jepson *et al.*, 2003). Filipodia formation in mammalian cells was previously linked to activation of a Cdc42::N-WASP::actin nucleation signalling pathway with cellular (including ecotopic expression of Map) and biochemical studies revealing Map to be a guanine-nucleotide exchange factor (GEF) that specifically activates Cdc42 (Huang *et al.*, 2009).

Plasmid expression of effector proteins - in EPEC or host cells (ectopically) - can lead to artefacts due to artificially high levels of the protein within the host cell so it is crucial to confirm the implicated functions of effectors under normal infection conditions. Moreover, while studies with non-polarised cultured cells have been extremely useful for identifying effectors, T3SS-dependent phenotypes, effector functions etc it is important to confirm *in vivo* relevance using, for example, cultured enterocyte models that morphologically and physiologically mimic EPEC's main target within the human GIT (Kenny and Dean, 2013).

1.11.1 Studies with non-polarised cells

As mentioned, studies on the identification and biology of effector proteins have come from studies using non-polarised cells, such as HeLa, despite them lacking key features of the target (enterocyte) cells. The main functions and/or features of EPEC effectors are outlined below.

1.11.1.1 Map

As mentioned, Map was named in relation to it accumulation within punctate structures in host cells, shown to be mitochondria, linked to organelle dysfunction

(Jepson *et al.*, 2003). Map was subsequently shown to target this host organelle via a classical N-terminal mitochondrial targeting sequence (MTS) enabling entry via the classical TOM/Hsp70 import system while organelle dysfunction was linked to features between residues 101-152 of this 203-residue protein (Kenny and Jepson, 2000; Papatheodorou *et al.*, 2006). However, most of Map's subversive activities have been found to occur outside the mitochondria linked to its role as a mimic of the host guanine nucleotide exchange factor (GEF) that activates the small RhoGTPase protein, Cdc42 (Huang *et al.*, 2009). Map carries a WxxxE motif needed to activate Cdc42 which recruits and activates N-WASP driving Arp2/3-mediated actin nucleation events producing filopodia at the infection site (Kenny, 2002; Huang *et al.*, 2009; Misra *et al.*, 2012). However, these filopodia are transient in nature due to Tir activity (in an Intimin-dependent manner) illustrating the concept of effector synergy/cooperativity and that Tir can have Intimin dependent functionality (Kenny, 2002).

1.11.1.2 EspF

Studies on the 206 residue EspF protein revealed that, like Map, it carries a classical N-terminal MTS with its import into mitochondria also linked to organelle dysfunction (Nagai *et al.*, 2005). Investigations on how EPEC inhibits its uptake by phagocytic cells revealed a central role for EspF which did not require it to target mitochondria (Quitard *et al.*, 2006). EspF is mostly composed of multiple poly-proline rich domains with each shown to possess motifs to binding N-WASP and sorting nexin 9 (SNX-9). EspF recruitment and activation of these host proteins leads to Arp2/3-dependent actin nucleation event linked to remodelling the host plasma membrane (Alto *et al.*, 2007). Most of EspF's known subversive activities, like Map's, relate to roles outside mitochondrial.

1.11.1.3 EspG

The first subversive activity linked to the 398 residue EspG protein was an ability to remodelling the actin cytoskeleton leading to microtubule instability (Elliott *et al.*, 2001; Matsuzawa *et al.*, 2004; Crepin *et al.*, 2005; Glotfelty and Hecht, 2012).

However, EspG has more recently been shown to act as a scaffolding protein and regulate GTPase signalling (Selyunin *et al.*, 2011) with inactivation of Arf GTPase disrupting endoplasmic-Golgi trafficking inhibiting protein secretion (Selyunin *et al.*, 2014).

1.11.1.4 EspH

The 176 residue EspH effector has been shown to be a RhoGEF inhibitor - through binding the DH-PH domain of RhoGEFs - resulting in their inactivation which has been linked to cell detachment and cytotoxicity (Dong *et al.*, 2010; Misra *et al.*, 2012). EspH activity has also been linked to modulating the levels of Map-induced filopodia and Tir/Intimin triggered pedestals (Braunstein *et al.*, 2003).

1.11.1.5 EspZ

The 98 amino acid EspZ effector, like Tir, integrates into the host plasma membrane in a hairpin loop topology with a short (10-amino-acid) extracellular loop flanked by two TMDs (Kanack *et al.*, 2005; Berger *et al.*, 2012). EspZ is required to prevent cytotoxic death of infected cells with multiple survival mechanisms proposed relating to it targeting mitochondria, controlling effector delivery levels or interacting with a type II transmembrane glycoprotein, CD98 (Shames *et al.*, 2010; Roxas *et al.*, 2012).

1.11.1.6 Tir

As mentioned, Tir (Translocated intimin receptor Tir) and Intimin are needed for intimate bacterial-host cell interactions and to induce pedestal formation (Kenny *et al.*, 1997b). Tir is a 550-residue protein that becomes inserted into the host cell membrane in a hairpin like conformation via two transmembrane domains (TMDs). The N-terminal (233 residues) and C-terminal (165 residue) domains are located within the host cytoplasm while the central (109 residue) extracellular domain acts as a receptor for Intimin binding (Abe *et al.*, 1999; Kenny, 1999). The membrane insertion process is linked to Tir being phosphorylated on serine residues by host kinases resulting in detectable shifts in apparent molecular mass from an

unmodified (T^0) to kinase-modified (T' and T'') forms (Kenny and Warawa, 2001). Tir is subsequently phosphorylated by host tyrosine kinases with modification of Tyr residue 474 generating the T''pY form (Kenny and Warawa, 2001). The latter phosphorylation event is linked to several host kinases - including Src, Abl, and Tec family members binding via their SH3 domain and a poly-proline rich region (PPR) (Phillips *et al.*, 2004; Bommarius *et al.*, 2007; Swimm and Kalman, 2008). Intimin binding to Tir leads to docking of the Nck adaptor protein, via its SH2 domain, to the phosphorylated Y474 residue (Campellone *et al.*, 2002) which recruit the neural Wiskott-Aldrich syndrome protein (N-WASP) and actin-related protein (ARP) 2/3 complex (Goosney *et al.*, 1999; Lai *et al.*, 2013) which polymerises actin forming pedestal-like structures characteristic of A/E lesions (Cepeda-Molero *et al.*, 2020) (Figure 10). Tir is likely to alter many host processes as it has been shown to interact with numerous host proteins (including cytoskeletal components, phosphatases, and ubiquitin ligases), some dependent on it being modified by host kinase or interacting with Intimin (Dean, 2011).

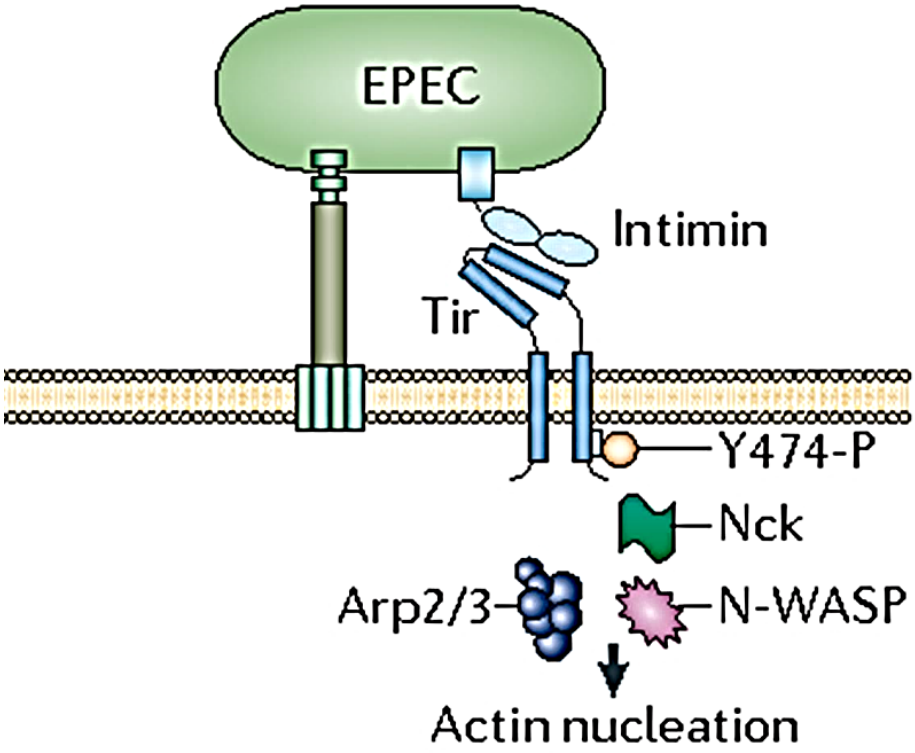


Figure 10 Schematic representation of actin nucleation induced by EPEC

EPEC Tir is translocated through T3SS in the host cell membrane and its extracellular portion binds the outer membrane protein intimin. Phosphorylation of EPEC Tir at Y474 recruits the host protein Nck which in turn recruits N-WASP. Image taken from Stevens *et al.* (Stevens *et al.*, 2006).

1.11.2 Nle effectors

As mentioned Nle effector activity is mainly linked to interfering with signalling cascades regulating transcriptional factors controlling the expression of anti-microbial, cell death and/or inflammatory responses (Figure 9)(McNamara *et al.*, 2001; Hemrajani *et al.*, 2010; Gao *et al.*, 2013; Cepeda-Molero *et al.*, 2020). Individual Nle effectors function by targeting proteins in one or more signalling pathways typically involving IκB and mitogen-activated protein kinase (MAPK) to prevent enterocytes either inducing an effective immune response and/or destroying an infected cell (Figure 9).

As previously mentioned the NF-κB (p50/p65) transcriptional factor remains in the cytoplasm until receptor signalling activates the IκB kinase complex (IKK) leading to IκB (inhibitory NF-κB) phosphorylation triggering its degradation which release the p50/p65 complex for nuclear entry to direct the transcription of NF-κB dependent genes (Misra *et al.*, 2012). IKK activity also activates Map kinases (p38, JNK, Erk) controlling the activity of other transcriptional factors (Figure 11). The first Nle effectors reported to inhibit innate signalling were NleC and NleD by being metalloproteases: NleC cleave p65, p50 and IκB with NleD cleaving p38 and JNK (Figure 11) (Baruch *et al.*, 2011; Creuzburg *et al.*, 2017).

In contrast, another Nle effector, named EspL, a cysteine protease that cleaves proteins such as RIP kinase 1 with a RHIM domain, interferes with immune signalling (Pearson *et al.*, 2017)(Figure 11). Other Nle effectors including NleE and NleB, function by inhibiting the activities of their target proteins (Figure 11)(Nadler *et al.*, 2010; Newton *et al.*, 2010; Gao *et al.*, 2013). NleH1 and NleH2 are serine/threonine kinase mimics, with auto-phosphorylation activity, that can alter the activity of a NF-κB co-factor, RPS3, thereby impacting gene transcription (Gao *et al.*, 2009; Biemans-Oldehinkel *et al.*, 2011; Pham *et al.*, 2012) (Figure 11). Indeed, the Tir effector can also interfere with immune signalling, by Intimin-dependent and -independent mechanisms (Ruchaud-Sparagano *et al.*, 2011; Yan *et al.*, 2013; Gao *et al.*, 2017).

Collective Nle effector activity, effectively, suppresses signalling pathways controlling the ability of the infected enterocyte to induce anti-infective, anti-inflammatory and cell death responses.

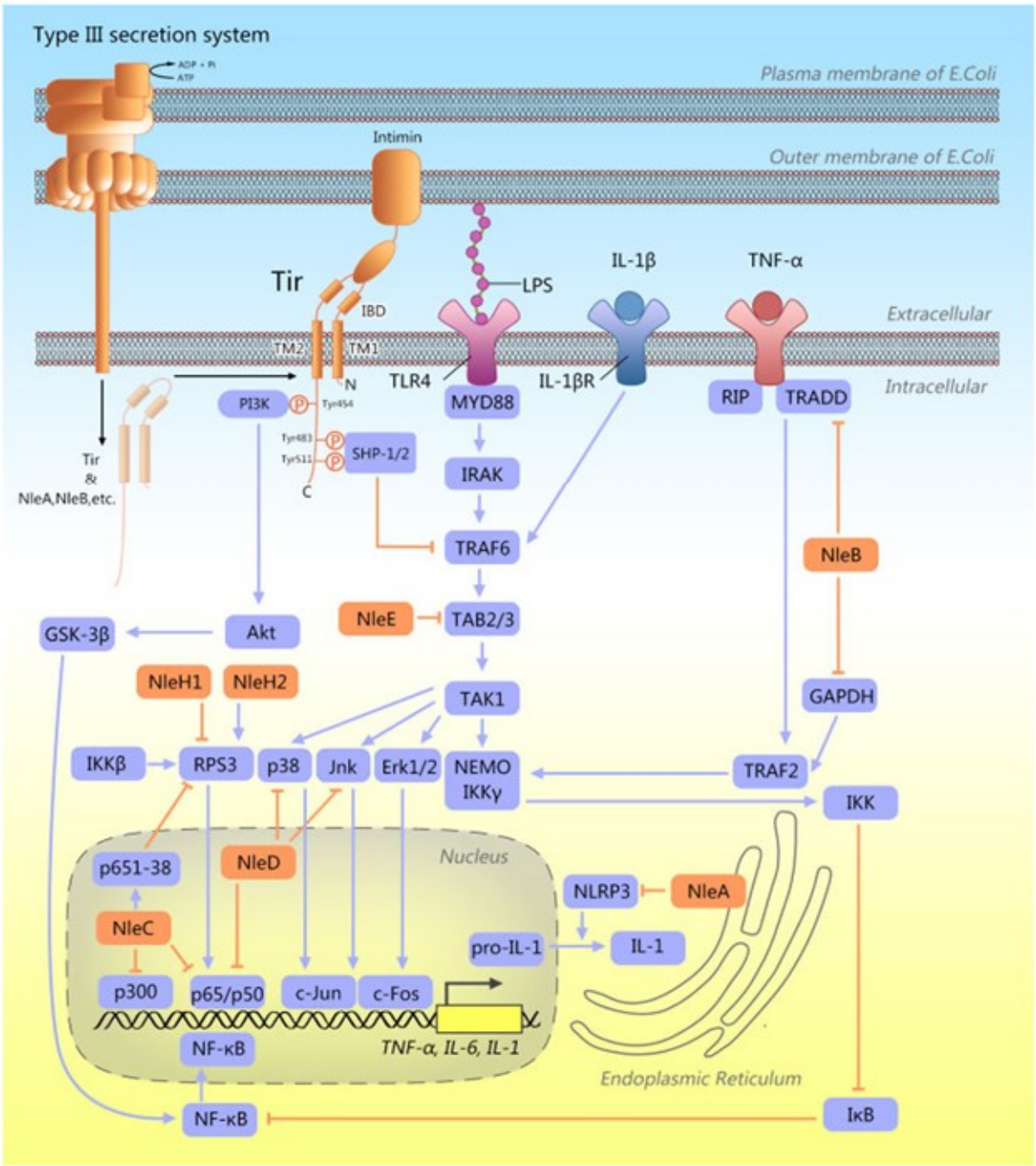


Figure 11 Overview on EPEC E2348/69 effector interference of immune signalling by infected enterocytes

Taken from Zhuang et al., (Zhuang *et al.*, 2017). See Figure 9, text, and review (Zhuang *et al.*, 2017) for explanations and/or further detail.

1.11.3 Studies with polarised cells

To understand the *in vivo* relevance of effector functionality to the disease process many groups use cultured enterocytes since they, as previously mentioned, morphologically and physiologically mimic EPEC's *in vivo* target (Dean *et al.*, 2013; Kenny and Dean, 2013). Such studies often examine aspects such as i) bacterial colonisation, ii) changes of the brush border (often by scanning and/or transmission electron microscopy), iii) alterations in transporter activity (transporter functional assay and/or epifluorescent/confocal microscopy), iv) disruption of cell-cell interaction (monitoring changes in transepithelial electrical resistance; TER), v) changes to host organelles or vi) specific subversive events such as Tir-Intimin triggered actin nucleation that produce pedestal like structures under the bacteria (epifluorescent/confocal microscopy) and vii) changes in the host cellular signalling (such as IKK and Map Kinase) pathways by western blot analyses (Dean *et al.*, 2013; Kenny and Dean, 2013). Key effector changes in epithelia of the human GIT are outlined below and summarised in Figure 12 & Figure 13.

1.11.3.1 EPEC induced Diarrhoea

1.11.3.1.1 Rapid onset of watery diarrhoea

It was proposed that EPEC's T3SS-dependent capacity to compromise sodium glucose co-transporter (SGLT-1) activity prior to the loss/effacement of microvilli may explain the rapid onset of watery diarrhoeal disease response as SGLT-1 is responsible for the daily uptake ~6 litres of fluid from the small intestine (Dean and Kenny, 2009) (Figure 12). This inhibitory activity was linked to non-essential roles for at least three LEE effectors (EspF, Map, Tir) and intimin proteins through a cooperative mechanism (Dean and Kenny, 2009).

1.11.3.1.2 Alteration of other transporter activity

The movement of water in and out of epithelial cells in mostly driven by ion gradients with EPEC found to reduce, in an EspF dependent manner, the

expression of NHE3 which is crucial for sodium ions (Na^+) absorption (Gawenis *et al.*, 2002; Hecht *et al.*, 2004; Hodges *et al.*, 2008). Decreased uptake of Na^+ activity is aggravated via reduced expression of sodium glucose co-transporter (SGLT-1) at the apical membrane activated through the collective action of Tir, Map and EspF (Dean *et al.*, 2006) Moreover, EspG and EspG2 dependent disruption of the micro-tubular network has been linked to reduced levels of the Cl/HCO3 exchanger *in vitro* and *in vivo* (Gill *et al.*, 2007) (Figure 12). Microtubule dysfunction results in the reduced uptake of chloride (Cl^-) ions and their accumulation in the lumen by changing the membrane targeting of the Cl/HCO3 exchanger DRA (downregulated in adenoma) (Gill *et al.*, 2007; Gujral *et al.*, 2015). The EPEC EspF and EspG effectors were reported to modulate epithelial aquaporin (AQP) expression altering water transport independent of ion distribution but relevance is questioned by AQPs being expressed in colonic epithelial cells (Agre *et al.*, 2002). EspG and EspF also reported in the redistribution of water channels (aquaporins) to intracellular compartments; distant from the apical and lateral membrane (Guttman *et al.*, 2007). EPEC infection has also been linked to reduced function and expression of the sodium dependent bile acid transported (ABST) which is crucial for the absorption of bile juice in the gut (Annaba *et al.*, 2012).

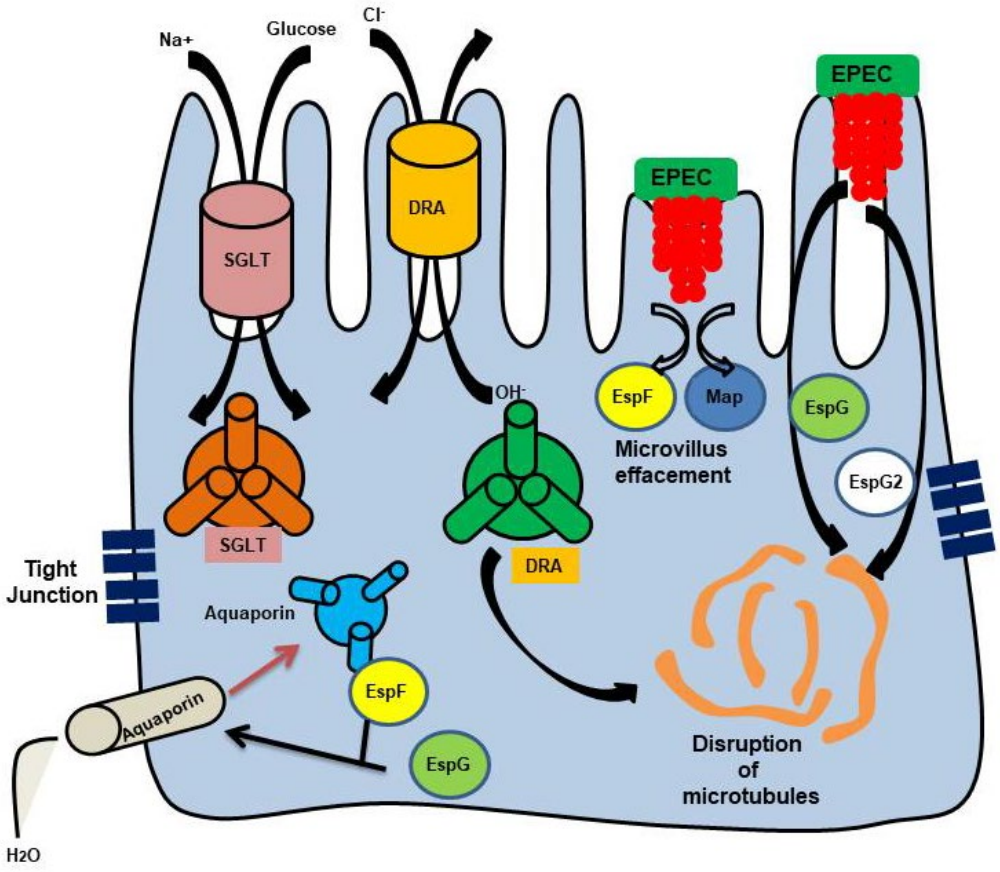


Figure 12 EPEC effector proteins altering epithelial cell function and inducing water loss and diarrhoea

In a multifactorial process, EPEC infection disrupts the absorption of ions, water and nutrients, depending on the collective behaviour of mainly the translocated LEE-encoded effector proteins. EPEC effectors interfere with absorption by loss of absorbent microvilli (effacement) caused by the cooperative activities of Map, EspF, Tir and Intimin outer membrane protein effectors. The decreased surface expression of sodium glucose also leads to these effectors. By re-distribution to intracellular vesicles, the transporter (SGLT1). Decreased transportation of water is mediated through the redistribution of apical water channels (Aquaporins) in the apical and basolateral membrane is depending on EspF and EspG effectors. The sodium hydrogen exchanger (NHE), which is interrupted by the effector EspF, is the main route of sodium uptake (Na+). The effectors EspG and its Nle homolog EspG2 mediate the disruption of chloride (Cl-) absorption through the chloride bicarbonate (HCO3-) exchanger, known as downregulated in adenoma (DRA). EspG and EspG2 block the microtubules of the host cell, preventing protein trafficking, thus reducing the expression of DRA at the host cell. Adapted from Viswanathan *et al.*, (Viswanathan *et al.*, 2009).

1.11.1.3 Microvilli Effacement

The effacement of microvilli (Figure 13) has been described as a two-step event with bacteria sinking into the microvilli requiring Tir and Intimin activities with EspF and Map activities driving the elongation-associated loss (effacement) of microvilli next to the colonising bacteria (Dean *et al.*, 2006). Studies with human intestinal tissue revealed a key role for EspF but not Map (Dean *et al.*, 2006) with subsequent *in vitro* studies showing Map's activity is silenced by Tir and Intimin co-operative activity (Dean *et al.*, 2013). Both Map and EspF's effacing activities depend on features linked to them triggering Arp2/3-dependent actin nucleation (Dean *et al.*, 2013). Moreover, the EspB translocator/effector proteins is also reported to contribute to microvilli loss through binding myosin destabilization actin bundles in the microvilli structure (Iizumi *et al.*, 2007)(Figure 13).

1.11.1.4 Disruption of epithelial tight junctions (TJs)

As mentioned, the enterocyte monolayer plays a role 'barrier' function in preventing gut lumen antigen from accessing host-privileged areas and thus preventing constant immune responses (Snoeck *et al.*, 2005). Crucial to this barrier function - and for preserving cell-cell adhesion and preventing paracellular diffusion (the passage of molecules between cells) - are tight junctions (TJs) (see Figure 1)(Alberts *et al.*, 2002). TJs composed of several transmembrane proteins i.e. claudins, occuldin, zonula occluden (ZO) and the junction adhesion molecules (JAM)(Heinemann and Schuetz, 2019). It is reported that EPEC alters both the structure and function of the intestinal epithelial barrier *in vitro* and *in vivo*. Permeability of apical junctional complexes is regulated by phosphorylation of myosin light chain (MLC) by MLC kinase (Manjarrez-Hernandez *et al.*, 1996). EPEC can activate MLC kinase, which induces the distention of the transmembrane tight junction proteins, which in turns increase paracellular permeability (Turner et al., 1997). EPEC also disrupts adherin junctions, through the EspB-dependent phosphorylation and activation of PKCα (Malladi *et al.*, 2004), resulting in the cytoplasmic redistribution of β-catenin and increase in paracellular

permeability (Malladi *et al.*, 2004). EPEC can, in an EspF dependent manner, directly alter tight junctional proteins leading to the redistribution a key component (occludin) which decreases, *in vitro* and *in vivo*, barrier function (McNamara *et al.*, 2001) (Simonovic *et al.*, 2000; Muza-Moons *et al.*, 2004; Shifflett *et al.*, 2005). Studies in our laboratory suggest disruption of barrier function is a complex process with roles for many effectors including Map, EspF, EspG and EspG2 whose activities are controlled by Tir and Intimin (Dean *et al.*, 2010). The research suggests that Tir silences the barrier disrupting activities of the EspG homologues with Intimin binding Tir leading to EspG effector activity resulting in the calpain-dependent loss of cell-substratum interactions. The Tir/Intimin interaction also enables Map and EspF effector activities to disrupt cell-cell interactions in a way that is influenced by the motifs required to activate the N-WASP-Arp2/3 actin nucleation pathway (Peralta-Ramírez *et al.*, 2008). Collectively, these effectors alter actin assembly and disrupt the microtubule networks, which leads to dysfunction of protein trafficking required to maintain TJ structure (Figure 13) (Peralta-Ramírez *et al.*, 2008; Guttman and Finlay, 2009; Thanabalasuriar *et al.*, 2010; Glotfelty and Hecht, 2012).

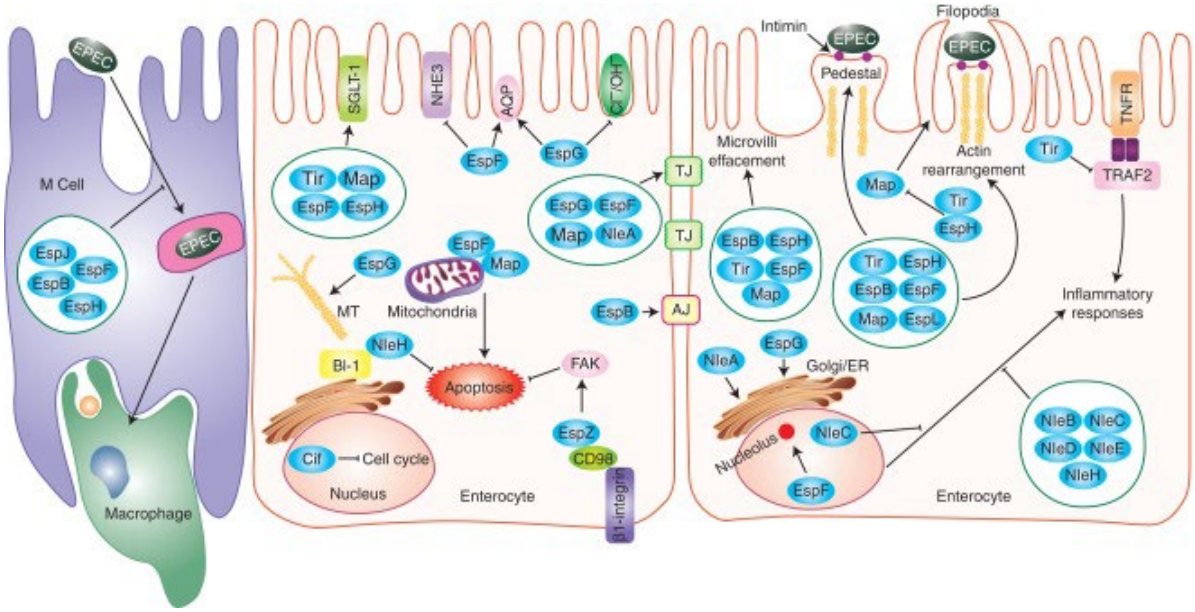


Figure 13 EPEC's T3SS effectors and their targets inside the host cells

Multifuntional and redundant role for effectors in EPEC alteration of enterocyte biology. Abbreviations**:** ER (endoplasmic reticulum); MT (microtubules); SGLT-1 (sodium D-glucose cotransporter); NHE3 (sodium hydrogen exchanger); AQP (aquaporin); Cl-/OH- (Cl-/OH-transporter); FAK (focal adhesion kinase); BI-1 (BAX-1 inhibitor); TNFR (tumour necrosis factor receptor); TRAF2 (TNF-associated factor 2). TJ (tight junction); AJ (adherent junction). Taken from Law *et al*., (Law *et al.*, 2013). See text, Figure 9 and/or review for further information.

1.12 EPEC interaction with M-cells

EPEC strains have also being reported to interact with M-cells within the GIT (Inman and Cantey, 1983; Siebers and Finlay, 1996). M-cells are phagocytic cells that internalise antigens (including bacteria) from the gut for presentation to underlying macrophages with a key role in controlling immune responses. Interestingly, EPEC inhibited its uptake by M cells *in vivo* (Inman and Cantey, 1983; Siebers and Finlay, 1996) suggesting that the pathogen may delay it recognition and thus emerging immune responses. *In vitro* studies with mouse-derived macrophages revealed EPEC has a T3SS dependent ability to inhibit its uptake via phosphoinositide 3 (PI-3 kinase) dependent pathways (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006). While some EPEC (~40%) were internalised by PI3 kinase-independent pathways (Goosney *et al.*, 1999; Quitard *et al.*, 2006) uptake in M-cells is almost exclusively PI-3 kinase dependent (Martinez-Argudo *et al.*, 2007). Several LEE effectors have been linked to inhibiting EPEC phagocytic events (Figure 13) (Law *et al.*, 2013). PI3 kinase activity is commonly examined by monitored the phosphorylation of a downstream effector, Akt (pAkt)(Celli *et al.*, 2001), However, the discovery that the EspF effector blocks PI3 kinase dependent uptake without preventing loss of the pAkt signalling (Quitard *et al.*, 2006) implied that other effectors can inhibit the activity of Akt kinase that regulate important host cellular processes.

1.13 The PI3K/Akt/mTOR signalling pathway.

1.13.1 PI3-Kinase and phosphoinositides

Ligand bindings to receptors can activate of PI3 kinase family members which phosphorylate the 3’ hydroxyl group of the inositol ring in phosphatidylinositol (PtdIns), PtdIns-4-phosphate (PtdIns4P) or PtdIns-4,5- bisphosphate (PtdIns (4,5)P2) (Vanhaesebroeck *et al.*, 2010) These inositides direct membrane recruitment of effector proteins that have lipid-binding domains such as the pleckstrin homology (PH), phox homology (PX) or FYVE (Vanhaesebroeck *et al.*, 2010). The typical structure of phosphatidylinositol is composed of a diacylglycerol

backbone linked to an inositol ring via a phosphate group (Vanhaesebroeck *et al.*, 2010) with phosphorylation at the free-OH groups located at 3,4,5 positions (Vanhaesebroeck *et al.*, 2010) (Figure 14).

There are three classes of PI3K isoforms (Class I, II and III) based of structural features and substrate specificity but only class I induce Akt activity (Vanhaesebroeck *et al.*, 2010). Class I PI3Ks function as a heterodimer consisting of a catalytic (p110) and a regulatory subunit to produce the secondary messenger, phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3). Based on their different regulatory subunit, this class is further subdivided into class IA (p110α, p110β, and p110δ) and class IB (p110γ). The catalytic subunit is composed of an N-terminal adaptor binding (ABD), Ras binding (RBD), C2, helical, and C-terminal kinase domains (Vanhaesebroeck *et al.*, 2010). Class IA and IB kinases function downstream of the receptor tyrosine kinase (RTKs) and G-protein coupled receptors (GPCRs) respectively (Shaulov *et al.*, 2017).

1.13.2 Protein kinase B (Akt)

The human genome encodes ~500 putative protein kinases, which correspond to 2% of all eukaryotic genes (Kostich *et al.*, 2002). The enormous number of targets regulated by protein kinases opposes this relatively small percentage of kinases (Enjalbert and Le Pechon-Vallee, 2003). It is estimated that nearly 30% of all cellular proteins may be modified by protein kinase activity (Manning *et al.*, 2002). Protein kinases form one of the largest gene families in eukaryotes and are crucial regulators of cellular function through their ability to phosphorylate Serine (Ser), Threonine (Thr) and/or Tyrosine (Tyr) residues to regulate signal transduction pathways (Manning *et al.*, 2002). Protein kinases have a major role in eukaryotic biology by controlling processes including cell metabolism, cell cycle progression, transcription and cytoskeletal rearrangement events (Manning *et al.*, 2002). Dysfunction of these kinases may lead to human disease including cancer, diabetes, neurodegenerative disorders, and bacterial infections (Testa and Bellacosa, 2001; Zdychova and Komers, 2005; Endicott *et al.*, 2012).

1.13.3 Akt Structure and Function

Akt was isolated from an AKR mouse T-Cell lymphoma and is the human homologue of the viral oncogene V-Akt encoded by the retrovirus Akt8 (Liao and Hung, 2010). As mentioned, Akt regulates numerous host cell processes including cell proliferation, transcription, translation, apoptosis and cellular metabolism (Pearce *et al.*, 2010; Bononi *et al.*, 2011). While first identified in relation to activation of insulin signalling in 1995, Akt function is best understood as a downstream effector of PI3K activity (Bononi *et al.*, 2011).

Akt shares homology to protein kinase A (PKA) and protein kinase B (PKB) and has three isoforms (Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ) which are closely related and highly conserved in mammals (Tobe *et al.*, 1999). Each Akt protein contains an N-terminal pleckstrin homology (PH), a central kinase and carboxyl-terminal regulatory domain (Liao and Hung, 2010). Although, the isoforms share some functions, studies have noted some have specific roles i.e. Akt1 in cell survival and proliferation; Akt2 in glucose homeostasis; and Akt 3 in brain development (Cho *et al.*, 2001; Tschopp *et al.*, 2005).

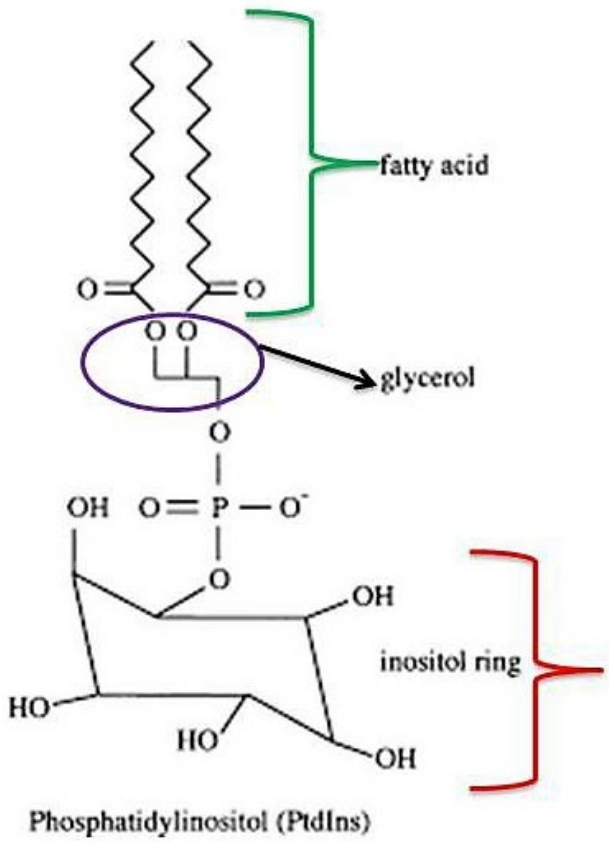


Figure 14 Schematic of phosphatidylinositol (PtdIns).

Phosphatidylinositol consists of two fatty acid chains attached to glycerol backbone at carbon positions 1 and 2. Inositol ring is attached to the backbone at carbon position 3 via a phosphate group. The free hydroxyl group (OH) on the inositol ring may be phosphorylated, on positions 3', 4'and 5'. Adapted from Vanhaesebroeck and Alessi *et al.* (Vanhaesebroeck *et al.*, 2010).

1.13.4 PI3K-dependent Akt phosphorylation

Akt is a serine/threonine kinase that plays a critical function in many mammalian processes including cell survival, cell proliferation, regulation of metabolism, motility, transcription, and cell-cycle progression (Fayard *et al.*, 2005). Dysfunction of this kinase is linked to various diseases including cancer, diabetes, neurodegenerative disorders and bacterial infection (Testa and Bellacosa, 2001; Zdychova and Komers, 2005).

As mentioned, activation of receptor tyrosine kinases (RTKs) or G proteins coupled receptors recruits and activates PI3K which converts PtdIns-[4,5]-bisphosphate (PI[4,5]P2) to PtdIns-[3,4,5]-trisphosphate (PI[3,4,5]P3), providing a second messenger that membrane recruits Akt by its N-terminal pleckstrin homology (PH) domain (Salinger *et al.*, 2009; Liao and Hung, 2010; Vanhaesebroeck *et al.*, 2012; Gao *et al.*, 2014)(Figure 15). Membrane localised Akt is then activated by phosphorylation on two residues, Thr308 by the phosphoinositide-dependent kinase 1 (PDK1) and Ser473 by the mammalian target of rapamycin complex 2 (mTORC2) (Diehl and Schaal, 2013; Gao *et al.*, 2014) (Figure 15). Activated Akt can then phosphorylate substrates in various cellular compartments to control processes including cell survival, cell proliferation and protein synthesis (Manning and Cantley, 2007).

1.13.5 Akt and protein phosphatases

Akt signalling is typically short-lived either through the de-phosphorylation of PIP3 by lipid phosphatases or pAkt by protein phosphatases. The lipid phosphatases responsible include the membrane localised phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and the SH2 domain containing inositol 5-phosphatase (SHIP) proteins that dephosphorylates PI[3,4,5]P3 to PI[4,5]P2, thereby preventing further Akt recruitment (Carver *et al.*, 2000; Hers *et al.*, 2011). The protein phosphatases include phosphatase 2A (PP2A) and PH domain leucine rich repeat phosphatase (PHLPP); these remove the phosphate group from the

Thr308 and Ser473 residues respectively (Bayascas and Alessi, 2005; Liao and Hung, 2010).

1.13.6 Dysregulation of PI3K/Akt signalling.

Dysregulation of Akt activity is linked to various human diseases such as cancer, diabetes, schizophrenia, depression, bipolar disorder and cardiovascular disease (Hers *et al.*, 2011). Dysregulation can also affect Akt-mediated inhibition of the pro-apoptotic protein Bad and Foxo which promotes neuronal cell survival (Datta *et al.*, 2000). Akt is a potential target for some human cancers as increased expression of the most common isoform, Akt2, is linked to 50% and 10% of colorectal and pancreatic cancers respectively (Hers *et al.*, 2011; Abdallah *et al.*, 2015). A major reason for dysregulated signalling is mutations that alter the expression of components regulating Akt activity (Hers *et al.*, 2011), including overexpression of upstream receptor signalling, mutation of PI3K regulators subunit and PTEN inactivation (Chalhoub and Baker, 2009). Akt dysregulation is also linked to neurodevelopmental, neurodegenerative, and neurocognitive disorders that include Alzheimer's disease (Griffin *et al.*, 2005; Lipton and Sahin, 2014). Thus, the key role of Akt dysfunction in many human diseases identifies it as a drug target for therapeutic treatment, which could be assisted by understanding how pathogens, like EPEC, inhibit Akt activity.

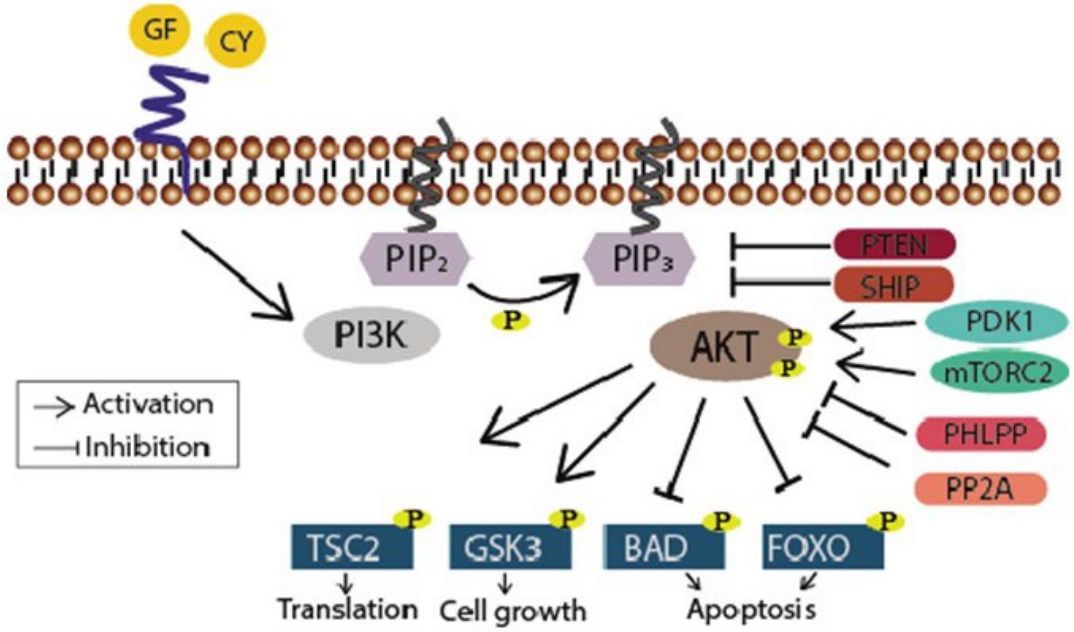


Figure 15 Schematic of PI3K/Akt pathway

Stimulation of receptors by, for example, growth factors (GF) or cytokines (CY), can activate PI3K (Phosphoinositide-3-kinase) resulting in phosphorylation of PtdIns-[4, 5]-bisphosphate (PI [4, 5] P2) (PIP2) to phosphatidylinositol-3, 4, 5-trisphosphates PIP3 to AKT recruitment, via pleckstrin homology (PH) domain binding PIP3. After binding of its pleckstrin homology (PH) domain to PIP_3, Membrane located Akt is then activated by phosphorylation on Thr308 (by phosphoinositide-dependent kinase1; PDK1) and Ser473 (by mammalian target of rapamycin complex 2; mTORC2). Activated AKT can then phosphorylate substrates in various cellular compartments to regulate processes including cell growth, protein translation, and apoptosis. Signalling is terminated either through de-phosphorylation of PIP3 by the lipid phosphatases (including PTEN or SHIP) and protein phosphatases (including PHLPP; PH domain leucine rich repeat phosphatase and PP2A; protein phosphatases 2A (PP2A) removing phosphates from the Ser473 and Thr308 residues. Image taken from Diehl *et al*., (Diehl and Schaal, 2013).

1.14 Prior studies on EPEC interference of PI3K/Akt signalling

Studies by a previous PhD student, Oliver Amin, (Amin, 2017), revealed that plasmid introducing LEE in non-pathogenic *E. coli* provided the strain an ability to inhibit its phagocytosis by cultured macrophages - a process dependent on LEE effectors (Quitard *et al.*, 2006). However, other data indicated that introducing LEE did not allow the strain to interfere with Akt signalling thereby implying that the responsible effectors were encoded outside LEE. Interestingly, inhibition of Akt signalling depended on EPEC expressing the LEE multi-substrate chaperone, CesT, suggesting that the responsible Nle effector(s) needs to interact with CesT for T3SS dependent delivery.

Moreover, screening available mutants ruled out key roles, individual or cooperative, for most known non-LEE-encoded T3SS substrates. However, the work did not address contributions for 4 T3SS substrates, EspC, NleJ, LifA, LifA-like protein. Studies on the inhibitory process suggested that infection-induced phosphorylation of Akt is reversed by EPEC T3SS-delivered effector(s) through events either inhibiting the activity of the kinases that phosphorylate Akt (on Ser 473 and Thr 308) or by removing the added phosphate groups through providing a Ser/Thr phosphatase or by activating host phosphatase(s) that regulate Akt signalling.

1.15 Aims and objectives

The first aim of this project was to identify the EPEC effector or effectors responsible for inhibiting Akt signalling and to confirm a key role for the CesT chaperone. To achieve these objectives this work focussed initially on the individual or cooperative roles of 4 unexamined T3SS substrates (EspC, NleJ, LifA, LifA-like). A second aim was to provide insight into the mechanism by which EPEC inhibits Akt signalling with the objective of providing support for a Ser/Thr phosphatase and determining whether subsequent dephosphorylation is indirect i.e., *via* a host phosphatase(s) that regulates Akt activity.

Chapter 2. Materials & Methods

2.1 Cell Culture

2.1.1 Mammalian cell culture

All experiments were carried out with immortalised mouse macrophage-like cells, J774.A1 (ATCC_TIB-67), grown in Dulbecco's minimal Eagle's medium (DMEM; Sigma Cat #D5796) with 10% Foetal calf serum (FCS; Sigma Cat #7524). Mammalian cell stocks (~10^6 cells) were stored in liquid nitrogen in a 10%DMSO 90% DMEM/FCS solution and, following thawing, were aseptically harvested within a Class 2 laminar flow hoods (BioMat2). Cells were routinely maintained in 75cm^2 culture flasks (SLS Ltd Cat #430641U) in a 5% CO_2 atmosphere at 37^0C (LEEC Research CO2 Incubator). When cells were 80-90% confluent they were harvested using a cell scraper (Sarstedt; Cat #83.183) with cell number counting using a haemocytometer (Hawksley) following the manufacturer's instructions. Cells were routinely seeded, at two days prior to infection, into 6, 12 or 24 well plates (Corning; #3506, #3512, #411080) to obtain 80-90% confluency on the day of experiment. Cells were passaged (1:6 dilution) a maximum of 30 times with media replaced every 2-3 days.

2.1.2 Bacterial strains

Strains used in this study are listed in Table 1-3. Strain stocks were maintained at -80^{o}C in Luria-Bertani (LB) broth containing 10% glycerol. When needed, a stock sample was streaked out onto LB agar plates (37^{o}C stock overnight) containing appropriate antibiotic. Antibiotics were used at a final concentration of 12, 50, 100, 25 and 25ug/ml for tetracycline (Tet), nalidixic acid (Nal), carbenicillin (Cab), chloramphenicol (Cmp) and kanamycin (Km) respectively. A single colony was used to inoculate LB broth, containing selective antibiotics when required, and grown overnight 37^0C without shanking (for host cell infection) or with shaking (250rpm) for molecular biology-related studies.

2.1.3 Measurement of Bacterial Optical Density

One millilitre of bacterial culture was placed into a plastic cuvette (Greiner Bio-one; Cat #613101) and the absorbance was measured at 600 nm (OD_{600}) using UV 1101 Biotech Photometer. High density cultures were diluted to make sure OD was less than <1 before measuring relative to the appropriate 'blank' solution (uninfected media) set to zero. 1 OD_{600} was taken to equal 10^9 bacteria.

2.2 Molecular Biology

2.2.1 Plasmids

Plasmids used and/or generated in this study are listed in Table 4.

2.2.2 Plasmid Purification

Bacteria grown overnight, with shaking, in LB containing appropriate selective antibiotic(s) were used for extraction of carried plasmids using Plasmid Mini Extraction Kit (New England Bio-labs; Cat # T1010L) following the manufacturer's instructions.

2.2.3 Polymerase Chain reaction (PCR)

PCR reactions were performed using isolated plasmid or total bacterial DNA (released by resuspending bacteria from a colony in 60µl dH_2O and incubating 5 min at 100^oC). PCR conditions are given in Table 3 and Table 4 and routinely contained, for a total 10µl volume, 1µl template DNA, 1x Taq buffer (New England Biolabs Cat # B90145), dNTPs (final conc. 1.25 µM from 200µM stock; New England Biolabs Cat #NO4475), Taq DNA Polymerase (0.625 units, England Biolabs Cat #M0493S) and appropriate primer set (final conc. 0.2µM from 10µM stock; Sigma). Primers used in the studies are listed in Table 7. Thermocycle reactions were carried out using a Techne TC-3000 or LABnet international thermocycler following manufacturer's instructions.

Chapter 2 Materials & Methods

The strains used in this study are listed in Tables 1,2 and 3 respectively. Table 1 shows all the strains acquired from Prof. Kenny lab. Each table mentioned the antibiotic selection and the gene description in Table 2 and 3.

Table 1 Strains derived from Kenny Laboratory stocks

Bacterial Strain	Description	Antibiotic	Source
DH10B (*E. coli K12*)	Laboratory *E. coli* K12 strain.	NA	Thermo Fisher Scientific
SY327 λpir	Laboratory strain containing λpir gene allows replication of R6K ori-based suicide plasmids (most suitable for transformation)	NA	(Donnenberg and Kaper, 1991)
SM10λpir	Laboratory strain containing λpir gene allows replication of R6K ori-based suicide plasmids (most suitable for conjugation)	Km^R	Thermo Fisher Scientific
EPEC E2348/69 (O127:H6)	Wild Type Strain	Nal^R	(Levine *et al.*, 1985) Brendan Kenny lab
cfm-14	T3SS mutant Transposon-disrupted *escN* gene	Km^R	(Donnenberg and Kaper, 1991)
Δ*cesT*	EPEC E2348/69 lacking LEE encoded chaperone, CesT	Km^R	S. Quitard, Unpublished
EPEC B171-8	Another Prototypic EPEC strain	None	(Puente *et al.*, 1996)
EHEC	Enterohemorrhagic *E. coli* O157:H7 EDL993 strain (lacks shiga-toxin genes)	Nal^R	(Dziva *et al.*, 2004)
EHEC Δ*escN*	As EHEC but lacks functional T3SS	Nal/Km^R	(Marchès *et al.*, 2008)
Citrobacter rodentium	Mouse-specific A/E pathogen	Nal^R	(Schauer and Falkow,1993)
Citrobacter rodentium* Δ*espA	*Citrobacter rodentium* lacking functional T3SS (Transposon-disrupted *espA* gene)	Nal/Km^R	(Mundy *et al.*, 2003)
RDEC-1	Rabbit-specific A/E pathogen	Nal^R	(Cantey and Blake, 1977)
RDEC-1Δ*espA*	RDEC-1 lacking functional T3SS (Transposon-disrupted *espA* gene)	Km^R	(Cantey and Blake, 1977)
Δ*core::Km*	EPEC E2348/69 LEE region encoding 3 effectors (EspH, Map, Tir) chaperones (CesT, CesF) and Intimin (*eae* gene) replaced by kanamycin gene	Nal^R/Km^R	(Dean *et al.*, 2010)
TOEA7	EPEC E2348/69 lacking all known non-LEE encoded effectors except EspC, NleJ, LifA and LifA-like protein	None	(Yen *et al.*, 2010)
TOEA7*Δcore*	Lacking genes *espH*, *cesF*, *map*, *tir*, *cesT* & *eae* [Intimin] from a TOEA7 strain.	Tet^R	(Amin, 2017)
ΔPP2	EPEC E2348/69 lacking entire PP2 region	Nal^R	S. Quitard, Unpublished
ΔPP4	EPEC E2348/69 lacking entire PP4 region	Nal^R	S. Quitard, Unpublished
ΔIE5	EPEC E2348/69 lacking entire IE5 region	Nal^R	S. Quitard, Unpublished
ΔPP2/IE5	EPEC E2348/69 lacking indicated regions	Nal^R	S. Quitard, Unpublished
ΔPP4/IE5	EPEC E2348/69 lacking indicated regions	Nal^R	S. Quitard, Unpublished
ΔIE5/PP2/PP4	EPEC E2348/69 lacking indicated regions	Nal^R	S. Quitard, Unpublished
Δ*LifA:Km*	EPEC E2348/69 with *LifA* gene (encodes Lymphostatin) disrupted by Km gene	Km^R	This study
ΔlifA-like/lifA:km	EPEC strain missing *LifA-like* and *LifA* genes	Sp^R /Km^R	This study
***Δ*IE2a**	EPEC E2348/69 lacking entire IE2 PAI	Nal^R/km^R	This study
EPECΔFragB	EPEC E2348/69 lacking IE2 'FragB' region immediately downstream of *LifA-like* gene	Nal^R/Cm^R	This study
EPECΔFragB/L	As EPECΔFragB but also lacking IE2 *LifA-like* gene	Nal^R/Cm^R	This study
EPECΔFragB/L/N	As EPECΔFragB/L but also lacking IE2 region with *nleE2*, *nleB2** & *espL** genes (* indicates pseudo gene)	Nal^R/Cm^R	This study

Table 2 Strains derived from Litvak Laboratory EPEC E2348/69 stocks

Bacterial Strain	Description	Antibiotic	Source
ΔIE2	Lacks entire IE2 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔIE3	Lacks entire IE3 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔIE5	Lacks entire IE5 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔIE6	Lacks entire IE6 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP2	Lacks entire PP2 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP3	Lacks entire PP3 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP4	Lacks entire PP4 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP5	Lacks entire PP5 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP6	Lacks entire PP6 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP7	Lacks entire PP7 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP8	Lacks entire PP8 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP9	Lacks entire PP9 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP10	Lacks entire PP10 pathogenicity island	Km^R	(Litvak *et al.*, 2017)
ΔPP13	Lacks entire PP13 pathogenicity island	Km^R	(Litvak *et al.*, 2017)

Table 3 Strains derived from Cepeda Laboratory EPEC E2348/69 stocks

Bacterial Strain	Description	Antibiotic	Source
EPEC WT	EPEC O127; H6 strain	Sp^R	(Cepeda-Molero *et al.*, 2017)
ΔescN	Lacks functional T3SS. Transposon-disrupted *escN* gene	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC9	EPEC strain missing 4 LEE effector genes (*map*, *espG espF* & *espH*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC8	As EPEC9 but also missing IE5 effector genes (*espG2*, *espC*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC7	As EPEC8 but missing IE6 effector genes (*espL*, *nleB1*, *nleE1* & *LifA*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC6	As EPEC7 but also missing but missing IE2 effector genes (*nleE2*, & *LifA-like*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC5	As EPEC6 but missing PP2 effector genes (*nleH1*, *cif**, *espJ*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC4	As EPEC5 but missing PP3 effector gene (*nleJ*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC3	As EPEC4 but missing PP4 effector genes (*nleG*, *nleB*, *nleC*, *nleH** & *nleD*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC2	As EPEC3 but missing PP6 effector genes (*nleA*/*espI*, *nleH2*, *nleF* & *espO**)	Sp^R	(Cepeda-Molero *et al.*, 2017)
EPEC0	As EPEC2 but missing 2 more LEE effector genes (*espZ* & *tir*)	Sp^R	(Cepeda-Molero *et al.*, 2017)
ΔlifA	EPEC missing *LifA* gene	Sp^R	(Cepeda-Molero *et al.*, 2017)
ΔlifA-like	EPEC missing *LifA-like* gene	Sp^R	(Cepeda-Molero *et al.*, 2017)
ΔlifA/lifA-like	EPEC strain missing *LifA* and *LifA-like* genes	Sp^R	(Cepeda-Molero *et al.*, 2017)

* indicates pseudo gene

2.2.4 Restriction enzyme digestion

Restriction enzyme digests were performed using enzymes and buffers from NEB (Maryland, USA) according to the manufacturer's instructions. Briefly, DNA digestion reactions routinely consisted of 1-1.5µg of DNA, 5-10 Units (U) of restriction enzyme and one tenth volume of 10x restriction buffer made up to a final reaction volume of 20-50µl with dH_2O. Reactions were generally incubated at the recommended temperature for at least 1 h at 37^0C.

2.2.5 Agarose gel Electrophoresis

Plasmid and/or PCR products were visualised on the appropriate percentage (routinely 0.7%) agarose gel in TAE (40mM Tris-acetate, 1mM EDTA) buffer supplemented with a fluorescent nucleic acid stain, GelRedTM (1:30,000; Biotium). The DNA samples were mixed with 10x loading dye and run alongside a 1 Kb DNA ladder (New England Biolabs) at 100 volts for an appropriate length of time. DNA bands were visualised with a UV transilluminator (Bio-Rad) and images captured (Image lab Software) as required.

2.2.6 DNA ligation

The Gibson Assembly method is an easy-to-use cloning method that allows the efficient cloning of multiple DNA fragments simultaneously. Cloning is based on the recombination of PCR amplified DNA fragments to a vector fragment by 20bp extension to the primers that corresponds to the target vector 'insertion' site (Gibson *et al.*, 2009). The inserts were obtained by PCR amplification using oligonucleotide primers (Table 5) designed using NEBuilder assembly tool (http: //nebuilder.neb.com/) and Q5 high-fidelity DNA polymerase (New England Bio-labs; Cat #M0493S) and recipient vector. The vector was pre-digested with restriction enzymes, routinely XbaI/SacI for pDS132 suicide vector and BamHI/SalI pACYC184. The vector and insert fragment were isolated using (New England Bio-labs; Cat #T1030) following manufacturer's instructions. The concentration of gel extracted DNA was quantified (Nano-Drop 100 spectrophotometer [Lab-tech]) following manufacturer's instruction. Vector and insert were added at a ratio 2:1

(50ng vector + 25ng of insert) with 5ul Gibson assembly master mix (New England Bio-labs; Cat #E2621S) in a 10ul final volume. The mixture was incubated 12-16 hrs at 50^0C. Before a portion of the ligation sample was run in 0.7%-agarose gel to check for a ligation product. If appropriate, 2-5ul of the remaining ligation product was transformed into commercial competent *E. coli* cells. Resultant clones were screened by PCR analysis for the presence of the specific fragment.

Table 4 shows the list of plasmids used in this study and provides a brief description of key encoded factor(s), the selection antibiotics (Cb^R, Km^R & Cm^R, indicates resistance to Carbenicillin, Kanamycin and Chloramphenicol respectively) and the source of each plasmid.

Table 4 Plasmids used in this study

Plasmid	Description	Antibiotic Selection	References
pACYC184	Cloning vector	Cm^R/Tet^R	(Chang and Cohen, 1978)
pBR322	Cloning vector	Cb^R/Tet^R	(Watson, 1988)
pSK (bluescript)	Cloning vector	Cb^R	(Sohaskey *et al.*, 1992)
pACYC-*tir*	pACYC184 vector contain a MCS, the *lacZα* reporter gene, and P15A *ori* carrying *tir* gene	Cm^R	(Madkour, 2017)
pSK-*cesT*::HA	pSK expressing the LEE chaperone, CesT, as a CesT::HA fusion protein	Cb^R	This study
pACYC-3'gto	pACYC184 carrying LEE *tir*, cesT and 3' end of *map* genes	Tet^R/Cm^R	(Dean and Kenny, 2004)
pACYC-Frag-B	pACYC184 carrying IE2 'Frag-B' region	Cm^R	This study
pACYC-Frag-1	pACYC184 carrying IE2 'Frag-1' region	Cm^R	This study
pACYC-Frag-2	pACYC184 carrying IE2 'Frag-2' region	Cm^R	This study
pACYC-Frag-3	pACYC184 carrying IE2 'Frag-3' region	Cm^R	This study
pACYC- Frag-4	pACYC184 carrying IE2 'Frag-4' region	Cm^R	This study
pDS132-ΔPP2	pDS132 suicide vector used to delete entire EPEC PP2 region	Cm^R	S. Quitard. Unpublished
pDS-ΔFragB	Region of above vector used to delete PP2 replaced with region to delete IE2 'Frag B' region	Cm^R	This study
pDS-ΔFrag/L	Region of vector used to delete PP2 replaced with region to delete IE2 'Frag B' region and *LifA-like* gene	Cm^R	This study
pDS-ΔFragB/L/N	Region of vector used to delete PP2 replaced with region to delete IE2 'Frag B' region, *LifA-like*, nleE2, *nleB2**, *espL** genes (* indicates pseudogene)	Cm^R	This study
pACYC-*nle*E2/*nleB2/ *espL****	pACYC184 carrying IE2 *nleE2*, *nleB2**, *espL** genes (* indicates pseudogene)	Cm^R	This study
p*lifA*	Encodes *LifA* tagged at C-terminus with 6X-His under rhamnose-inducible promoter	Km^R	(Cassady-Cain *et al.*, 2016)

pΔDXD	As pRham-*LifA*-6X-His but with DXD motif of putative glycosyltransferase domain changed to AAA	Km^R	(Cassady-Cain *et al.*, 2016)
pΔCHD	As pRham-*LifA*-6X-His but with CHD motif of putative cysteine cysteine-protease domain changed to AAA.	Km^R	(Cassady-Cain *et al.*, 2016)
p*cesT*	pACYC184 expressing CesT chaperone	Cm^R	(Madkour, 2017)

Table 5 Standard PCR reaction mixture

A) Q5 Hot start high fidelity DNA polymerase B) Taq DNA polymerase

A) 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	

B) 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	

Table 6 Standard PCR reaction condition

A) Q5 Hot start high-fidelity DNA polymerase and B) Taq DNA polymerase.

A) Step	Cycles	Temperature and Time
Initial denaturation	1	98^0C for 30 seconds
Denaturation Annealing Extension	30	98^0C for 10 seconds 53-68^0C for 30 seconds 72^0C for 30-60 seconds
Final Extension	1	72 C for 2 min

B) Step	Cycles	Temperature and Time
Initial denaturation	1	95^0C for 30 seconds
Denaturation Annealing Extension	30	95^0C for 30 seconds 53-63^0C for 1 min 68^0C for 1 min/kb
Final Extension	1	68^0C for 2 min

Table 7 Oligonucleotide Primers used in this study

	Name of the Oligo	Oligonucleotide sequence 5'-3'
1	***nleE1* Forward Primer**	CCAGTATGTATACCAGCAGTTCATGGTAAG
2	***nleE1* Reverse Primer**	ATTGGGCGTTTTCCGGATATAACTG
3	***nleE2* Forward Primer**	TTATTTCCACAGGCATGTAG
4	***nleE2* Reverse Primer**	TCCTGTTACTAATACTCAGGG
5	***espGorf3* Forward Primer**	TAGGTATAACCCTATGCCTGTGTTC
6	***espGorf3* Reverse Primer**	AACAAATTCAGGCTGACACAGTACC
7	***nleF* Forward Primer**	AAGGGGGTTTTGATATGTTACCAACAAGTG
8	***nleF* Reverse Primer**	CCACGAGGCATTTCATTGCTCGTAG
9	***espH* Forward Primer**	CCCTTTGGCAACCGTAAAGC
10	***espH* Reverse Primer**	AAATATCGTCCCCAGAACAG
11	***espF Forward Primer***	ATGGAATTAGTAACGCTGCTTCTACAC
12	***espF Reverse Primer***	TTGGTTACCCTTTCTTCGATTGCTCATAG
13	***espG* Forward Primer**	ACAAAAACTATGGCTGACGCATCAC
14	***espG* Reverse Primer**	TTCAGCGCATGACATCTCATCCCAG
15	***espJ* Forward Primer**	AAATAACCACCACTCCCACACCAGCGAAAC
16	***espJ* Reverse Primer**	ATACCAAATGCGTTTTTTTGTGGGTTATAC
17	***nleD* Forward Primer**	GCTTTATTATCGGGTTTGGTGACCGCCTTG
18	***nleD* Reverse Primer**	AATAAGAGCTGAGTCGTGCGGGTAG
19	***LifA* Forward Primer**	CGCAGGATGACCTAGTTTC
20	***LifA* Reverse Primer**	CCTGGATTCCTTCTGTATTG
21	***LifA-like* Forward Primer**	GGTATCGAAGAGATTGTCAG
22	***LifA-like* Reverse Primer**	CTATCGCTTCCTGTATAGCC
23	***cesT* Forward Primer**	GCTCTAGACAACGTTGCAGCATGGGTAA
24	***cesT* Reverse Primer**	GCGAATTCTCATGTTTGGGCTCCACCAC
25	***tir* Forward Primer**	CCGCCACTACCTTCACAAAC
26	***tir* Reverse Primer**	GCGTTGGTGCGGCATTTACAG
27	**Frag-A Forward Primer**	GCGACCACACCCGTCCTGTGGGACATTATTGCCATATCCA
28	**Frag-A Reverse Primer**	AAGGCTCTCAAGGGCATCGGGACTCAAAACAAACTCAGATG
29	**Frag-B Forward Primer**	GCGACCACACCCGTCCTGTGCATATATGCCCGGTATGAA
30	**Frag-B Reverse Primer**	AAGGCTCTCAAGGGCATCGGGGCAATATGTAGTTCACTGC
31	**Frag-C Forward Primer**	GCGACCACACCCGTCCTGTGGCAATATGTGTCACTTTTACC
32	**Frag-C Reverse Primer**	AAGGCTCTCAAGGGCATCGGCAAAGATGGCTCTGCTAAG
33	**Frag-D Forward Primer**	GCGACCACACCCGTCCTGTGGCCGATATTGATCCAGTT
34	**Frag-D Reverse Primer**	AAGGCTCTCAAGGGCATCGGGAGTTCTACACATTGGTCAAG
35	**Frag-E Forward Primer**	GCGACCACACCCGTCCTGTGCAAATGTCGGAAACAGTAAC
36	**Frag-E Reverse Primer**	AAGGCTCTCAAGGGCATCGGTAGTGGGAATGTACGCATAG
37	**Frag-F Forward Primer**	GCGACCACACCCGTCCTGTGCAACCAGTGAACATATTTCC
38	**Frag-F Reverse Primer**	AAGGCTCTCAAGGGCATCGGCTGAAACCGGTTTATATGC
39	**Frag-1 Forward Primer**	CGCGGATCCCGAGTATTTTTAGTCATCCTG
40	**Frag-2 Reverse Primer**	CGCGGATCCGACTACGAACTCGAACCACAATC
41	**Frag-3 Forward Primer**	CGCGGATCCATAGTTCGGACATAAGCCCCCG
42	**Frag-4 Reverse Primer**	CGCGGATCCGACTCAAAACAAACTCAGATGAG
43	**Frag-1-4 Forward Primer**	CGGCTACGGGAACTCTCGGAAGCAGCTGCGCA
44	***LifA-CHD* Forward Primer**	GCAGCCAGAACATTGAT
45	***LifA-CHD* Reverse Primer**	ATCAGGCTATAGGTGCG
46	***LifA-DXD* Forward Primer**	CCGCATTTCAATTAAAGATG
47	***LifA-DXD* Reverse Primer**	TCTGCATCACTGATTTCATTATAG
48	**IE2 Forward Primer 1**	GCGTCGACAAGGAGTTATACAC
49	**IE2 Reverse Primer 1**	GGGGATACCTGGTTAATGC
50	**IE2 Forward Primer 2**	CGTTGCCGTATACACACTTTC
51	**IE2 Reverse Primer 2**	ATGCTCTGACTCTTGCCTGAG
52	**Frag-B Forward Primer 1**	GGTTAAAAAGGATCGATCCTGGCTGAGTTTCAAAGCTATC
53	**Frag-B Reverse Primer 1**	ACAGTTTATCCGACTGATCGCATTGTAAC
54	**Frag-B Forward Primer 2**	CGATCAGTCGGATAAACTGTTTGGTTGC
55	**Frag-B Reverse Primer 2**	GTGGAATTCCCGGGAGAGCTCTTTCCTGTCAATGACATC
56	**Frag-B/L Forward Primer 1**	GGTTAAAAAGGATCGATCCTGGCTGAGTTTCAAAGCTATC
57	**Frag-B/L Reverse Primer 1**	CTAAACCTTTGCAGCGACTGATCGCATTGTAAC
58	**Frag-B/L Forward Primer 2**	CAATGCGATCAGTCGCTGCAAAGGTTTAGATATTAAC
59	**Frag-B/L Reverse Primer 2**	GTGGAATTCCCGGGAGAGCTAGAAGCTCAGCAACTTGTG
60	**Frag-B//N Forward Primer 1**	GGTTAAAAAGGATCGATCCTGGCTGAGTTTCAAAGCTATC
61	**Frag-B/L/N Reverse Primer 1**	CATAGAAAGGCGACTGATCGCATTGTAAC
62	**Frag-B/L/N Forward Primer 2**	CAATGCGATCAGTCGCCTTTCTATGAGTTTTACACC
63	**Frag-B/L/N Reverse Primer 2**	GTGGAATTCCCGGGAGAGCTACACAAAATGACAGCTGTG

2.2.7 Bacterial Transformation

2.2.7.1 Heat-shock method

Commercial NEB turbo competent *E. coli* (High Efficiency; New England Biolabs; Cat # C2984H) were thawed on ice for 10 minutes before 25µl was placed in a pre-chilled 1.5 ml Eppendorf tube. 10-100ng (1-2µl) of DNA (usually ligation mix; see below) was added and gently mixed by pipetting. The suspension was kept on ice for 30 min followed by a brief heat-shock by placing at 42˚C for 30 seconds before returning to ice for another 5 mins. Then 950µl of pre-warmed SOC medium was added, gently mixed with pipette and incubated (with shaking; 225-250 rpm) at 37˚C for 1-hour prior to plating 50 and 450µl onto LB agar plates containing appropriate antibiotic(s). Plates were incubated overnight at 37 ^{0}C. Colonies were screened for the transformed plasmid by PCR analysis (see below).

2.2.7.2 Electroporation Method

Bacterial strains grown with shaking overnight in LB broth were diluted 1:50 into 100 ml LB broth, containing antibiotic when needed, and grown with shaking (Stuart Scientific incubator; Cat #00119; 225-250rpm) to an optical density (OD_{600}) of 0.6-0.8. The culture was placed on ice with gentle shaking for 5 min to rapidly chill the solution. All manipulations were carried out with ice cold reagents. The bacteria were pelleted (2800 g, 15 min, 4^{o} C) in 50 ml tubes (Star lab UK Ltd, #E1450-0200) and resuspended in 50ml deionised water (dH_2O). The centrifugation step was repeated and resulting pellet re-suspended in 50ml (dH_2O). After repeating the centrifugation step, the pellet re-suspended in 10% glycerol (ice cold). A final centrifugation step was followed by re-suspending the pellet in 300ul 10% glycerol which as aliquoted (40ul) into 1.5 ml tubes (Sarstedt; Cat #72.690.001) for use or snap frozen in liquid nitrogen for storage (-80^{o}C).

Electrocompetent cells were thawed on ice before adding plasmid DNA (1-2 µl; 25-50ng/µl) to a 40 µl aliquot and incubated for 5-10 min on ice. The suspension was transferred to a 2mm electroporation cuvette (Cell projects #EP-102) for electroporation (Gene Pulser II Bio-Rad with following setting: 2.5kV, 200Ω, 25mF

for 4.5μs) followed by immediate, gentle, resuspension in 1ml super optimal broth with catabolite repression (SOC; 0.5% Yeast Extract, 2% Tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ & 20mM Glucose) media pre-warmed to 37°C. After a minimum 1h recovery period at 37°C, 1 to 1000μl was plated on LB agar plates containing selective antibiotics and incubated (37^0C) for 12-16 hours. Individual colonies were screened for plasmid introduction by PCR and/or western blot analyses (see below).

2.2.8 Construction of EPEC gene/gene complex knockout mutants

Disruption or knockout of target EPEC genes using the standard allelic exchange protocol (Donnenberg and Kaper, 1991) followed by phenotypic characterisation of the resulting mutant strain. Briefly, SM10 *λpir* (Km^R) strains carrying the appropriate, usually pDS132-based (Cm^R), suicide vectors were co-incubated with the recipient strain i.e., EPEC (Nal^R) overnight at 37^0C on a LB agar plate. Next day, bacterial cells were scraped off from different areas and grown to stationary phase in LB broth contacting Nal antibiotic. Bacterial were then added to Nal/Cm plates to select for transconjugants (suicide vector incorporates in EPEC genome by single cross-over recombination at target gene). Next day, a single colony was inoculated to LB broth and grown to stationary phase (provide change for suicide vector to be excised from genome by recombination) before plating on LB agar plates containing 5% sucrose for overnight incubation at 30^0C. Five percent sucrose kills cell still carrying the suicide vector. Single clones were streaked on LB agar plates containing Nal, Cm or no antibiotic and incubated overnight at 37^0C. Colonies growing on Nal, LB but not Cm plates were screen for gene disruption by PCR analysis.

2.2.9 DNA sequencing

The gene of interest was PCR amplified using Q5 high fidelity DNA polymerase (New England Bio-labs; Cat # M0493S) with appropriate oligonucleotide pair. PCR

products were cleaned up using GenElute™ PCR Clean-Up Kit (Sigma Cat #1020-1KT) following manufacturer's instructions and quantified using (Nano-drop 100 spectrophotometer; Lab-tech) prior to sending for sequencing along with appropriate oligonucleotides (Table 5). Obtained DNA sequence was analysed by performing pairwise alignment programme (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) with native gene sequence.

2.3 Bacterial infection-related

2.3.1 J774.A1 infection

J774.A1 macrophage (~10^5) were seeded into 12 well plates 2 days prior to infection, as described previously. The cells were serum starved ~16h prior to infection by replacing the media with DMEM containing only 0.5% FCS. Overnight standing LB bacterial cultures (OD_{600} 0.6-0.8) were diluted into DMEM (1:10) for 2h at 37°C in 5% CO_2 atmosphere induces expression of T3SS and other virulence proteins (Rosenshine *et al.*, 1996a) before taking OD_{600} measurements. The macrophages were infected with bacteria at a multiplicity of infection (MOI) of 200:1 (bacteria: macrophage) for the required period.

2.3.2 Isolation of protein fractions from J774.A1 cells

Infections were stopped by washing three times with ice cold PBS (137mM sodium chloride [NaCl], 2.7mM potassium chloride [KCl], 10mM disodium phosphate and 1.8mM monopotassium phosphate; pH 7.4) which removes non-adherent bacteria. Total cells extracts were obtained resuspending cells in 120µl of sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Laemmli, 1970). In some experiments, cells were fractionated into cytoplasmic (contains host cytoplasm proteins and T3SS-delivered effectors), membrane (contains host membrane proteins and T3SS-delivered effectors) and insoluble (contains host nuclei, cytoskeleton, and adherent bacteria) fractions using ice-cold solutions. Briefly, post-infection, cells were washed three times with 1X PBS and

drained well before adding Saponin Lysis Buffer (PBS containing 0.2% Saponin [(BD7; Cat # 6168150), 1mM sodium fluoride (NaF; ICN Biomedical; Cat #194864), 1mM sodium orthovanadate ($NaVO_4$; ICN Biomedicals; Cat # 159664) plus a protease inhibitor cocktail (PIC; Sigma; Cat #1003013902)]. The samples were centrifuged (13000 g, 5min, 4^0C) in 1.5ml microfuge tubes, with supernatant (cytoplasmic fraction) isolated before adding 5X SDS-PAGE sample buffer to final 1X conc. The pellet was washed (gentle addition/removal of 1 ml PBS) before suspending in Triton X-100 (BioRad; Cat # 9002-93-1) Lysis Buffer (as saponin Lysis Buffer but also contains 1% Triton X-100) and centrifuging as before. The supernatant (membrane fraction) was isolated before adding 5X SDS-PAGE sample buffer to final 1X conc. The remaining pellet (insoluble fraction) was gently washed, as before, and re-suspended in 1X SDS-PAGE sample buffer. Samples were heated (100^{o}C, 5min), vigorously vortexed for immediate use or stored at -20^{o}C.

2.3.3 Sodium dodecyl sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE (routinely 6% or 10%) gels (Laemmli, 1970) were prepared for use in a BioRad mini-gel system and, generally, loaded with 10µl samples alongside 2-4µl of protein marker (Bio-Rad; Cat #161-0373). The gels were resolved in SDS-PA gel running buffer (25mM Tris-HCl, 192mM glycine, 0.1% w/v SDS: pH 8.3) at 200 Volts for 1h. The proteins were either visualised by Coomassie staining or wet transferred to nitrocellulose (GE Healthcare Cat #15269794) using a Bio-Rad Proteane 11 wet transfer system in 1X transfer buffer (25mM Tris, 192mM glycine; pH 8.3) for 1 at 110 Volts. Transfer efficiency/quality were assessed by adding a reversible strain (Ponceau Red; Sigma; Cat #P7170) and aided membrane cutting into sections to probe for specific proteins. After washing away the stain - with TBST (150mM NaCl, 10mM Tris-HCl; pH 7.5) the membranes sections were blocked for 1hr at room temperature (on rotating shaker) in TBST/Tween-20 (TBST with 0.05% v/v Tween-20) containing 5% w/v skimmed milk (Marvel). The membranes were removed from blocking buffer, washed 3x 5min with TBST and incubating overnight at 4^0C with

the appropriate primary antibody (Table 8) diluted in TBST. Post incubation, the membranes were washed 3x 5 min with TBST and incubated 1h (at room temperature) with appropriate secondary alkaline phosphatase (AP; Jackson Immuno research Cat #11-055-144) or horse radish peroxidase (HRP; Jackson Immuno Research Cat # .111-035-003) conjugated antibody diluted in TBST (Table 8). The membranes were washed (3 x) with TBST with bound AP-conjugated secondary antibodies visualised by using NBT/BCIP substrate (Promega-UK Ltd; Cat #53771), following manufacturer's instructions, and for HRP-conjugated antibodies the ECL Buffer (Thermo scientific; Cat #34079), following manufacturer's instructions, with chemo-luminescence signal captured using Gel-Doc machine (Bio-Rad System) and image J gel analysis software.

2.3.4 Immunofluorescence microscopy

Hela cells (ATCC-CCL-2^{TM}) – derived from human cervical epithelial cancer - were seeded 2 days prior to infection onto sterile 13mm glass coverslips (VWR Cat No. 631-1578) to ~80-90% confluency on the day of infection. Post-infection, the cells were washed with ice cold 1x PBS and fixed for 30 min at room temperature with freshly prepared 2.5% paraformaldehyde (Chem Cruz; Cat No. SC 281692) in ice cold 1x PBS. Following fixation, the cells were washed three times with 1x PBS and incubated for 1h with 20-25µl of 1x PBS containing 1% (v/v) TritonX-100, tetramethyl-rhodamine-isothiocyanate (TRITC)-phalloidin (1µg/ml [1:100]; final concentration 10ng] to detect polymerised actin; fluoresces in 'Red' channel) and 4'6-diamidino-2-phenylinodole (DAPI; fluoresces in 'Blue' channel) to strain DNA. Coverslips were washed three times in 1x PBS before mounting onto glass slides on 20µl of FluroSave reagent (Millipore Cat no. 345789-20). The samples were examined using a Zeiss Axioskop epifluorescent microscope with images taken using a Hamamatsu C4742-95 charge-coupled device camera and Improvision software. Fiji ImageJ software were used for analyses.

2.3.5 Immunoprecipitation

J774.A1 cells were seeded at approximately 3x 10^5 cells/ml in 10 cm dishes (Sarstedt, Cat #83.3921) 2 days prior to infection. On the day of infection cells were 80-90 % confluent (~1x 10^6 cells in 10 cm dishes), infected with EPEC WT and T3SS mutant for 2 hr. Post-infection, the cells were washed twice with cold PBS and lysed in 250µl cold 1% Triton-X-100 Lysis buffer (see section 2.3.2) and sample centrifuged (13000 g, 5min, 4^0C) to obtain soluble fraction (contains host cytoplasmic and membrane proteins plus T3SS-delivered effector proteins). A sample (50ul) was taken as an 'input' reference and added to 5x SDS sample buffer to final 1x conc. Thirty microlitre of pan-Akt antibodies conjugated to magnetic beads (Table 6) were added and incubated (on a rotating wheel) overnight at 4°C. The beads were magnetically separated with a sample (200ul) of the supernatant to provide a reference 'output' that was added to 5x SDS sample buffer (to final 1x conc). Beads were pelleted using a separation rack. To give a reference 'output,' the beads were magnetically separated with a sample (200ul) of the supernatant, which was added to 5x SDS sample buffer (to final 1x conc). Beads were extracted using magnetic separation rack. The bead was washed 3 times with ice-cold lysis buffer (500ul) and kept on ice between washes. The pellet was resuspended with 30µl 1x SDS sample buffer and briefly vortex to mix. The sample was heated to 95-100^0C for 5 min, briefly centrifuged and the supernatant transferred to a new microfuge tube. This was processed for Western blot analysis as described (section 2.3.3) with appropriate primary and HRP-conjugated antibodies. Table 8 lists the antibodies and conditions used for immunofluorescence (IF) and/or western blot (WB) analyses.

Table 8 Antibodies used for western blot and immunofluorescence studies

Primary antibody	Origin	Dilution	Company and Cat #
Tir	Rabbit	1:5000	Kenny Lab
β-Actin	Rabbit	1:10000	Sigma; A2066
Pan-Akt	Mouse	1:500	Cell Signalling; Cat # 2920
p-AktSer473	Rabbit	1:500	Cell Signalling; Cat # 9271
p-AktThr308	Rabbit	1:500	Cell Signalling; Cat # 4060
p-AktSer473	Mouse	1:500	Cell Signalling; Cat # 4051
LifA	Rabbit	1:10000	Stevens Lab, Edinburgh
O-GlcNAc	Mouse	1:500	Santa Cruz, Cat # SC 59623
Akt (Pan) Magnetic bead conjugate	Rabbit	1:6	Cell Signalling; Cat # 11848

Secondary antibody	Origin	Dilution	Company and Cat #
Anti-Rabbit IgG-Horse radish peroxide (HRP)	Goat	1/5000	Jackson Immuno Research; Cat # 111-035-003
Anti-mouse IgG-Horse radish peroxide (HRP)	Mouse	1/5000	Jackson Immuno Research; Cat # 111-005-146
Anti-Rabbit IgG- Alkaline Phosphatase (AP)	Goat	1/2500	Jackson Immuno Research; Cat # 111-055-144
Phalloidin-TRITC	NA	1:100	Sigma; Cat # P1951.1MG
DAPI	NA	1:1000	Invitrogen; Cat # D1306

Chapter 3. Identifying EPEC effector(s) with role in pAktSer473 loss

3.1 Introduction

A previous study on the T3SS dependent mechanism by which EPEC interferes with Akt signalling found no role for the LEE and all 13 examined Nle effectors suggesting roles for the 4 unexamined T3SS Nle substrates (EspC, LifA, LifA-like and/or NleJ) or other yet unidentified effector(s) (Amin, 2017). Thus, initial studies would focus on define roles, individual or collective, for these 4 proteins alone or with other LEE and/or Nle effectors.

Studies with an available EspC mutant did not reveal any impact on the inhibitory process (O. Amin unpublished) suggesting EspC does not play a key role or functions in a redundant manner with other effectors. NleJ, is a 191aa protein encoded on a P2-like prophage (PP3) predicted to have a C-terminal phosphoribosyl transferase domain (Deng *et al.*, 2012). NleJ does not have homologs in other A/E pathogen members such as EHEC or *C. rodentium* but has 50% identity with a SopEΦ phage-encoded protein in *Salmonella enterica* (Deng *et al.*, 2012). LifA (also known as *Efa1*) is the largest known T3SS substrate (3223 aa) and is conserved to different degrees in genome-sequenced A/E pathogen strains including rabbit EPEC (RDEC-1) *Citrobacter rodentium* and some enterohemorrhagic *E. coli* (EHEC) strains (Deng *et al.*, 2012). In the EHEC O157 EDL933 strain the *lifA/efa1* gene is truncated into two open reading frames, Z4332 and Z4333 respectively (Hayashi *et al.*, 2001; Stevens *et al.*, 2004). Z4332 encoded the N-terminal 433 amino acid residues (99.9% identical to that of LifA) and Z433 aligns with a 275 amino acid region in the middle portion of LifA (Deng *et al.*, 2012). However, EHEC O157:H7 also encodes a LifA-like homolog, named ToxB, on a plasmid (Makino *et al.*, 1998) exhibiting 28% sequence identity with LifA/Efa1. ToxB is considered a functional homolog of EPEC LifA, at least in relation to it also being a lymphostatin – a feature that led to the identification of LifA; lymphocyte inhibitory factor A (Klapproth *et al.*, 2000). The Efa1 (*Escherichia coli* factor for adherence) nomenclature came from the studies by another group reporting the gene product to act as an adhesion (Nicholls *et al.*, 2000a). In EPEC, the LifA/Efa1-encoding gene is located on the IE6 while the LifA-like homolog (~28% identity) is encoded on IE2 (Iguchi *et al.*, 2009; Deng *et al.*, 2012).

As mentioned EPEC has acquired several prophages (PP1-13) and integrative elements (LEE, IE1a, IE1b, IE2-6) with known effectors encoded, mostly in clusters, on 9 PP and IE regions (Iguchi *et al.*, 2009) (Figure 16). As PPs and IEs can be as large as 60kb (Iguchi *et al.*, 2009) it is possible they encode cryptic T3SS-dependent effectors. Interestingly an *in-silico*-based approach by a Kenny group member, Dr Paul Dean, identified putative effectors with examination of β-lactamase tagged versions supporting the T3SS-dependent delivery of 6 into host cells (Unpublished). This supports the idea that EPEC may encode other, currently unknown, effectors and possible substrates to interrogate roles in the inhibition of Akt activity. Furthermore, previous research revealed that the effector or effectors required for EPEC to inhibit Akt signalling require the function of the LEE multi-substrate chaperone, CesT (Amin, 2017). This relationship, if confirmed, could be used to identify novel CesT binding partners by, for example, mass spectrometry approaches as the possible responsible effector(s).

The aim of the work in the work described in this chapter was to identify the EPEC effector or effectors responsible for inhibiting Akt signalling in the J774.A1 macrophage-like cell model.

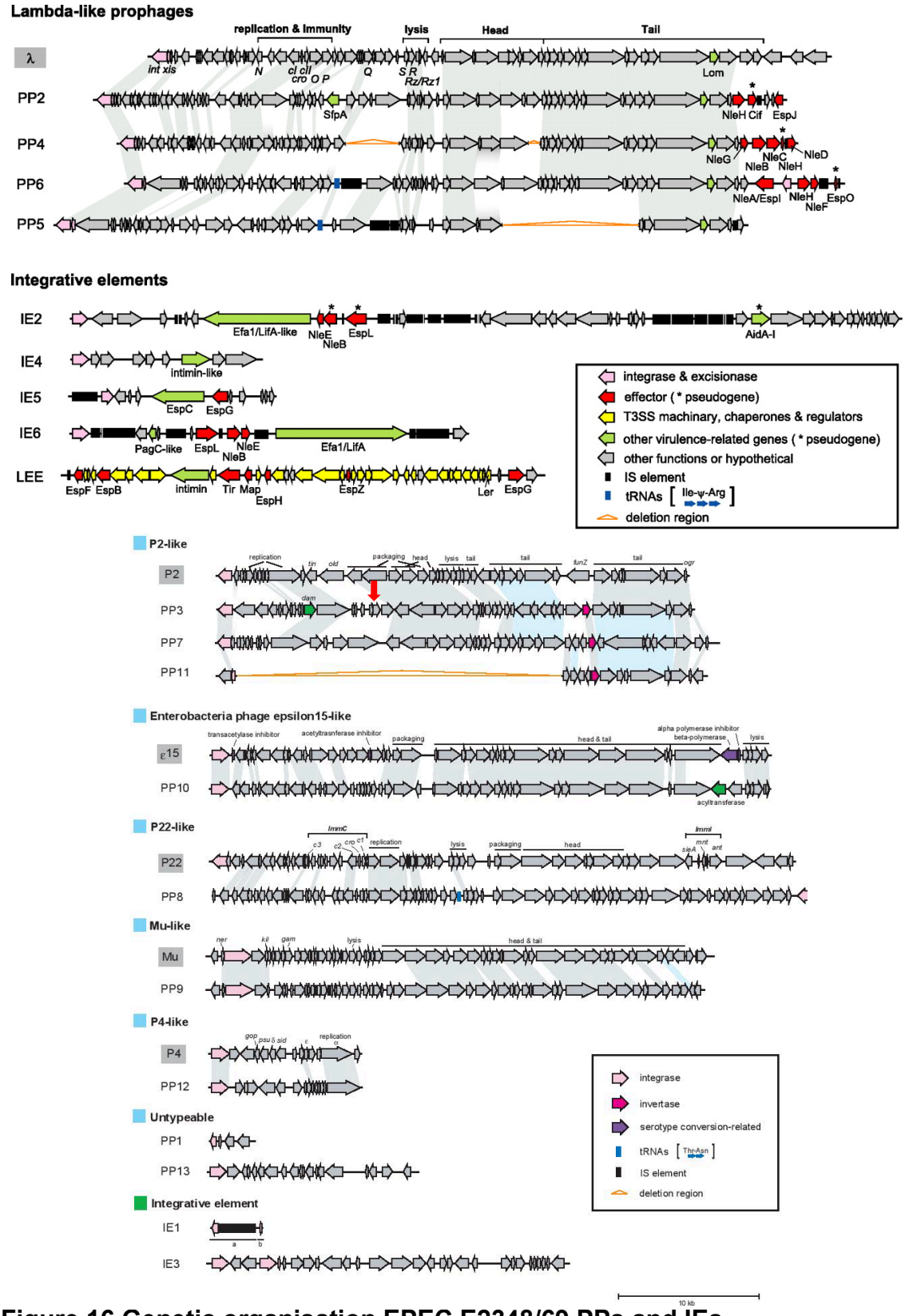


Figure 16 Genetic organisation EPEC E2348/69 PPs and IEs

Position of the NleJ-encoding gene (red arrow) on PP3

3.2 Results

3.2.1 Re-establishing the Akt phosphorylation assay

To enable screening for the responsible effector/s, it was first necessary to re-establish the infection model and detection assay (Celli *et al.*, 2001; Quitard *et al.*, 2006; Amin, 2017).Thus, J774A.1 macrophages were left uninfected or infected with pre-activated bacteria (grown 3hr in DMEM to induce the T3SS) at a multiplicity of infection (MOI) of 200:1 (bacteria to host cells). These studies used EPEC and a T3SS mutant, *cfm-14* (cannot deliver effector proteins; Chapter 2; Table 1) over a 120 min infection period. Following removal of non-adherent bacteria, the remaining cellular proteins were extracted in sample buffer and samples resolved (SDS-PAGE gels) for western blot analysis (See Material and Methods; Section 2.3.3).

Western blot probing for the Tir effector is used as a marker of T3SS functionality (Kenny *et al.*, 1997a) as Tir is the first and most abundantly delivered EPEC effector with delivery revealed by shifts in apparent molecular weight due to phosphorylation by host kinases (Kenny *et al.*, 1997a). The unmodified Tir form within bacteria (T^0) migrates as a ~78kDa protein with kinase modification leading to the T'' (~90kDa) form (Kenny *et al.*, 1997a). Probing the isolated samples revealed host kinase modification of Tir to the T'' form in cells infected with EPEC but not the T3SS mutant (Figure 17) supporting strain genotype.

Consistent with previous studies (Celli *et al.*, 2001; Quitard *et al.*, 2006; Amin, 2017) infection with both strains induced Akt phosphorylation on Ser473 (pAktSer473) that was sustained over the 120-minute T3SS mutant infection but declined to background levels, by 60 min post-EPEC infection (Figure 17). Probing for the host protein, β-actin, and revealed similar gel lane loadings illustrating pAktSer473 signal loss was due to a T3SS-dependent process. These findings were highly reproducible (as illustrated in subsequent experiments) and hence the work shows re-establishment of a robust model to study the T3SS-dependent loss of pAktSer473 signal with J774.A1 macrophages.

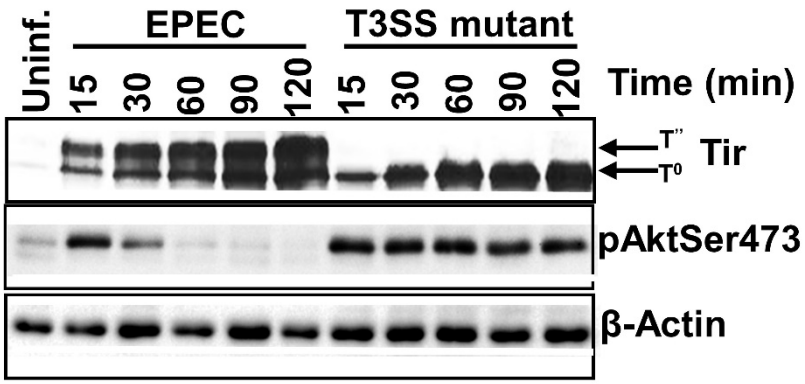


Figure 17 Assay for monitoring T3SS-dependent inhibition of Akt signalling.

J774A.1 monolayer were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending the macrophages in sample buffer. Samples were resolved on a 10% SDS-PA gel, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC and a T3SS mutant, *cfm-14*. The data is representative of multiple (>3) independent experiments (not shown but see other figures).

3.3.2 Role for CesT chaperone in loss of pAktSer473 signal

Previous studies suggested that the CesT chaperone is needed for infection-associated loss of pAktSer473 signal (Amin, 2017). Thus, experiments were done to support this finding as it could provide a means to identify the responsible effector/s by, for example, isolating CesT binding partners for identification by mass spectrometry. However, the interpretation of the previous studies were complicated by the CesT-deficient strains inducing pAktSer473 signal loss by an additional mechanism i.e. cytotoxic death as CesT is needed to deliver the 'anti-death' LEE effector, EspZ (Amin, 2017). These previous studies focused on *cesT*::Km and *core*::Km (lacks LEE region encoding 3 effectors [EspH, Map, Tir], 2 chaperones [CesF/CesT] and Intimin surface protein) mutants. However, preliminary studies suggested a *core*::Km derived mutant, TOEA7Δ*core::*Km, did not induce this cytotoxicity mechanism and thus behaved like the T3SS mutant (O. Amin; unpublished). The Δ*core*::Km mutant lacks the LEE region encoding 3 effectors (EspH, Map, Tir), 2 chaperones (CesF, CesT) and Intimin surface protein with TOEA7Δ*core:*Km mutant also lacks genes for 13 Nle effectors (Madkour, 2017). Thus, studies were undertaken to determine if re-introducing *cesT* restored the strain's ability to interfere with Akt signalling.

This work used plasmids available in the lab that encode CesT - as a HA-epitope tagged CesT::HA variant which functionally substitutes CesT (B. Kenny personal communication) or only Tir (as T3SS functionality marker). The plasmids were introduced separately or together into the TOEA7Δ*core::*Km mutant for a time course infection study (see Materials and Methods; Section 2.3.1). The control strains, EPEC and T3SS mutant, reproduced the previously obtained Tir, pAktSer473 and actin signals (Figure 18A versus Figure 17) supporting assay reproducibility. Examining samples from TOEA7Δ*core::*Km mutant infected cells revealed, as expected, no Tir band unless the strain carried the Tir-encoding plasmid (Figure 18B). Introducing both the Tir and CesT-encoding plasmids led to the detection of unmodified and T'' modified Tir forms (Figure 18C) revealing the TOEA7Δ*core::*Km mutant has a functional T3SS (Figure 18C). Probing for pAktSer473 illustrated the TOEA7Δ*core::*Km mutant strain carrying none or only one plasmid retained a sustain pAkt signals over the 120 minute infection period

i.e. behaved like the T3SS mutant (Figure 18A-C). By contrast, the TOEA7Δ*core::*Km strain carrying both plasmids was linked to loss of pAktSer473 signal at the final (90 and 120 minute) time points (Figure 18C). These data support the idea (Amin, 2017) that EPEC's T3SS-dependent ability to interfere with pAktSer473 signalling depends on it expressing the CesT chaperone. However, data preliminary data indicated a role for Tir with CesT (see Supplementary Figure 1) despite previous studies ruling out a need for Tir in pAktSer473 signal loss (Amin, 2017). A role for Tir may only be apparent in specific genetic backgrounds.

To support a role for CesT and/or Tir, studies were also performed with a plasmid (pACYC-3'gto) carrying both *tir* and *cesT* genes in their natural context i.e., adjacent to each other, no tags and same copy number. Consistent with previous work (Amin, 2017), this revealed a plasmid associated increase in pAktSer473 loss supporting a role for CesT and/or Tir. Additional studies to confirm this possibility were not done due to other work identifying the responsible effectors (see below).

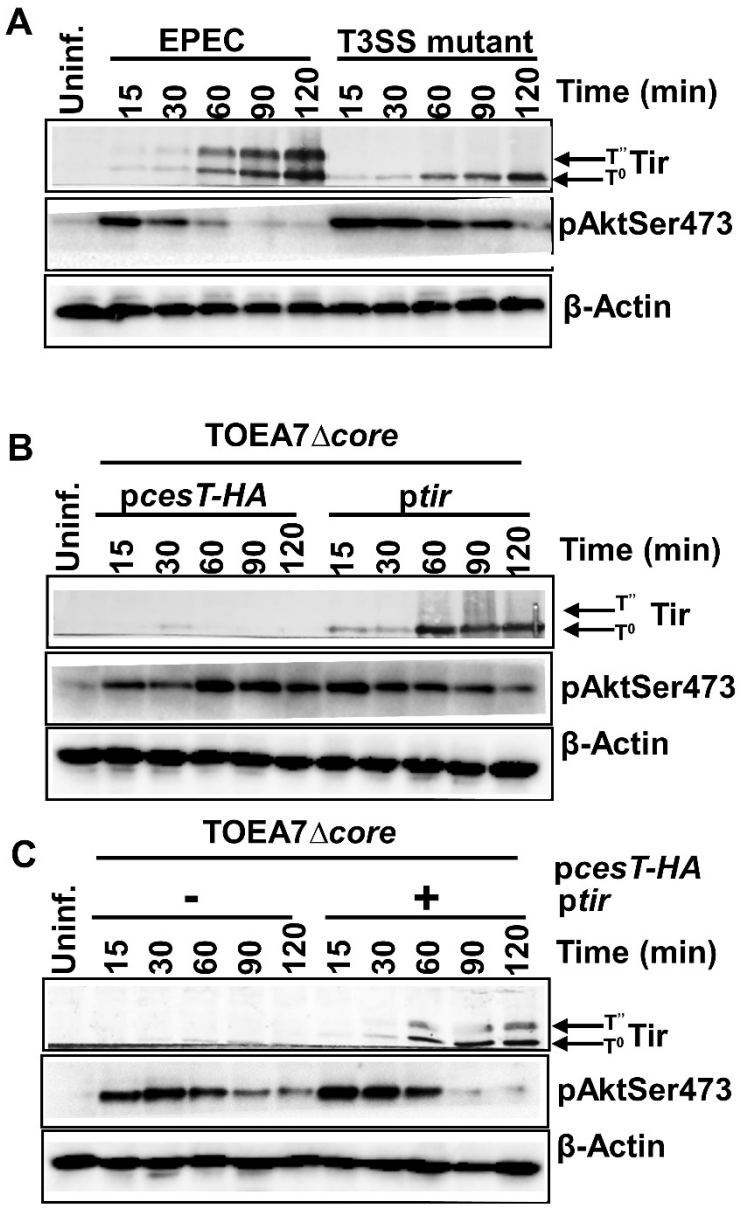


Figure 18 CesT and Tir-dependent loss of pAktSer473 signal.

J774A.1 monolayer were left uninfected or were infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS (*cfm-14*) and TOEA7Δ*core* (lacks 14 Nle and 6 LEE proteins [3 effectors, CesT & CesT chaperones and Intimin surface protein]) mutants. The TOEA7Δ*core* mutant carried no (-) plasmid or indicated plasmids (+) encoding CesT (p*cesT*-HA) or Tir (p*tir*). Roles for the plasmid-encoded factors are supported by an additional infection study (see supplementary figure 1).

3.2.3 T3SS-dependent pAktSer473 signal loss is a shared feature of other A/E pathogens

A second strategy to identify effectors inhibiting Akt signalling was to determine if other A/E pathogens - whose genome sequence and thus effector repertoire is known - behaved like EPEC E2348/69. Hence infection studies were carried out with genome sequenced strains available in the Kenny group i.e. another prototypic EPEC strain, B171-8 (O111: NM), rabbit EPEC (RDEC-1 O15:KH), enterohaemorrhagic *E. coli* (EHEC O157:H7 EDL933; variant lacking genes encoding the Shiga-like toxin) and *Citrobacter rodentium* (Cantey and Blake, 1977; Schauer and Falkow, 1993; Puente *et al.*, 1996; Mundy *et al.*, 2003; Dziva *et al.*, 2004; Marchès *et al.*, 2008). This work included isogenic T3SS mutants for each strain except B171-8, latter not available within the lab.

J774A.1 infection time course experiment were carried out, as before (with pre-activated strains, MOI 200:1) and samples isolated over a 120 min period for Western blot analyses. Probing samples from the EPEC B171-8 infected cells for pAktSer473 revealed a rapid loss of infection induced signal unlike the negative control i.e., EPEC E2348/69 T3SS mutant (Figure 19A). Probing with antibodies against Tir from EPEC E2348/69, unsurprisingly, detected the B171-8 Tir variant (83% identical) revealing both unmodified and kinase-modified forms thereby illustrating EPEC B171-8 has a functional T3SS. The antibodies also detected Tir in *Citrobacter* and RDEC-1 with the kinase-modified form in extracts from cells infected with the parental but not T3SS mutant strains (Figure 19B & C) supporting strain genotype. The obtained pAktSer473 and actin signals are consistent with both *Citrobacter* and RDEC-1 having a T3SS-dependent mechanism that interferes with Akt signalling (Figure 19B & C). While EHEC infection was linked to pAktSer473 signal loss there was a similar, though less dramatic decrease in cells infected with the Δ*escN* (T3SS-deficient) variant (Figure 19D) suggestive of an additional T3SS-independent component. As the anti-Tir antibodies do not detect Tir from EHEC O157 (Kenny, 1999) there is no support for the *ΔescN* mutant genotype i.e., lacks a functional T3SS. Similar findings were obtained in an additional time course infection with EHEC and *ΔescN* mutant strains. Importantly strain genotype was supported by microscopy studies on infected HeLa cells

(Figure 20) as this revealed EHEC, but not the *ΔescN* mutant could nucleate actin nucleation – a process dependent on Tir delivery into host cells.

Collectively, these studies suggested that the examined A/E pathogen strains may all encode a common effector (or effectors) that can inhibit Akt signalling though EHEC may encoded T3SS-dependent and -independent inhibitory mechanisms. Previous work had ruled out roles for all EPEC LEE effectors and most non-LEE-encoded effectors (Amin, 2017). Of the remaining known EPEC T3SS substrates - EspC, NleJ, LifA, LifA-like protein - the examined A/E pathogens (Dautin, 2010) encode EspC and LifA homologues (Vijayakumar *et al.*, 2014; Bease, 2020). However, EspC was not needed for EPEC to inhibit Akt signalling (O. Amin; unpublished) suggestive of possible roles for LifA-homologues in the different strains.

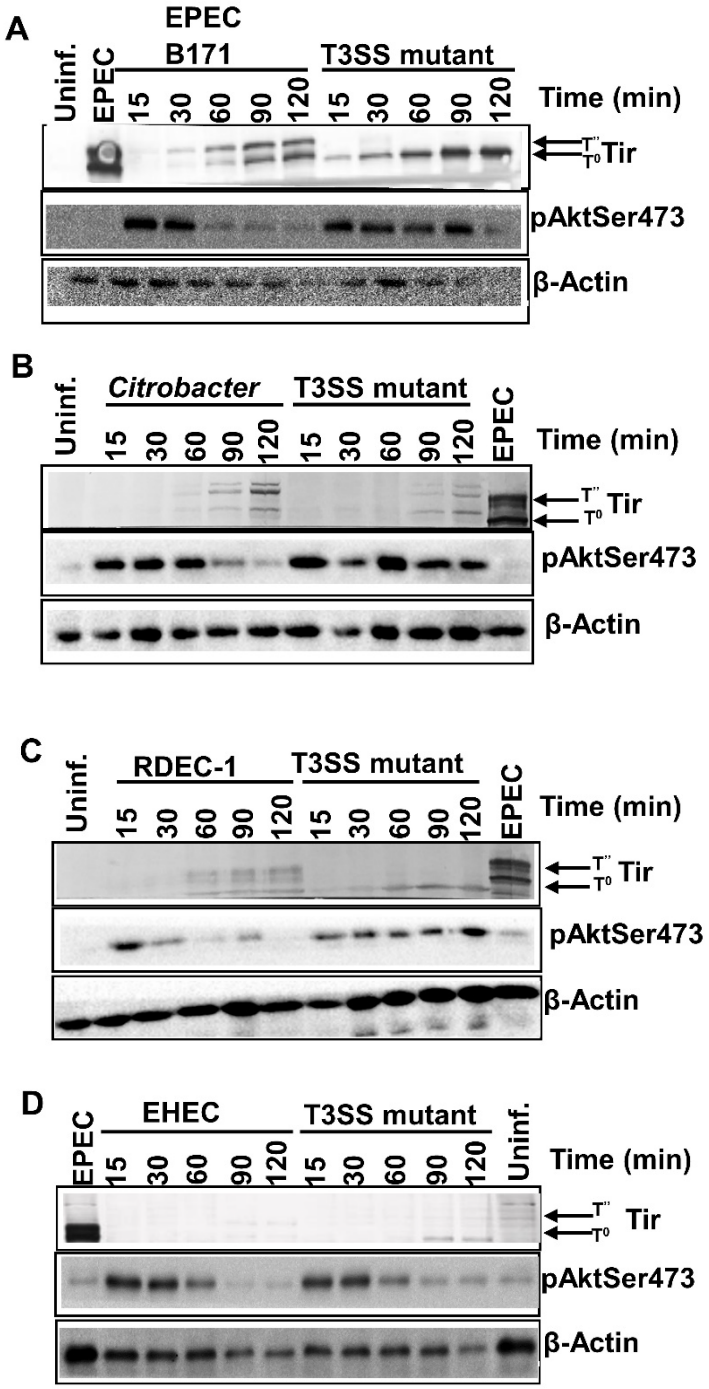


Figure 19 Other A/E pathogens inhibit Akt signalling.

J774A.1 cell were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') EPEC Tir forms. Strains used were EPEC B171-8, *Citrobacter rodentium* (*Citrobacter*), rabbit EPEC O15:KH (RDEC-1) and enterohaemorrhagic *E. coli* O157:H7 EDL993 (but lacks genes encoding Shiga-like toxins; EHEC) and T3SS-deficient variant of each strain except EPEC B171 (used EPEC E2348/69 T3SS mutant). These data are supported by an additional time-course infection study (see Supplementary Figure 2).

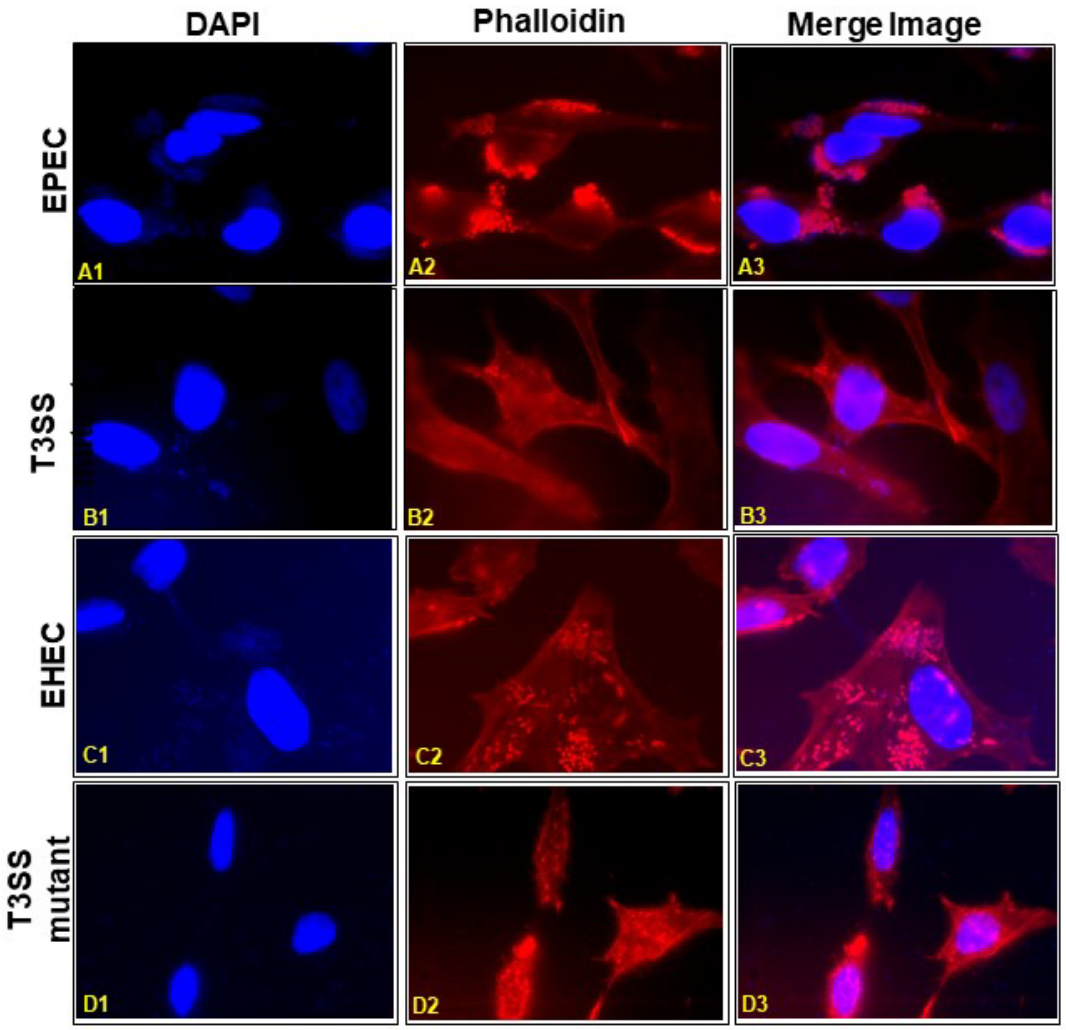


Figure 20 The EHEC ΔescN mutants lacks a functional T3SS

HeLa cells were infected with indicated EPEC and EHEC strains for 3 hours. The infection was stopped by washing away non-adherent bacteria and fixing cells in a 1% paraformaldehyde solution. The cells were incubated with detergent (to permeabilise the host membrane) that also contained fluorescently labelled phalloidin (binds polymerised actin; Red) and DAPI (stains host and bacterial DNA; Blue). The fluorescent signals were detecting by florescence microscopy. A1-D1: DAPI signal; A2-D2: Polymerised actin; A3-D3: Both (merged) signals. Strains used were EPEC E2348/69, EHEC O157:H7 (EDL933) and their T3SS deficient mutant strains (*cfm-14* and *ΔescN* respectively). These data suggests that EHEC but not its T3SS-deficient variant can trigger actin polymerisation events.

3.2.4 EPEC does not require LifA to inhibit Akt signalling

A third strategy to identify the effector/s responsible for inhibiting Akt signalling was to assess the role of the four unexamined effectors, EspC, NleJ, LifA and LifA-like protein. As mentioned, studies with an available Δ*espC* mutant argued against EspC having a key role (O. Amin; unpublished). Strains lacking *nleJ* or *lifA*-like genes were not available. Thus, initial studies focused on LifA, as a suicide vector was available (Klapproth *et al.*, 2000) to disrupt the gene by replacing an internal fragment (1980bp of 9669bp) with a kanamycin resistance-encoding gene (Klapproth *et al.*, 2000). This suicide vector was used (See Material and Methods; Table 5) to generate an EPEC E2348/69 *lifA::*Km mutant with the gene disruption event supported by PCR analysis (Figure 21A). The *lifA*::Km mutant had no obvious defect in binding HeLa cells and inducing Tir-dependent actin nucleation events as reported (Klapproth *et al.*, 2000)(not shown).

A time course infection study with J774.A1 macrophages revealed similar Tir, pAktSer473 and actin signal in samples obtained from cells infected with EPEC or the *lifA*::Km mutant (Figure 21B). This finding argues against a critical role for LifA in the inhibitory process. However, the *lifA*::Km gene could, in theory, still produce the first ~1900 residues. The N-terminus of T3SS effectors carries the export signal and, for CesT substrates, CesT chaperone binding site (Little and Coombes, 2018). Thus, this large polypeptide could be delivered into cells where it might interfere with Akt signalling.

3.2.5 An integrative element (IE5) and two prophages (PP2, PP4) are not needed for EPEC to inhibit Akt signalling.

All known EPEC effectors are encoded on horizontally acquired DNA regions (Iguchi *et al.*, 2009) i.e., prophages (PP) and integrative elements (IE). Thus, another strategy to identity responsible effector/s that inhibit Akt signalling was to screen available mutants lacking entire PP and/or IE elements to determine if the regions (4->60kB) encode needed factor/s. The entire PP2, PP4 and IE5 regions had previously being deleted - individually and in all combinations (S. Quitard; unpublished). Previous studies ruled out key roles for effectors encoded on these

regions i.e., NleC, NleD, NleG, NleH1, NleH2, EspJ, EspG2, EspC (Amin, 2017) so the undertaken time-course infection assays were examining roles for other PP/IE genes. Probing samples showed all examined strains, except the T3SS mutant, delivered Tir into the macrophages linked to loss of pAktSer473 signal i.e., they behaved like EPEC (Figure 22). These data discounted a critical role for all IE5, PP2 and PP4 genes for EPEC to inhibit Akt signalling.

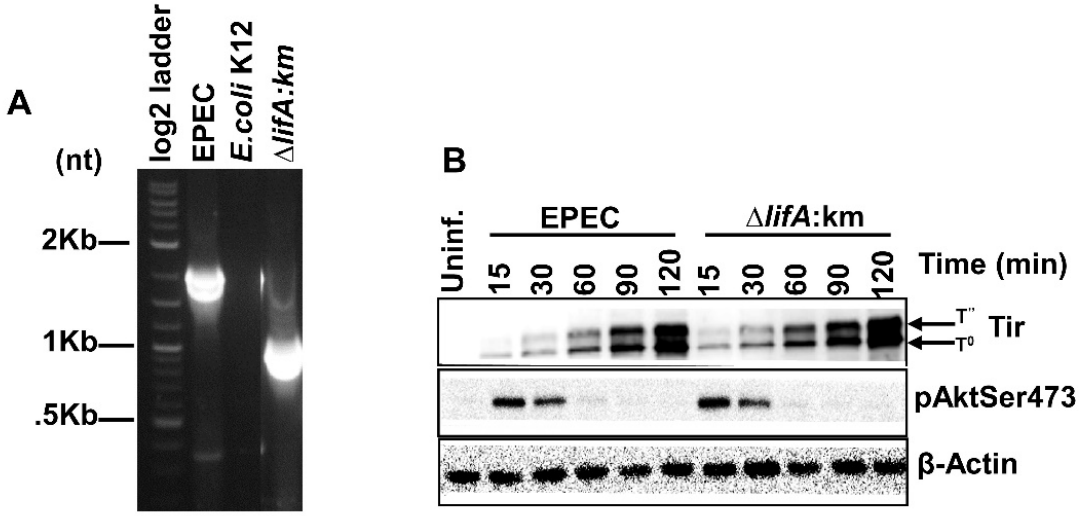


Figure 21 An intact lifA gene is not required for EPEC to inhibit Akt signalling

A) PCR support for generation of an EPEC E2348/69 Δ*lifA*::Km mutant using *lifA* specific oligonucleotides flanking the region where a ~1900bp internal fragment was replaced by a smaller DNA fragment which encodes kanamycin resistance. The PCR products were resolved on a 0.7% agarose gel and visualised (gel red stain) alongside indicated molecular mass markers. B) J774A.1 cells were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') Tir forms. Strain used were EPEC, *LifA*-deficient (Δ*LifA*::Km) mutant and, for PCR studies (in A) non-pathogenic K12 *E.coli* (*E. coli* K12).

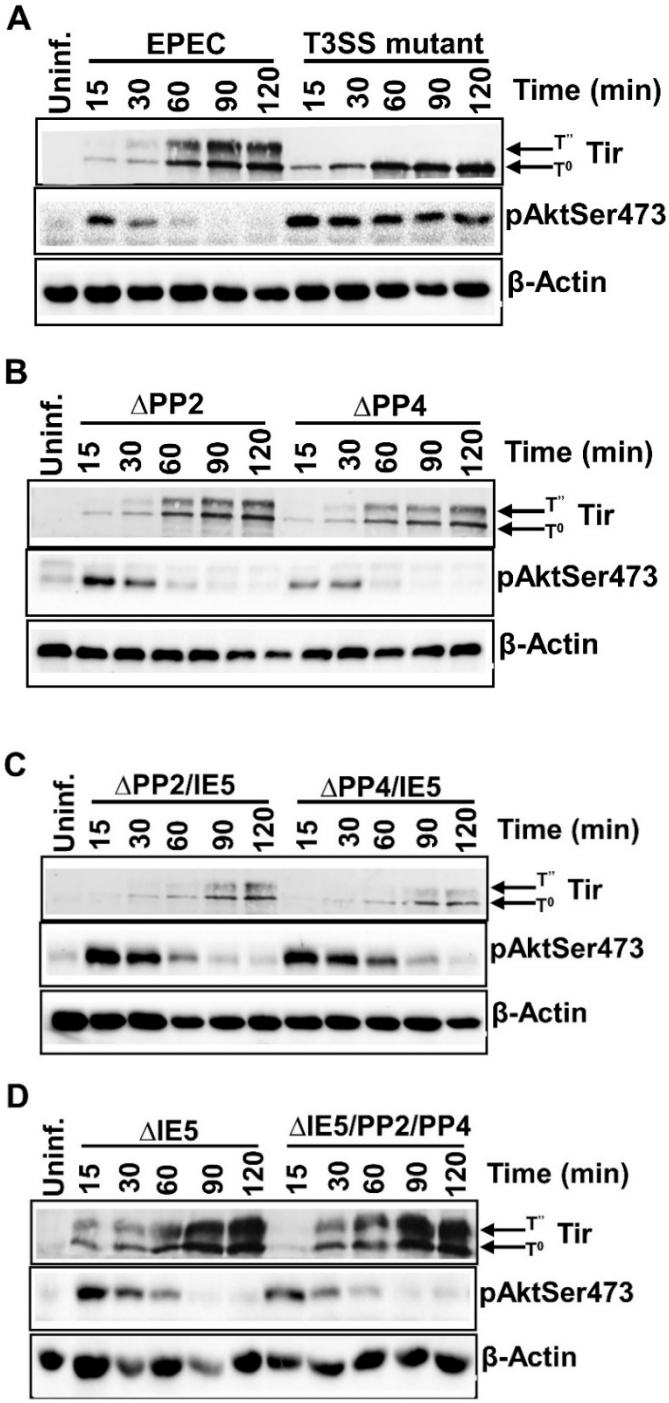


Figure 22 IE5, PP2 and PP4 regions are not needed for EPEC to inhibit Akt signalling

J774A.1 cell were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS mutant and strains lacking the entire IE5 (ΔIE5), PP2 (ΔPP2) or PP4 (ΔPP4) DNA regions in indicated combinations i.e., single, double and/or triple mutants.

3.2.6 Linking IE2-encoded factors with inhibition of Akt signalling

The approach of screening strains lacking PP and/or IE regions was greatly aided by a publication describing a bank of EPEC E2348/69 mutants lacking individual PP or IE regions (Litvak *et al.*, 2017). The bank of 16 mutants were initially screened at just two infection time point (60 and 120 min) to rapidly determine if any had obvious defects in inhibiting Akt signalling.

Western blot analysis of isolated samples for pAktSet473 (Figure 23A) revealed prominent defects for ΔIE2 (lacks ~61kb; includes genes for NleE2 and the LifA-like protein) and ΔPP10 mutants (encodes no known effectors). Less prominent defects were evident for the ΔPP13, ΔIE5 and ΔIE6 mutants but the interpretation was questioned by the high pAktSer473 signal background in control uninfected cells (Figure 23B). Of note previous work found no role for IE5-encoded factors (Figure 23). Probing for Tir revealed the ΔPP10 mutant had an effector delivery defect (Figure 23B) and thus was excluded from further studies. To support the findings, the mutants of most interest were re-examined in a time course study. This work revealed three mutants (ΔPP13, ΔIE5, ΔIE6) behaved like EPEC i.e., showing similar Tir and pAktSer473 signal profiles though a delay in pAktSer473 signal loss was evident (Figure 24). By contrast, the ΔIE2 mutant behaved like the T3SS mutant despite its ability to delivery Tir (Figure 24B). PCR analyses supported strain genotype as showed ΔIE2 lacks *nleE2*, ΔIE6 lacks *nleE1* and ΔIE5, like EPEC, has both *nleE1* and *nleE2* genes (Figure 25). This work is suggestive of an important role for one or more IE2-encoded factors with no key need for factors encoded on the other 15 examined PP and IE elements. The IE2 region encodes 2 known T3SS substrates - NleE2 and LifA-like protein - but previous work ruled out a key role for NleE2 in the inhibitory process (Amin, 2017). Thus, the findings highlight a key role for the LifA-like protein or other IE2-encoded protein/s.

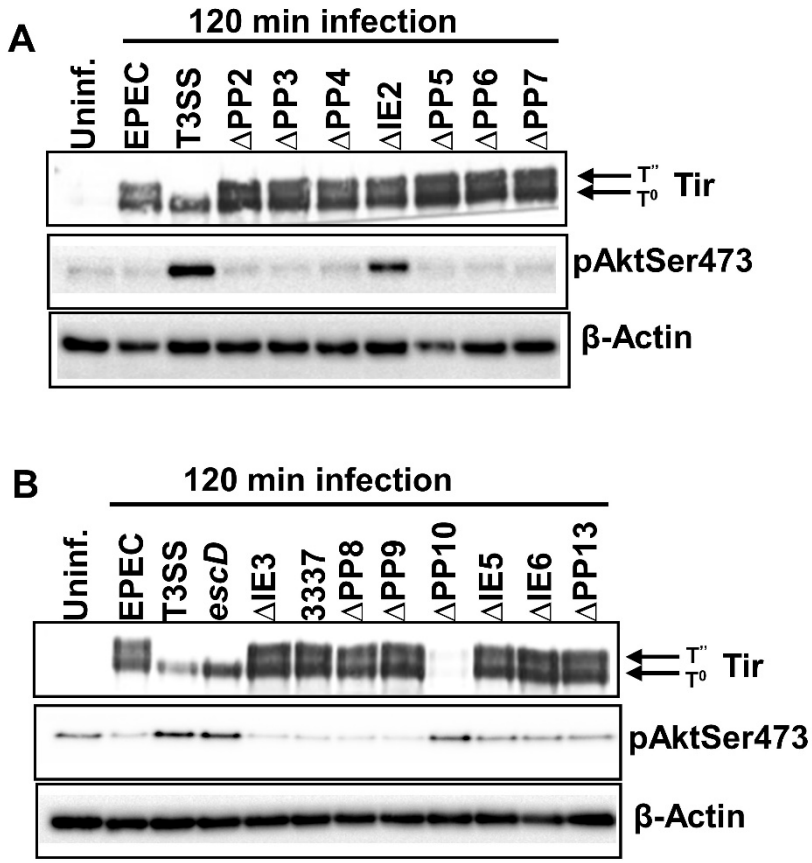


Figure 23 Screens indicate roles for IE and PP factors in EPEC mechanism to inhibit Akt signalling

J774A.1 macrophages were left uninfected or infected (MOI 200:1) with indicated pre-activated strains for 60 (not shown) and 120 minutes before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC E2348/69, T3SS mutants (*cfm14* and *escD*) and E2348/69 strains lacking indicated prophage (PP) or integrative element (IE) regions (Litvak *et al.*, 2017).

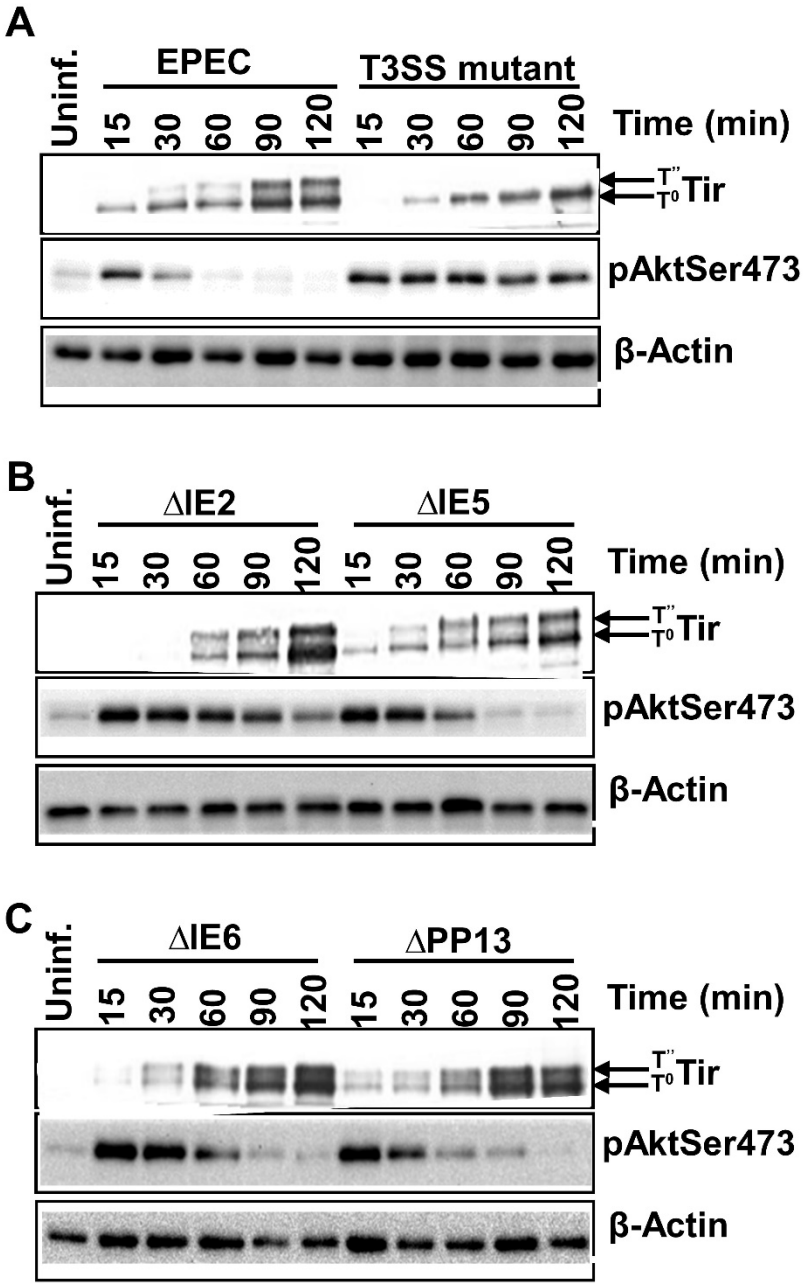


Figure 24 Major contribution of IE2-encoded factor for EPEC to inhibit Akt signalling.

J774A.1 macrophage were left uninfected or infected (MOI 200:1) with indicated pre-activated strains for 90 (not shown) and 120 minutes before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC and mutants lacking a functional T3SS (T33S) or horizontally-acquired IE2 (ΔIE2), IE5 (ΔIE5), IE6 (ΔIE6) or PP13 (ΔPP13) regions (Litvak *et al.*, 2017). The findings are supported by an additional infection experiment (see Supplementary Figure 3).

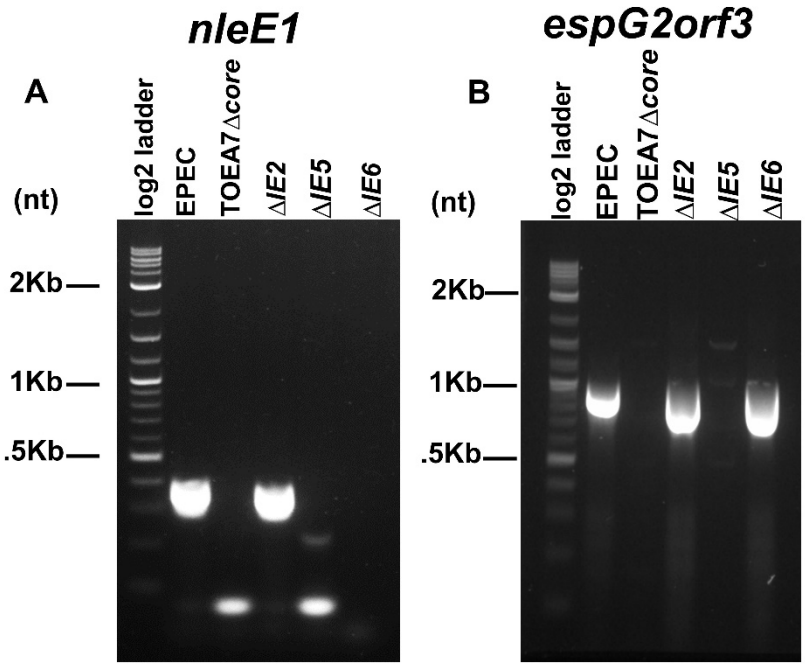


Figure 25 PCR support for use of ΔIE2 and ΔIE6 mutants.

PCR analysis of indicated strains with oligonucleotides specific for a gene on IE6 (*espG2*/*orf3*) and IE2 (*nleE2*). The PCR products were resolved on a 0.7% agarose gel and visualised (gel red stain) alongside indicated molecular mass markers. Strains examined were EPEC, TOEA7Δ*core* mutant (lacks 14 *nle* effector genes including *nleE1* and *nleE2*), ΔIE2 (lack IE2 region and thus *nleE2*), ΔIE6 (lack IE6 region and thus *nleE1*) and ΔIE5 (lack IE5 region and has both *nleE* genes.

3.2.7 Key role for IE2-encoded factor(s)

To determine if the LifA-like effector was required for EPEC to inhibit Akt signalling studies took advantage of a publication describing studies with EPEC E2348/69 mutants lacking all known EPEC Nle effectors including LifA-like protein (Cepeda-Molero *et al.*, 2017). In the published study, the effectors (and pseudo-effector encoding) genes were deleted in a sequential manner (see Table 9); firstly 4 LEE effectors (Map, EspF, EspG, EspH) generating EPEC 9, then those on IE5 (EPEC8), followed by those on IE6 (EPEC7), then IE2 (EPEC6), then PP2 (EPEC5), then PP3 (EPEC4), then PP4 (EPEC3) and finally PP6 (EPEC2). The EPEC2LEE is same as EPEC2 but with the 4 LEE effector genes (EspB, EspZ and Tir) added back.

Initial infection studies focused on the strains linked to the loss of both *lifA*-like genes. Hence, time course studies were carried out with EPEC8, EPEC7 (lacks *LifA*), EPEC6 (lacks *LifA* and *LifA*-like) and, as a further control, EPEC5 (lacks *LifA*, *LifA*-like and additional *nle* effector genes). Probing the samples isolated from infected J774A.1 cell revealed all strains, except the T3SS mutant, delivered Tir as evidenced by detecting the kinase modified (T'') form (Figure 26). Importantly, probing for pAktSer473 revealed similar profiles for EPEC, EPEC8 and EPEC7 strains while EPEC6 and EPEC5 behaved like the T3SS mutant (Figure 26). Probing for actin showed that pAktSer473 signal loss was not due to gel loading issues (Figure 26). PCR studies supported strain genotype by revealing the expected presence or absence of *lifA* and/or *lifA*-like genes (Figure 27). Strain genotype was further supported by confirming absence of an effector gene specific to each PP or IE region (Figure 28). Previous work indicated that EPEC inhibition of Akt signalling does not require i) an intact *lifA* gene (Figure 21), ii) the IE6 region (carries *lifA* gene; Figure 27) or iii) 13 examined Nle effector encoding genes (Amin, 2017). Thus, our findings suggest that the LifA-like protein may be key for EPEC to inhibit Akt signalling.

Pathogenicity Island (PAI)	Gene	EPEC WT	EPEC 8	EPEC 7	EPEC 6	EPEC 5	EPEC 4	EPEC 3	EPEC 2	EPEC 2 LEE
IE5	*espG 2/ orf3*	+	-	-	-	-	-	-	-	-
	espC	+	-	-	-	-	-	-	-	-
IE6	*nleE1*	+	+	-	-	-	-	-	-	-
	nleB1	+	+	-	-	-	-	-	-	-
	espL	+	+	-	-	-	-	-	-	-
	efa-1/ lifA	+	+	-	-	-	-	-	-	-
IE2	*nleE2*	+	+	+	-	-	-	-	-	-
	*nleB**	+	+	+	-	-	-	-	-	-
	*espL**	+	+	+	-	-	-	-	-	-
	efa-1/ lifA-like	+	+	+	-	-	-	-	-	-
PP2	*nleH1*	+	+	+	+	-	-	-	-	-
	Cif	+	+	+	+	-	-	-	-	-
	espJ	+	+	+	+	-	-	-	-	-
PP3	*nleJ*	+	+	+	+	+	-	-	-	-
PP4	*nleG*	+	+	+	+	+	+	-	-	-
	nleB	+	+	+	+	+	+	-	-	-
	nleC	+	+	+	+	+	+	-	-	-
	nleD	+	+	+	+	+	+	-	-	-
	nleH	+	+	+	+	+	+	-	-	-
PP6	*nleA*	+	+	+	+	+	+	+	-	-
	nleF	+	+	+	+	+	+	+	-	-
	nleH2	+	+	+	+	+	+	+	-	-
	*espO**	+	+	+	+	+	+	+	-	-
LEE	*espF*	+	-	-	-	-	-	-	-	+
	espG	+	-	-	-	-	-	-	-	+

Table 9. Genotype of effector deficient EPEC strains

The seven-known effector-encoding pathogenicity islands (PAI) are listed alongside the PAI-specific genes probed for in PCR analyses. The expected presence (+)/ absence (-) of the PAI specific effector gene in each indicated EPEC strain is given. EPEC2LEE is same as EPEC2 except the four deleted LEE genes have been reintroduced (Cepeda-Molero *et al.*, 2017). Pseudogenes are indicated by * and includes *cif* on PP2.

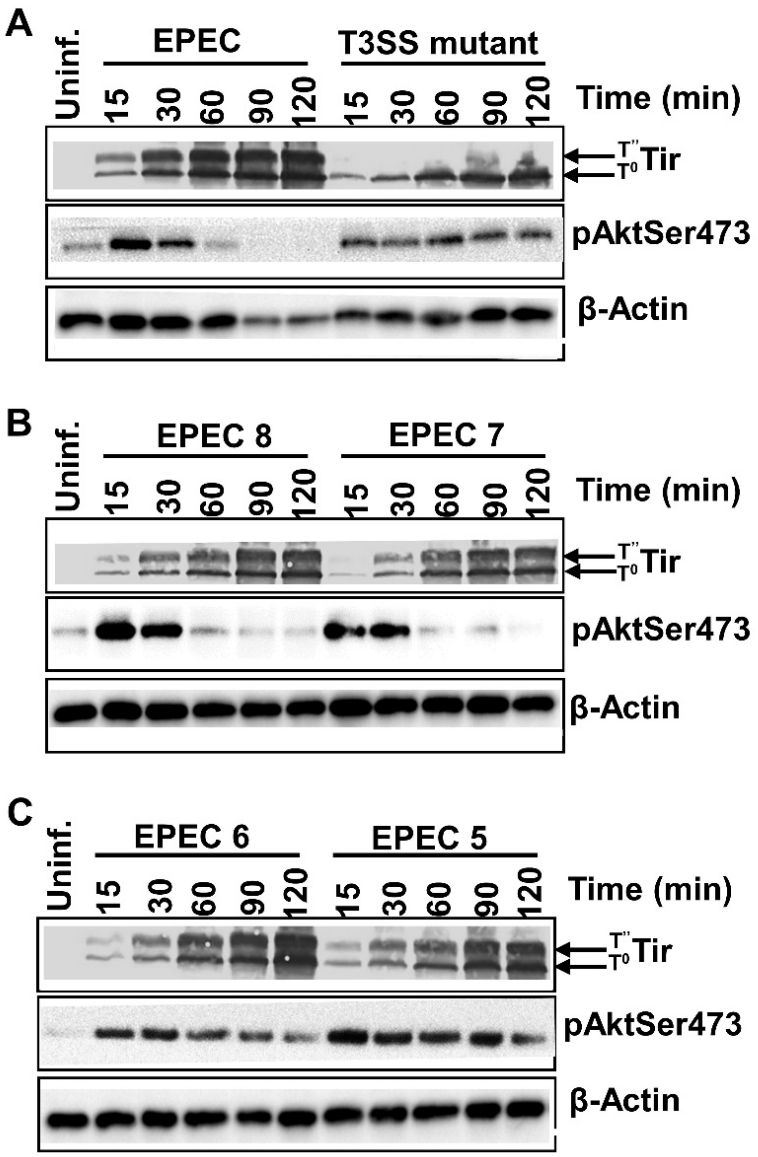


Figure 26 EPEC6 multi-effector deficient strain cannot inhibit Akt signalling

J774A.1 macrophage were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS), EPEC8 (missing *map*, *espF*, *espH*, *espG*, *espG2*, *espC*); EPEC7 (as EPEC8 but also missing *espL, nleB1, nleE1, lifA*); EPEC6 (as EPEC7 but also missing *nleE2* and *lifA*-like); EPEC5 (as EPEC6 but also missing *nleH1* and *espJ*). The findings are supported by an additional infection experiment (see Supplementary Figure 4).

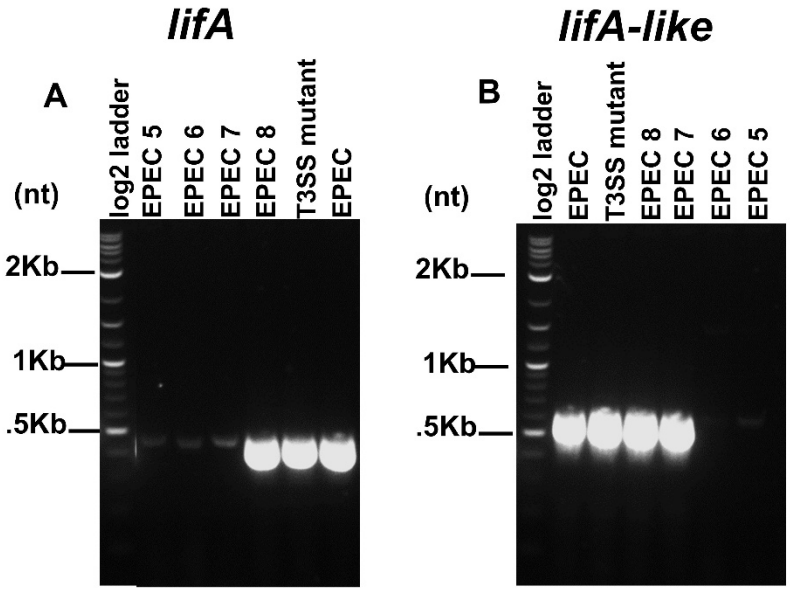


Figure 27 Confirming presence/absence of lifA genes in EPEC mutants

PCR analysis of indicated strains using oligonucleotides specific for internal (small) region of *lifA* and *lifA*-like genes. The PCR products were resolved on 0.7% agarose gels and visualised (gel red stain) alongside indicated molecular mass markers. Strains used were EPEC, a T3SS mutant (*escN*), EPEC8 (missing *map*, *espF*, *espH*, *espG*, *espG2*, *espC*); EPEC7 (as EPEC8 but also missing *espL, nleB1, nleE1, lifA*); EPEC6 (as EPEC7 but also missing *nleE2* and *lifA*-like) and EPEC5 (as EPEC6 but also missing *nleH1, espJ*).

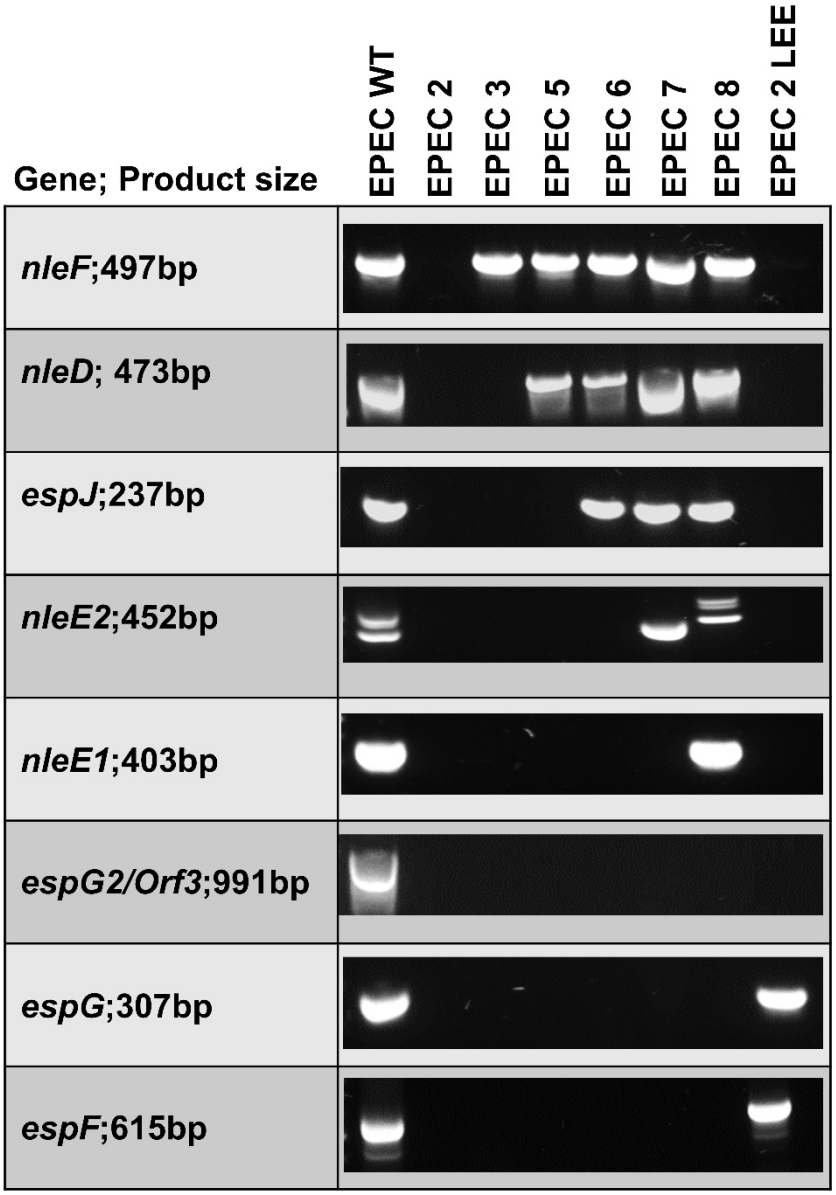


Figure 28 PCR support for genotype of effector-deficient mutant strains

PCR analysis of indicated strains using oligonucleotides specific for an effector gene specific to each PP and IE (see Table 2). The PCR products were resolved on 0.7% agarose gels and visualised (gel red stain) alongside indicated molecular mass markers. Strains used were EPEC, EPEC8 (missing *map*, *espF*, *espH*, *espG*, *espG2*, *espC*); EPEC7 (as EPEC8 but also missing *espL, nleB1, nleE1, lifA*); EPEC6 (as EPEC7 but also missing *nleE2* and *lifA*-like); EPEC5 (as EPEC6 but also missing *nleH1, espJ*); EPEC3 (as EPEC5 but lacks *nleJ* [as per EPEC4] and *nleG*, *nleB*, *nleC*, *nleD*), EPEC2 (as EPEC3 but also lacks *nleA*, *nleH2*, *nleF*) and EPEC2LEE (as EPEC2 but has *map*, *espF*, *espG* and *espH* genes reintroduced (Cepeda-Molero *et al.*, 2017).

3.2.8 Redundant roles for LifA and LifA-like effectors

To explore the importance of the LifA-like protein in the inhibitory process, time course studies were undertaken with available strains (Cepeda-Molero *et al.*, 2017) lacking only *lifA*, only *lifA*-like or both *lifA* genes. Probing for Tir revealed all but the T3SS mutant strain had a functional T3SS (Figure 29). An apparent delay in Tir delivery, by ~30 minutes, was found for each l*ifA* gene-deficient strains (Figure 29) though this was inconsistent (see for example Supplementary Figure 5&6). Probing for pAktSer473 unexpectedly showed both single mutants behaved like the EPEC with a T3SS mutant-like profile for the double mutant (Figure 29). Probing for actin uncoupled the pAktSer473 signal loss from gel loading issues (Figure 29) with strain genotype supported by PCR analysis of bacteria collected at the end of infection period (Figure 30). This work suggested there are key, but redundant roles for both LifA (encoded on IE6) and LifA-like (encoded on IE2) proteins in EPEC's ability to inhibit Akt signalling.

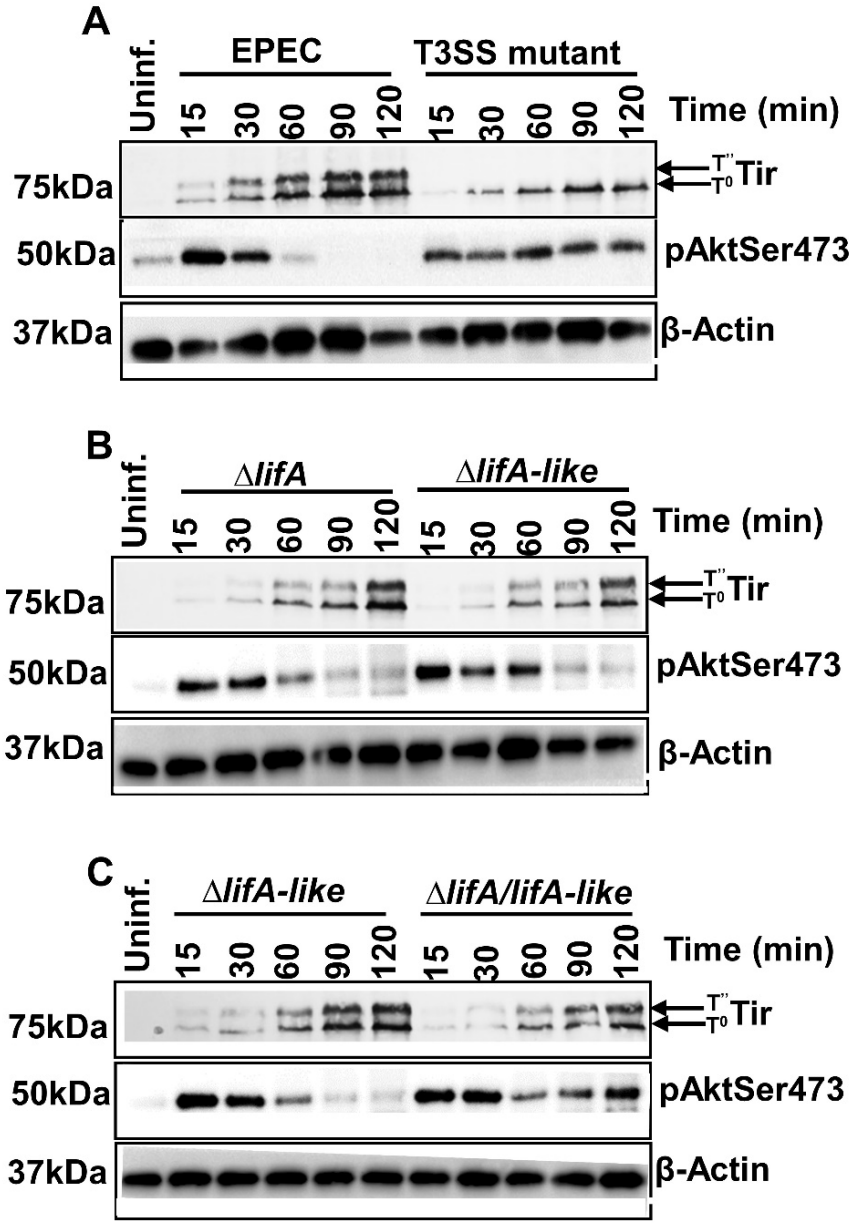


Figure 29 Key but redundant role for LifA homologues in loss of pAktSer473 signal

J774A.1 cell were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS) and strains lacking *lifA* (Δ*lifA*), *lifA*-like (Δ*lifA*-like) or both (Δ*lifA*/*lifA*-like) proteins. These data was supported by an additional experiment (see for example Supplementary Figure 5&6 and Chapter 5 data).

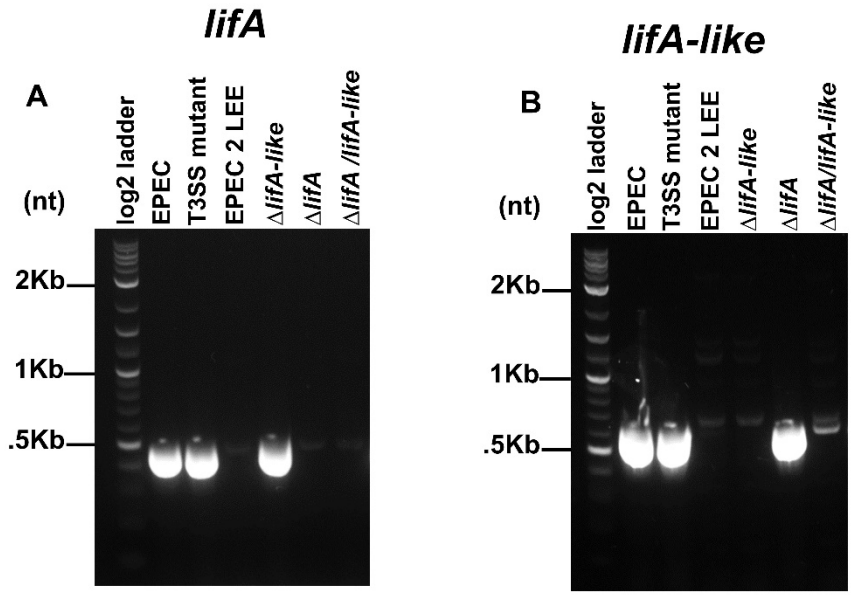


Figure 30 PCR support genotype of lifA single and double mutants.

PCR with indicated strains using oligonucleotides specific for a (small) internal fragment of *lifA* (on IE6) and *lifA*-like (on IE2) genes. PCR products were resolved on a 0.7% agarose gel and visualised (gel red stain) alongside indicated molecular mass markers. Strains examined were EPEC, *escN* (lack functional T3SS), EPEC2LEE (lacks all known non-LEE effectors), Δ*lifA* (lacks *lifA*), Δ*lifA*-like (lacks *lifA*-like protein) and Δ*lifA*/*lifA*-like (lacks *lifA* & *lifA*-like proteins) mutants.

3.2.9 The *lifA*::Km gene product does not inhibit Akt signalling

LifA and LifA-like protein redundancy could explain why the *lifA*::Km mutant behaved like EPEC in the pAktSer473 assay (Figure 21). As mentioned, the *lifA*::Km mutant could theoretically express a large N-terminal domain for T3SS delivery into cells to interfere with Akt signalling. To determine if the *lifA*::Km gene product can inhibit Akt signalling the *lifA*::Km suicide vector was used to disrupt *lifA* in the EPEC Δ*lifA*-like mutant (Cepeda-Molero *et al.*, 2017) to generate a Δ*lifA*-like*/lifA:*Km double mutant (See Material and Methods). This new strain was used in a standard time-course infection study with J774A.1 macrophage. Examining the anti-Tir signals revealed all strains, except the T3SS mutant, had a functional T3SS with all *lifA*-deficient mutants linked to similar or even greater levels, of Tir delivery compared to EPEC (Figure 31). As expected, the pAktSer473 profiles from macrophages infected with the *lifA*-like single mutants were similar to those from EPEC infected cells (Figure 31). However, cells infected with the Δ*lifA/lifA*-like (Cepeda-Molero *et al.*, 2017) and, newly-generated, Δ*lifA*-like*/lifA*::Km double mutants had sustained pAktSer473 signals like the T3SS mutant (Figure 31). Strain genotype was supported by PCR analysis of bacteria collected at the end of infection period (Figure 32). This finding is consistent with redundant roles for both LifA homologues in the inhibitory process and reveals that the interrupted *lifA*-like gene product does inhibits Akt signalling; latter could be due to not being delivery or lacking features needed to interfere with Akt signalling.

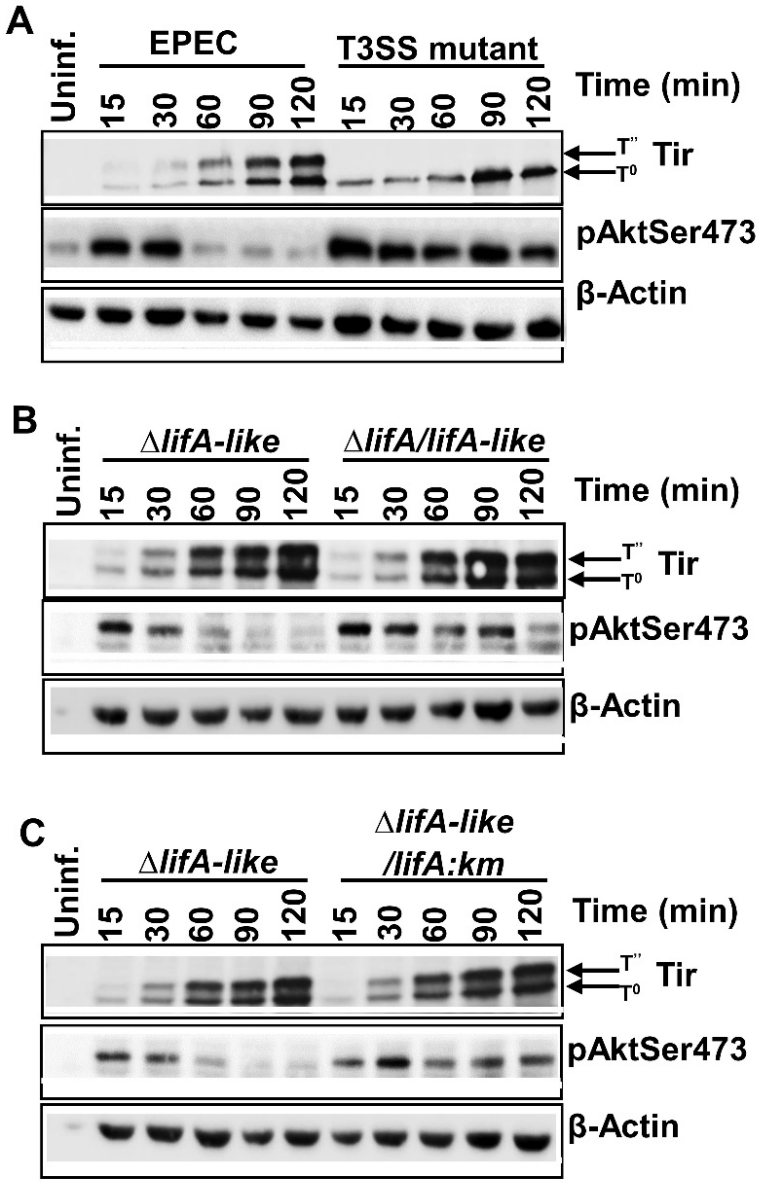


Figure 31 Non-functionality of lifA-Km disrupted gene supports redundancy hypothesis.

J774A.1 cells were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS) and strains lacking *lifA* (Δ*lifA*), *lifA*-like protein (Δ*lifA*-like) or both proteins i.e. Δ*lifA*/*lifA*-like and, newly-generated, Δ*lifA*-like/*lifA*::Km mutants. Data is from a single experiment, Use of the Δ*lifA*-like/*lifA*::Km mutant was supported by growth in presence of kanamycin.

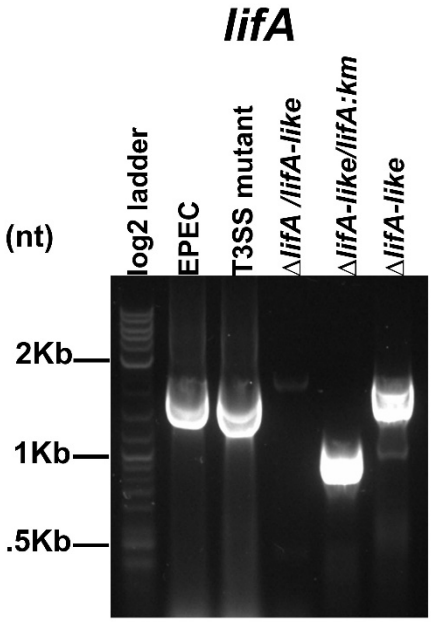


Figure 32 PCR support genotype of lifA-like single and *lifA*-like/*lifA*:*km* mutants

PCR with indicated strains using oligonucleotides specific for a (large) internal fragment of *lifA* (on IE6) and *lifA*-like (on IE2) genes. PCR products were resolved on a 0.7% agarose gel and visualised (gel red stain) alongside indicated molecular mass markers. Strains examined were EPEC, *escN* (lack functional T3SS), EPEC2LEE (lacks all known non-LEE effectors), Δ*lifA* (lacks *lifA*), Δ*lifA*-like (lacks *lifA*-like protein) and Δ*lifA*-like/*lifA*:Km (lacks *lifA*-like & *lifA*:km proteins) mutants.

3.3 Discussion

This study achieved the aim of identifying the responsible effector(s) needed for EPEC to inhibit Akt signalling by clearly showing key, but redundant roles for both LifA homologues. This finding explains why the previous search did not identify the responsible effector (Amin, 2017) as it did not examined roles for the LifA homologues.

Homology to known bacterial effectors is a typical method for identifying putative effectors (Tobe *et al.*, 2006). This approach on other genome sequenced A/E pathogens revealed each strain has 7 LEE effectors with a highly overlapping, but distinct, number of Nle effector-encoding genes: 20 in another prototypic EPEC strain (B171), 16 in rabbit EPEC (RDEC-1), 22 in *Citrobacter rodentium* and 44 in EHEC O157:H7 (Zhu *et al.*, 2001; Ogura *et al.*, 2008; Iguchi *et al.*, 2009; Petty *et al.*, 2010). My studies with these strains revealed T3SS dependent inhibition of Akt signalling by all these A/E strains with, possibly, an additional T3SS-independent inhibitory mechanism for EHEC O157:H7. A share T3SS-dependent inhibitory activity suggested the strains might have a common responsible effector. Previous studies argued against roles for all but 4 known EPEC E2348/69 T3SS substrates with the other examined A/E pathogens encoding homologues for EspC and LifA proteins (Deng *et al.*, 2012; Vijayakumar *et al.*, 2014). A need for EspC was not found for the EPEC inhibitory process (Amin, 2017) suggesting that inhibition of Akt signalling is possibly due to LifA homologues. EPEC E2348/69 encodes LifA and LifA-like proteins (Iguchi *et al.*, 2009), EPEC B171 has two LifA homologues (Ogura *et al.*, 2008), RDEC-1 has one (Zhu *et al.*, 2001), *Citrobacter rodentium* has two (Petty *et al.*, 2010) and EHEC O157:H7 has a partial, 2-*orf*, LifA-encoding gene and a plasmid-encoded homologue, ToxB (Hayashi *et al.*, 2001; Deng *et al.*, 2012). ToxB has lymphostatin activity as first described for LifA (Stevens *et al.*, 2004). Given our findings with EPEC E2348/69 we suggest that LifA homologues in other A/E pathogens enable them to interfere with Akt signalling in a T3SS-dependent and possibly, for EHEC O157:H7, also a T3SS-independent mechanism. Future studies are needed to verify this prediction.

Little progress was made on the strategy to delete the four unexamined effectors individually and collectively with each other and all other EPEC effectors due to the

screening of PP/IE mutants identifying the responsible effector. Previous research (Amin, 2017) ruled out key roles for all but four known T3SS substrates - EspC, NleJ, LifA, LifA-like - with preliminary work discounting EspC (O. Amin, unpublished) suggesting it is mediated NleJ, the LifA homologues or, yet to be identified, effectors. As mentioned NleJ is not encoded by other A/E pathogens herein shown to inhibit Akt, making LifA homologues the best candidate to inhibit Akt signalling. Studies with an available suicide vector (Klapproth *et al.*, 2000) suggested LifA was not needed for the inhibitory process but this interpretation was questioned by the possibility the gene encodes a large N-terminal (AA) protein region which may be delivered into cells to inhibit Akt signalling. Indeed, later studies revealed LifA does inhibit Akt signalling, unlike the truncated protein encoded by the *lifA::*Km gene, with the *lifA::*Km strain inhibiting Akt signalling due to a redundant role with the LifA-like protein. These discoveries negated the need to generate suicide vectors to knock out the *nleJ*, *lifA*-like, *lifA* and *espC* genes, individually and collectively, in EPEC (or EPEC lacking all other known Nle effector genes) to interrogate their roles for inhibiting Akt signalling.

It was likely that the responsible effectors would be encoded on the horizontally acquired element (PP or IE) with the first screening approach using mutants available within the Kenny laboratory. Thus, studies examined mutant missing IE5, PP2 and PP4 (individually and in all combinations) with a plan to sequentially knock out additional PP/IE regions until find a mutant defective in the inhibitory process. This screen argued against key roles for 8 Nle effectors - encoded on IE5 (EspC/EspG), PP2 (NleH/EspJ) and PP4 (NleB/NleC/NleD/NleG/NleH) – consistent with previous studies (Amin, 2017), EspC and any gene product on these 15-54kb IE/PP regions. The need to delete other PP and/or IE regions was not required due to a timely publication reporting the existence of EPEC E2348/69 mutants lacking, individually, most IE and PP regions (Litvak *et al.*, 2017). Screening these 16 mutant strains implicated a key role for factors on IE2 (~61Kb region encoding 2 known T3SS substrates; LifA-like and NleE2) and possible role for factors on IE6 (~30Kb region encoding 4 known T3SS substrates; LifA, NleE1, NleB and EspL). As the need for EPEC to express the Nle and/or EspL effectors had already been discounted (Amin, 2017) the findings suggested possible roles for the LifA homologues or other IE-encoded factors. Of note, another mutant

(lacks PP10) could not inhibit Akt signalling but this was linked to very little Tir delivery. Future studies could examine if the ~40Kb PP10 region encodes factors that influence EPEC binding, Tir expression or delivery levels and/or T3SS functionality.

While these approaches implicated LifA proteins in the inhibitory process, demonstrating their role was possible by being able to access EPEC E2348/69 strains lacking, in a sequential manner, all known effectors including the LifA homologues (Cepeda-Molero *et al.*, 2017). It should be noted that these strains did not lack other PP/IE genes just the known T3SS substrates. The screen was restricted to strains linked to the loss of both LifA homologues - noting *lifA* was deleted before *lifA*-like (Cepeda-Molero *et al.*, 2017). The examined *lifA* double mutant lacked 4 LEE effectors and 9 Nle effectors previously reported not to be needed for EPEC to inhibit Akt signalling (Amin, 2017). Interestingly, the multi-effector strain lacking only one LifA inhibited Akt signalling like EPEC while the mutant lacking LifA and LifA-like homologues behaved like the T3SS mutant. This finding was similar to that of the mutant lacking the entire IE2 region (encodes the T3SS substrates LifA-like and NleE2) thereby implicating a critical role for LifA-like but not LifA protein; latter still encoded by the ΔIE2 mutant. Surprisingly, studies to verify this with mutants lacking just one or both LifA homologue genes (Cepeda-Molero *et al.*, 2017) revealed redundant roles that were supported in repeated experiments. However, in some experiments the data was not clear due to some loss of pAkt signal in the final time point samples, but this was also found in some experiments with the T3SS mutant that cannot inhibit Akt signalling (e.g., Figure 18A, 19A). This inconsistent, T3SS-independent loss of pAkt signal may reflect variability between experiments due to different levels of bacterial growth, macrophage health or sample processing issues. The redundancy idea was supported by showing that the *lifA* single mutant lost its ability to inhibit Akt signalling when the *lifA* gene was exchanged for a disrupted (*lifA*::Km) version. The latter result also discounted the idea that the *lifA*::Km gene - potentially expresses a N-terminal ~2000 residue truncated variant - could still produce a polypeptide encoding sufficient information for T3SS delivery into host cells to inhibit Akt signalling . Further experiments could determine if the N-terminal domain is in fact expressed and/or delivered into host cells.

Uncovering redundancy between the LifA and LifA-like proteins was very surprising as the IE/PP mutant screen revealed a key role for factor(s) on IE2 (~61kB). As the ∆IE2 mutant retains an intact *lifA* gene (on IE6) this suggested that IE2 factor(s) may be required for LifA to inhibit Akt signalling. Indeed, this prompted studies that are reported in Chapter 4.

A final point worth mentioning relates to the results that support the multi-effector chaperone, CesT, playing a key role in the inhibition of pAkt signalling (Amin, 2017). However, the findings need additional supportive experiments and to examine the unexpected role for Tir, with CesT, as previous studies had ruled out the need for Tir with or without 14 Nle effectors (Amin, 2017). The Tir finding might be specific to the genetic background of the used complex multi-mutant, TOEA7∆*core* strain. Nevertheless, the work implies that the LifA homologues requires CesT functionality suggesting it may aid their stability and/or promote their delivery to the T3SS for transfer into the macrophages. Antibodies specific to LifA are available (Bease, 2020) so CesT's role in these events could be examined and/or studies could test for predicted CesT-LifA interactions. Indeed, the availability of LifA-related reagents (Bease, 2020) promoted studies to examine CesT's role in LifA expression/delivery and how LifA inhibits Akt signalling (see Chapter 5).

In conclusion, the studies provided in this chapter clearly show that EPEC's T3SS dependent ability to inhibit pAkt signalling depends on the LifA homologues which act in a redundant way. This inhibitory activity also appears to require EPEC to express its multi-substrate effector chaperone, CesT. The work also opens other aspects for study including i) the apparent need for IE2-encoded factors to enable LifA to inhibit Akt signalling, ii) to determine if and how EHEC inhibits Akt by T3SS-dependent and -independent mechanisms, iii) a possible (indirect) role of PP10 encoded factors in the inhibitory process and iv) a predicted key role for LifA homologues in other A/E pathogens to inhibit Akt signalling.

Chapter 4. Role of IE2 Pathogenicity island in EPEC's ability to inhibit Akt

4.1 Introduction

Previous work (Result chapter 3; section 3.2.6) surprisingly suggested that the IE2 (~60kb) encodes a factor or factors needed for LifA to inhibit Akt signalling. EPEC E2348/69 has 8 integrative elements (IE1a, IE1b, and IE2-IE6) distributed across the entire genome (Iguchi *et al.*, 2009). Before investigating further, it was necessary to understand about the pathogenicity island (PAI) and about integrative elements (IEs).

Comparison of published bacterial genome sequences concluded that bacterial genomes consist of conserved *"**core gene pool**"* and *"**flexible gene pool**"* specific to strains (Hacker *et al.*, 2004). The latter is often referred as *"**genomic islands**"* (GEIs), which can be acquired by lateral gene transfer and are related to mobile genetic elements, such as integrative elements, bacteriophages and transposons (Burrus and Waldor, 2004; Hacker *et al.*, 2004). Whole GEIs and/or genes encoded on them confer advantages whether it is to survive better in the environment or, in relation to pathogenicity, a host. Thus, the identification and functional analysis of GEIs are essential for understanding strain-specific virulent features (Ogura *et al.*, 2008). When GEIs contains virulence genes, such as cycle inhibiting factor (Cif) in EPEC2348/69, these are often referred as pathogenicity islands (PAIs) (Dobrindt *et al.*, 2004). PAIs were first discovered as chromosomally associated genes in Uropathogenic *E. coli* (UPEC)(Low *et al.*, 1984). Exchange of mobile genetic elements between strains provide diversity to *Escherichia coli* species (Roche *et al.*, 2010). Most of the PAIs are located on the chromosomes, however, some also found on bacterial plasmids and bacteriophage (Hacker and Kaper, 1999). A simplified schematic of a bacterial PAI is shown in (Figure 33).

PAIs generally occupied large genomic regions, ≥10-200kb. However, various strains can also carry smaller insertion elements (<10kb) which may also encode virulence factors and called islets (Franco *et al.*, 1999; Novick *et al.*, 2010). PAIs are often associated with transfer RNA (tRNA) genes and this association suggested generation of PAIs by horizontal gene transfer (Hacker and Kaper, 2000). PAIs can also be flanked by small directly repeated (DR) sequences and often carry cryptic genes or genes encoding factors such as transposes, integrases, and insertion sequences (IS) elements (Hacker and Kaper, 2000).

As mentioned, (Chapter 1) EPEC LEE is a 35kb PAI containing genes which enables effacement of the brush border microvilli causing infantile diarrhoea (Elliott *et al.*, 1998). Besides LEE, EPEC virulence factors have been found on mobile genetic elements i.e. integrative elements (IEs), prophages (PPs) and plasmids (Iguchi *et al.*, 2009). EPEC E2348/69 has DNA regions relating to 13 prophages (PP1-PP13) and 8 integrative elements (IE1a, IE1b, and IE2- IE6). Bioinformatics revealed a subset of these PP and IE regions to encode non-LEE-encoded (Nle) effectors (Iguchi *et al.*, 2009).

Of interest to the work in this chapter is IE2 (~61kB) which has genes for 2 known effectors (NleE2, LifA-like) and pseudogenes for 2 other effectors (NleB, EspL). EPEC encodes two NleE and two LifA homologue proteins: NleE2 has an internal, in frame, deletion of 56 residues compared to NleE1 (encoded on IE6) with LifA-like a 599 residues, in frame, deletion compared to LifA (also encoded on IE6) (Dean and Kenny, 2009; Iguchi *et al.*, 2009). NleE2 and LifA-like protein were initially considered not to be expressed or function as an internal loss of 56 residues in NleE2 and 599 residues in LifA-like protein may hinder their translocation into host cells or functionality (Iguchi *et al.*, 2009; Nadler *et al.*, 2010). However more recent worked showed both proteins can be expressed and/or can subvert host cellular biology (Mühlen *et al.*, 2011; Cepeda-Molero *et al.*, 2017). Importantly IE2 has many genes for putative, transposes, or proteins of unknown function (Iguchi *et al.*, 2009)(Figure 34).

The aim of the work in this chapter was to firstly confirm that the IE2 region carries factors needed for EPEC to inhibit Akt signalling before undertaking studies to identify the responsible factor(s) and/or their role in the inhibitory process.

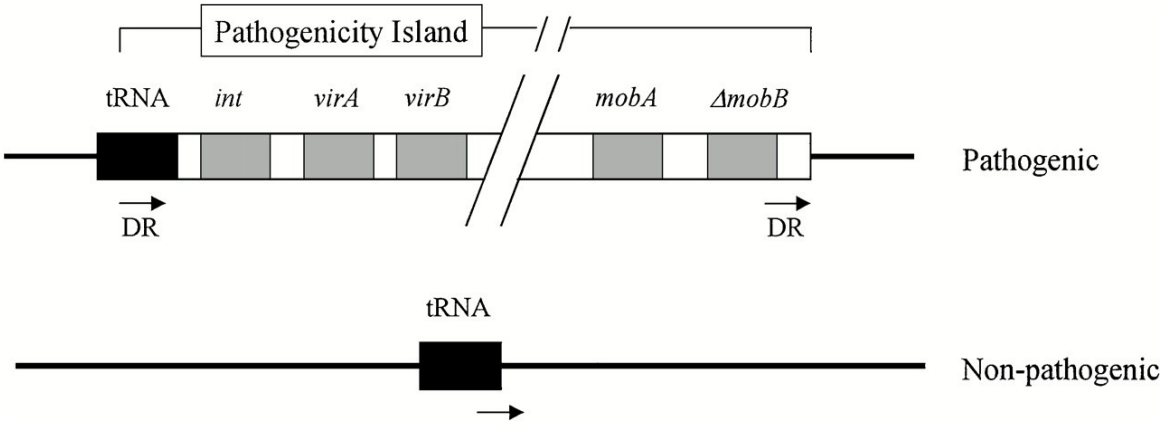


Figure 33 Schematic of a Pathogenicity Island (PAI)

The thin bold line represents regions of the core genome. The box shows the acquired genes with arrows the direct repeats at the end of the PAI. Abbreviations used are DR; direct repeats, *int;* integrase, *vir*; virulence associated genes, mob; mobility genes (includes integrases, transposes, and other proteins), Δ*mob*; pseudo-mobility gene. Image taken from a publication by Hacker and colleagues (Hacker and Kaper, 2000).

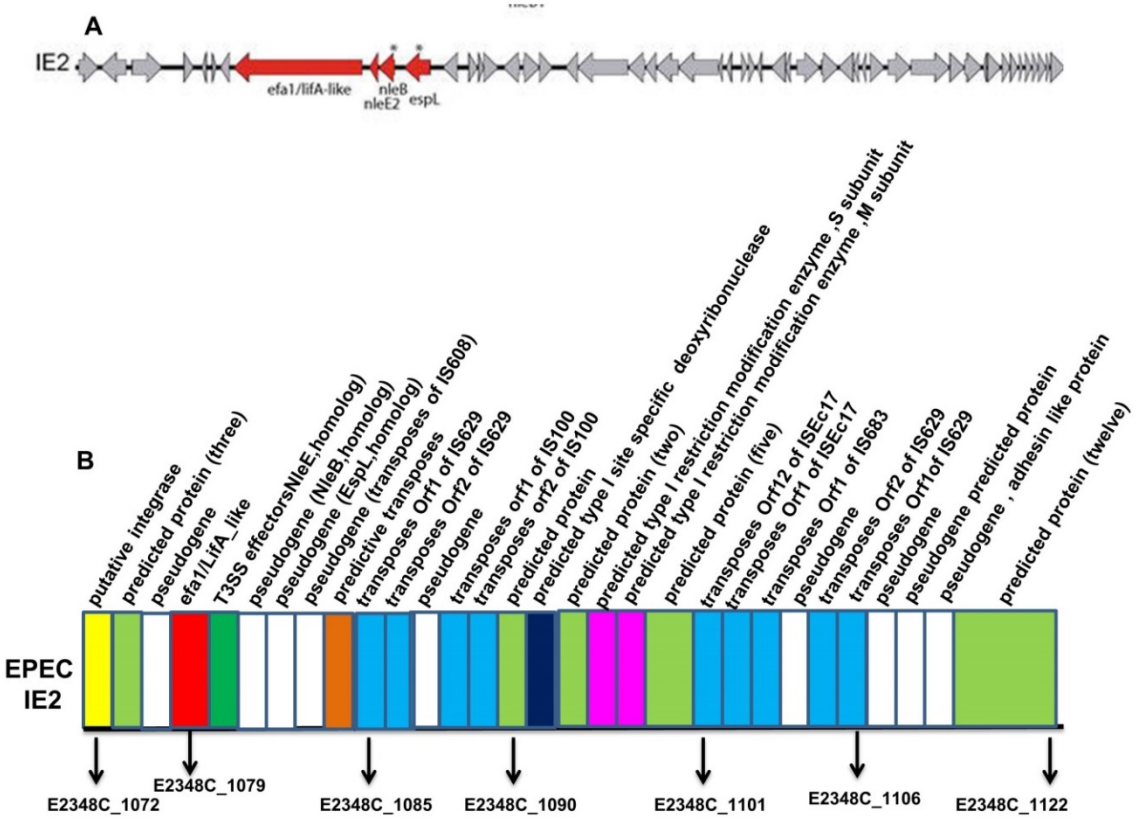


Figure 34 Genetic map of IE2 Pathogenicity Island

A- Nle effectors efa1/*LifA-like*, NleE2 and pseudogene espL* and nleB* (labelled in red) encoded in integrative element (IE2)**. B-** Schematic representation (this study) of the IE2 gene map with accession no representing predicted proteins (green colour) pseudogenes (white), transposes (Blue) and putative integrases (yellow).

4.2 Results

4.2.1 IE2 encodes factors needed for LifA to inhibit Akt signalling

Previous work (Chapter 3, section 3.3.6) revealed that the ΔIE2 deletion mutant had little, if any ability to inhibit Akt. This finding was puzzling as the strain encodes LifA with the earlier work uncovering redundant roles for both LifA proteins. While the EPEC E2348/69 genome has been reported to be stable (Nisa *et al.*, 2013) studies have revealed the presence of unplanned genetic alterations (Cepeda-Molero *et al.*, 2017)(Kenny lab, unpublished); latter refers to an undocumented 50kb deletion in the EPEC E2348/69 strain used to generate the Nle effector-deficient strains, TOEA1- TOEA7 (Yen *et al.*, 2010). Hence it was decided to regenerate the ΔIE2 mutant to determine whether the loss of Akt inhibitory activity was due to absence of the IE2 region or unknown alterations.

The strategy was to PCR amplify clone the region (~1kb) immediately upstream and downstream of the deleted IE2 region - from the provided ΔIE2 mutant - into a suicide vector to delete the IE2 region from the parental EPEC E2348/69 strain used in the Kenny Lab. Of note, the original ΔIE2 mutant had been generated by replacing the IE2 region with a gene encoding kanamycin resistance (Litvak *et al.*, 2017). Hence, briefly, the ΔIE2::Km region was cloned using oligonucleotides designed to bind upstream and downstream of the IE2 region allowing PCR amplification of a ~2.5Kb fragment (includes kanamycin gene plus IE2 flanking regions; Figure 35A) (Chapter 2, Table 7). The oligonucleotides also had extensions complementary to the target suicide vector enabling insertion, using the Gibson recombination-based ligation kit (see Materials and Methods; section 2.4.2). Generation of this plasmid (pDS-ΔIE2) was aided by digesting a pDS132 variant (pDS-ΔPP2) with SacI and XbaI restriction enzymes (Figure 35B) which removed a previously cloned ΔPP2-related DNA fragment and provided the vector with linear ends that aid the recombination ligation event. Successful ligation was evidenced by detecting an additional slower migrating DNA (Figure 35C). The ligation product was introduced, by electroporation (see Materials and Methods), into *E. coli* SY327 (λpir) with PCR screening supported the generation of the pDS-ΔIE2 plasmid (Figure 35D). The pDS-ΔIE2 plasmid was isolated and transferred

into SM10 (λpir) with PCR screening (Figure 35E) supporting the generation of the new ΔIE2::Km mutant, named ΔIE2a.

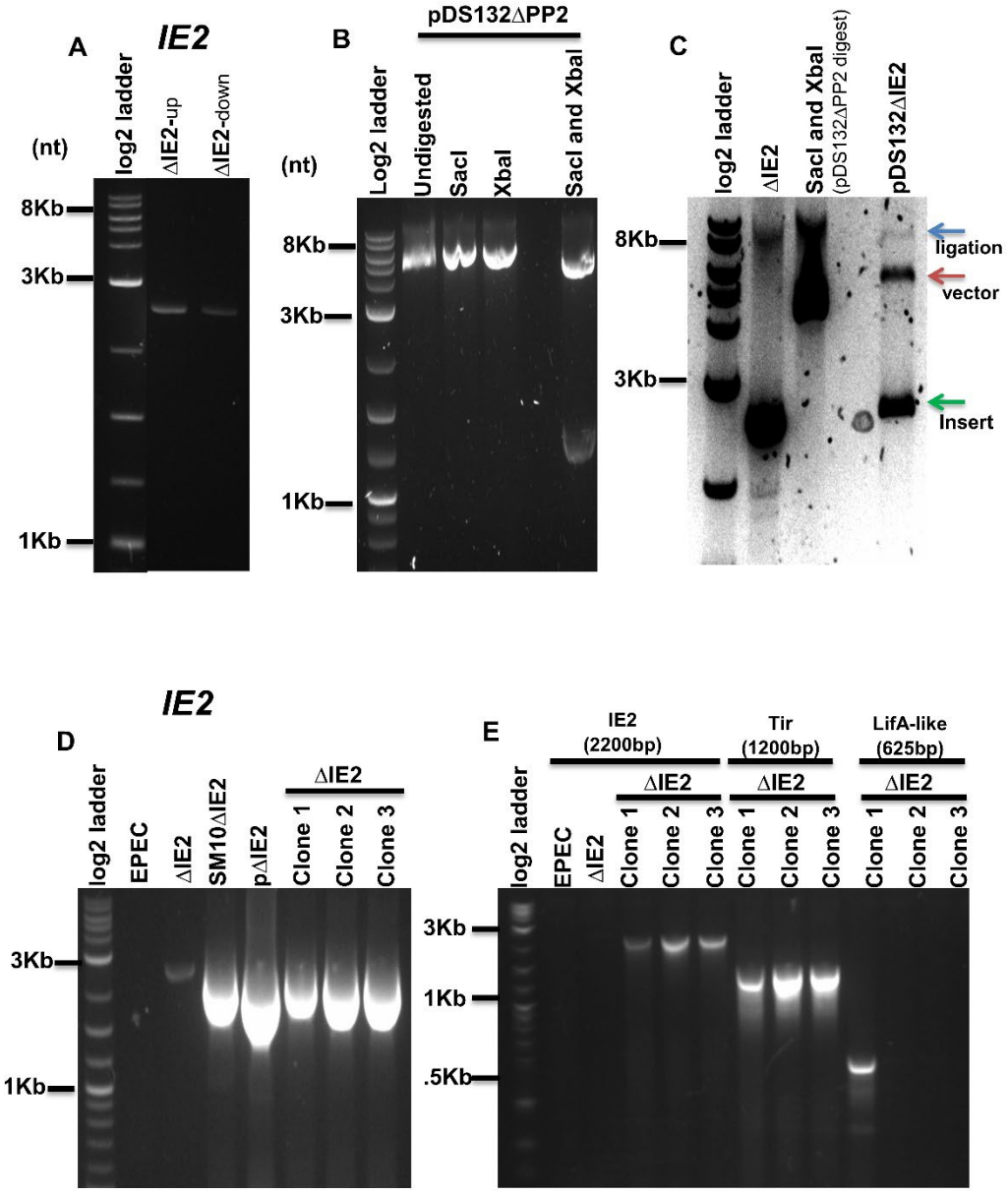


Figure 35 Construction of ΔIE2 mutant

A) PCR amplification of a single band of expected size for ΔIE2::Km region (~2200bp), B) pDS132 carrying an insert (ΔPP2) was digested with SacI/XbaI restriction enzymes to excise the ΔPP2-related region and the ~4Kb vector fragment was isolated for, in C), ligation - using Gibson Assembly protocol - with the PCR generated ΔIE2::Km region-related fragment. D) PCR screening with oligonucleotides flanking the IE2 region supports cloning of the ΔIE2::Km region into the suicide vector. E) PCR analysis of 3 potential IE2 deficient strains reveal all 3 EPEC (have *tir* gene) and have the ΔIE2-Km region (IE2 oligo pair) with 2 lacking the IE2 *lifA*-like gene.

Time course infections were carried out with EPEC, the T3SS-deficient, ΔIE2 and ΔIE2a mutant strains to determine if the new ΔIE2 mutant inhibited Akt signalling. Hence, J74A.1 macrophage were left uninfected or infected with the pre-activated strains (MOI 200:1) prior to isolating total protein extract over the 120 min infection period. Probing for Tir revealed all but the T3SS mutant had a functional T3SS (Figure 36). As expected, infection with EPEC led to a transient pAktSer473 signal with sustained signal for cells infected with, as before (Chapter 3, Figure 17), the T3SS and ΔIE2 (Figure 36) mutants. Importantly, the new ΔIE2a strain also led to a sustained pAktSer473 signal (Figure 36B). Similar gel loadings were illustrated by probing for host actin (Figure 36). This finding supports the idea that the IE2 (~61Kb) region encodes factors needed for the expression, delivery and/or Akt inhibitory function of the IE6-encoded LifA effector.

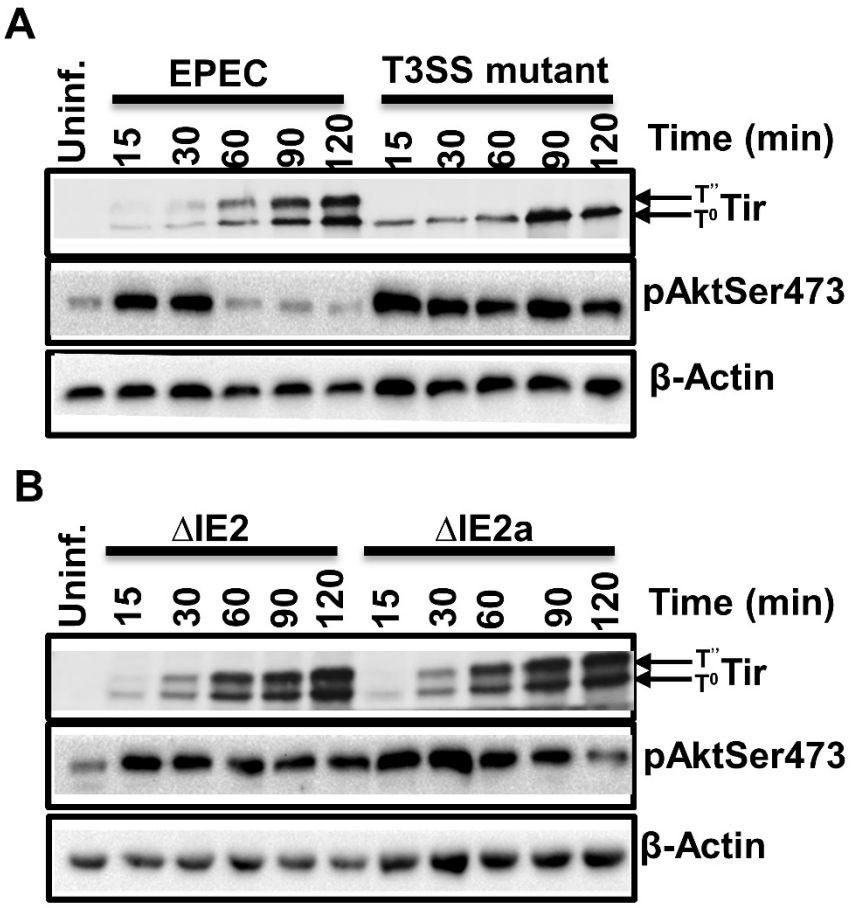


Figure 36 The ΔIE2 and IE2a mutants do not inhibit pAktSer473 signalling

J774A.1 monolayer were left uninfected or were infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS (*cfm-14*), ΔIE2 and ΔIE2a mutants. Data is representative of two independent experiments (see Supplementary Figure 7).

4.2.2 Search for IE2 factors needed for LifA to inhibit Akt signalling.

Examining the IE2 region for potential open-reading frames (*orfs*) revealed two regions dominated by potential pseudogenes separating areas with many, potential, protein encoding genes (Figure 37). As effector genes are often clustered together (Dean and Kenny, 2009) most interest was at the area just upstream and downstream of the known effector-encoding region. While the downstream region (~12kB) has many potential protein-encoding genes the upstream region (~6kb) relates to pseudogenes. The next, upstream, protein encoding region (~13Kb) is separated from the remaining protein encoding region (~10Kb) by another pseudogene (~9kB) area (Figure 37).

As each protein-encoding region is large (10-13kB) each was cloned seperately, but overlapping, fragments (Frag A-D; Figure 37). As genes from multiple regions may be involved, the fragments from each region would be cloned into different vectors for introduction of one or both into the ΔIE2a mutant. The latter also provided the possibility of introducing 2 fragments from different regions. Oligonucleotides were designed to clone the indicated (Figure 37) overlapping regions - fragment-A (~5.8Kb), fragment-B (~5.5Kb), fragment-C (~7.6Kb), fragment-D (~7.6Kb), fragment-E (~6Kb) and fragment-F (~6Kb). Fragment A, C and D were to be cloned into pACYC184 with the other fragments cloned into pBR322 (Cb resistance).

The cloning strategy is illustrated for fragment B (Figure 38) which was obtained from EPEC6 where IE2 lacks the *lifA*-like/*nleE2*/*nleB*/*espL* gene/pseudogenes (Cepeda-Molero *et al.*, 2017). Thus, oligonucleotide specific to a region ~5Kb downstream of the *lifA-like* stop codon and ~0.5 Kb upstream the pseudo-*espL* gene start codon was used to PCR amplify the ~5.5Kb region, this ~5.5Kb fragment B (frag-B) region was introduced into pACYC184, using the Gibson recombination kit, through a homologous region provided by extensions on the oligonucleotides (Chapter 2, Table 7; Section 2.2.6). Cloning the ~5.5Kb frag-B region into pACYC184 was supported by PCR studies (Figure 38D).

Attempts to clone all 6 regions (fragment A-F) produced 6 ligation products (not shown) that were introduced into commercial, chemically competent, K12 *E. coli*

but plasmid carrying colonies were only obtained for three. PCR colony screening confirmed the presence of fragment B (Figure 38), E and F (not shown). The plasmids were isolated and introduced, by electroporation, into the ΔIE2a mutant but only the fragment B carrying plasmid (pFrag-B) was successfully introduced (Figure 38D; data not shown).

Studies examined whether the sought-after factor was encoded on fragment B. Hence, macrophages were infected with the ΔIE2a strain and the ΔIE2a strain carrying pFrag-B, alongside control strains. As usual, EPEC and T3SS mutant infections induced transient and sustained pAktSer473 signals respectively (Figure 39A). As before, the ΔIE2a mutant has linked to a sustained pAktSer473 signal though some decrease was evident at final two time points that were not due to loading differences (Figure 39B). Importantly, the ΔIE2a/pFrag-B strain produced an EPEC-like pAktSer473 profile (Figure 39B). Probing for Tir confirmed all strains, except the T3SS mutant, had a functional T3SS (Figure 39A & B). This finding was supported by an additional experiment (see Supplementary Figure 8) with strain genotype supported by PCR analyses (Figure 39C-D). Collectively, these findings suggest that factor(s) encoded on the Frag-B region provide the ΔIE2a mutant an ability to inhibit Akt signalling.

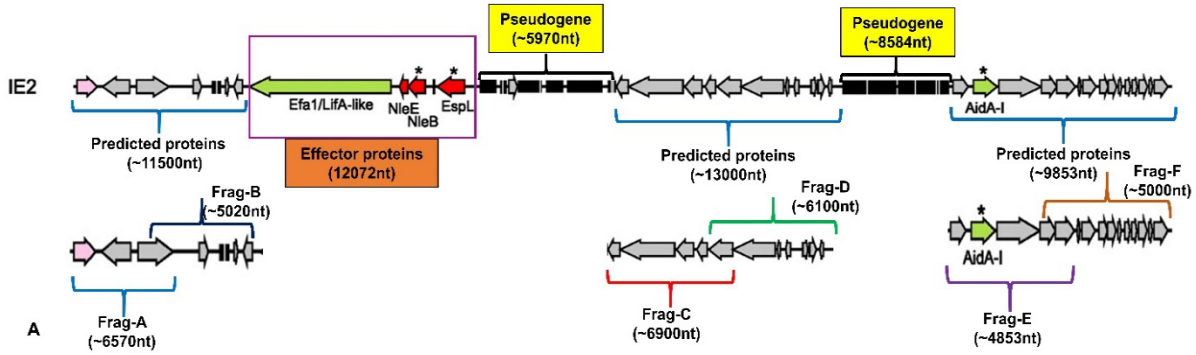


Figure 37 Schematic of predicted protein-encoding open reading frames

Schematic showing predicted protein-encoding genes, pseudogenes and Nle gene cluster region. The regions for cloning are indicated with each region to be cloned as two overlapping fragments: A-F with sizes given (in bracket).

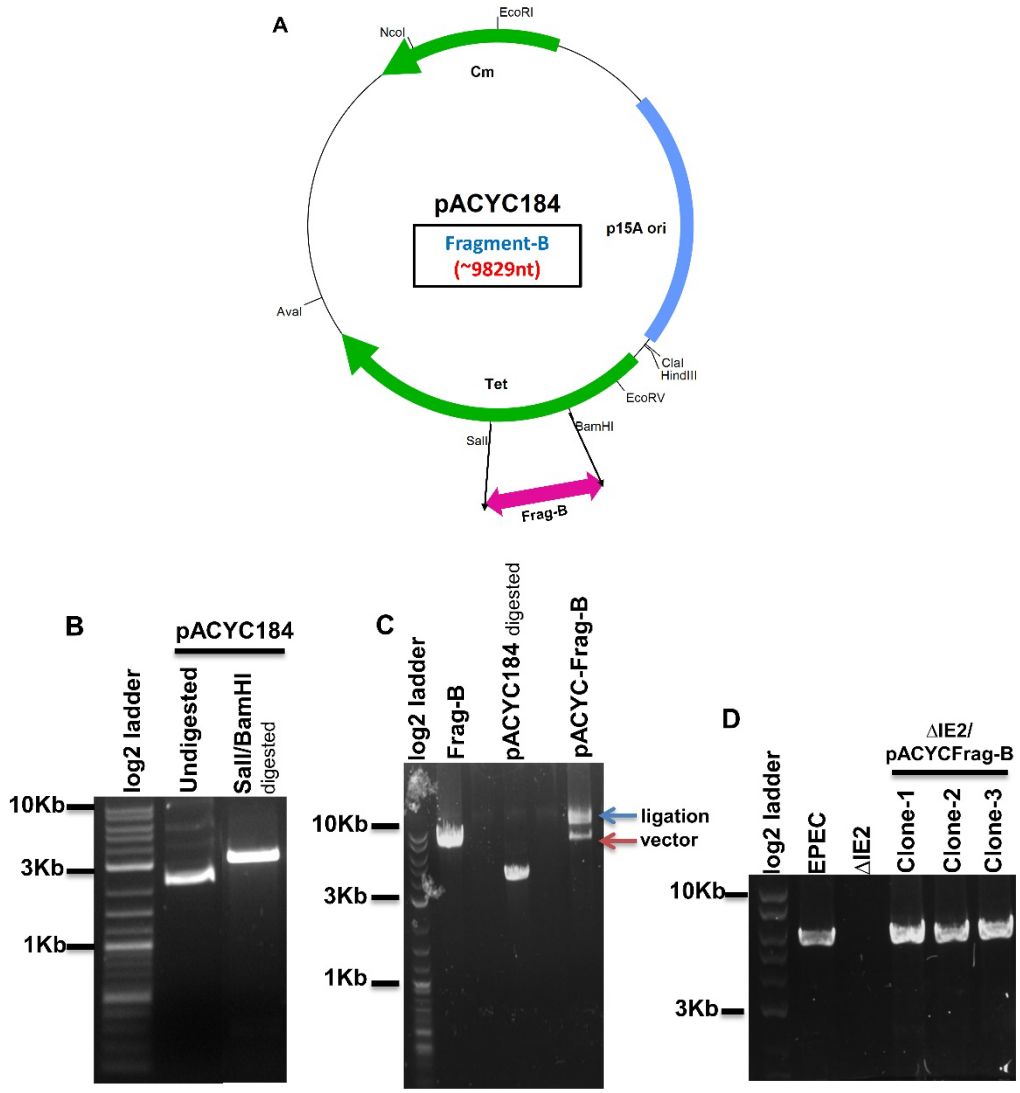


Figure 38 Generating pACYC-Frag-B

Agarose gel data illustrating steps in generating pACYC-Frag-B (A-D). A) Schematic of pACYC184 plasmid carrying EPEC Frag-B region, B) pACYC184 digestion with BamHI and SalI restriction enzymes to isolate ~4Kb vector fragment, which was used, in C), with PCR fragment in the Gibson Assembly protocol to produce pACYC-Frag-B that was verified, in D), by PCR with fragB specific oligonucleotides. The PCR used DNA from EPEC, ΔIE2a mutant and ΔIE2a mutant colonies putatively carrying the pACYC-Frag-B plasmid.

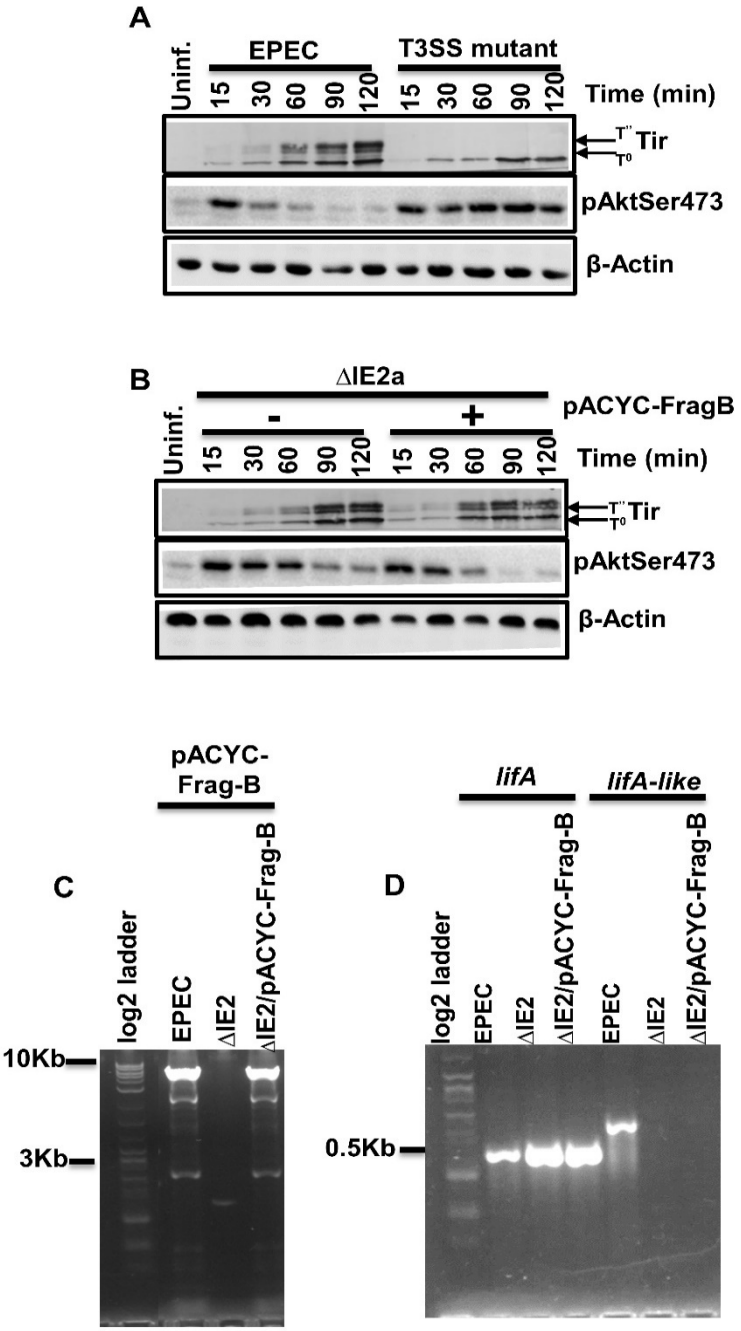


Figure 39 Fragment B encodes factor/s enabling ΔIE2a to inhibit Akt signalling

A & B) J774A.1 cell were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') Tir forms. The findings are supported by an additional experiment (see Supplementary Figure 8). C & D) PCR analysis of strains (collected post experiment) with oligonucleotides specific for *lifA*, *lifA*-like and a Frag-B gene. The PCR products were resolved on a 0.7% agarose gel and visualised (gel red stain) alongside indicated molecular mass markers. Strains used were EPEC, the T3SS mutant, ΔIE2a (has *lifA* but not *lifA*-like gene) without or, when indicated, the pACYC-Frag-B plasmid.

4.2.3 Search for Fragment B *orf*s restoring the Akt inhibitory activity of the IE2 mutant

To identify the location of the putative responsible factor/s on frag-B, it was decided to make vectors with smaller (3' end truncated) frag-B regions. Bioinformatics analyses of the fragment B revealed many potential open reading frames (>75 amino acids) on both strands (Figure 40). Oligonucleotides were designed (See materials & methods; Table 7) with a shared forward primer at 5' of Frag-B and 4 specific reverse primers to PCR amplify progressively smaller regions (see Figure 40A). Examining the obtained PCR products revealed single bands of expected sizes, ~5.5, ~4.5, ~3.6 and ~1.7 kb (Figure 40 & 41). The oligonucleotides (See Chapter 2; Table 7) carried extensions providing restriction enzyme sites (BamHI or SalI) allowing cloning into the same sites of pACYC184 (Figure 40 & 41). The resulting plasmids were named pFrag-1, pFrag-2, pFrag-3, and pFrag-4 (see Figure 40 & 41) which were isolated and introducing into the ΔIE2a mutant.

To assess the impact of the different fragment B regions on the ΔIE2a mutant's ability to inhibit Akt signalling, the standard time course infection was carried out. The initial study only examined strains carrying two of these plasmids, pFrag-4 (smallest fragment) and pFrag-2. Probing for Tir revealed that introducing the plasmids into the ΔIE2a mutant did not impact on Tir delivery or modification processes but restored the strain's ability to inhibit Akt (Supplementary Figure 8). This positive result led to an additional time course infection with the ΔIE2a mutant strain carrying, individually, each plasmid. Again, probing for Tir showed that all but the T3SS mutant had a functional T3SS with plasmid introducing having no obvious impact on Tir expression or delivery levels (Figure 42A-C). Consistent with the previous findings, all plasmid-carrying strains inhibited Akt signalling to a similar, EPEC-like, level (Figure 42A-C). Together this work suggests that factor or factors encoded the smallest fragment - Frag 2(1.7Kb) - are needed for EPEC to inhibit Akt signalling presumably via the IE6-encodied LifA as the mutants lack the IE2-encoded LifA-like protein.

Bioinformatics analysis of the Frag-1 region revealed a single large 537 amino acid Orf with a protein blast search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealing it encodes an N-terminally truncated version of an IE2-encoded 611 residue,

predicted (http://www.psort.org/psortb/), cytoplasmic protein. Including the upstream (vector sequence) in the bioinformatics analysis revealed a fortuitous 5' in-frame fusion with a vector *orf* with a fortuitous 5' in-frame fusion to the vector Tet gene providing a start codon to potentially produce a 592-residue fusion protein (not shown). However, as mentioned, there are also multiple small Orfs (>75 residues) predicted on this 1.7kB fragment (Figure 37A) and the responsible factor might not be a protein but a regulatory RNA (Waters and Storz, 2009). Time constraints did not allow the planned cloning of i) the entire 611 residue gene, ii) a C-terminal HA-tagged variant to allow Western blot detection of a gene produce , its location within EPEC or host cells and, if appropriate, determine if T3SS-dependent delivery into host cells and iii) the C-terminal HA-tagged variant carrying an internal stop codon to specifically examine the role for the 611-residue protein; noting stop codon would have minimal impact on other putative o*rfs* or possible regulatory RNAs.

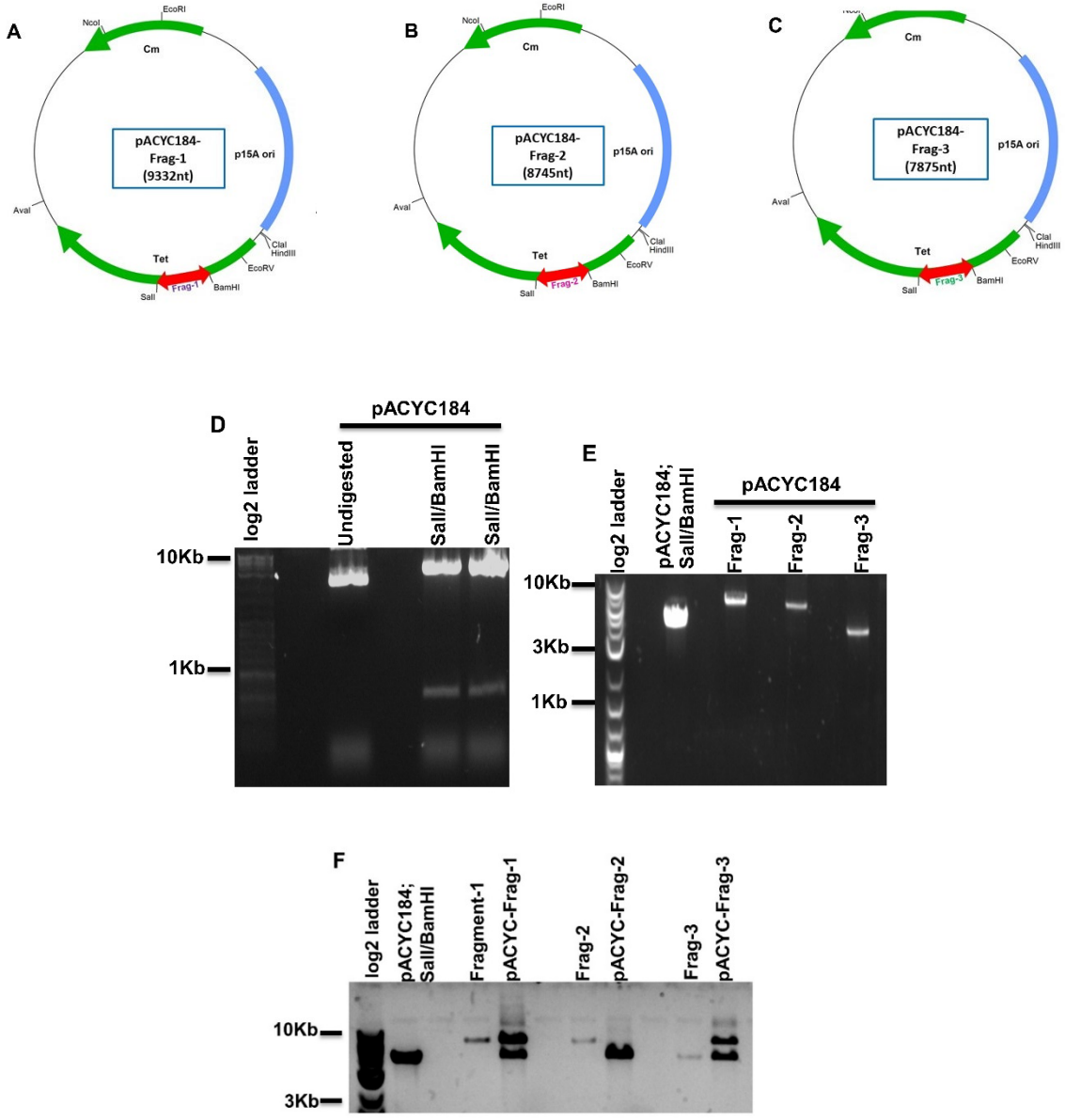


Figure 40 Sub cloning Frag-B regions into pACYC-184

Schematic of frag-B and sub-regions (Frag. 1-3) to cloned into pACYC184. D-F Agarose gel data illustrating steps in generating the plasmids D, pACYC184 digested BamHI/SalI to isolate ~4Kb vector fragment for ligation with, E, PCR amplified Frag-1, Frag-2 & Frag-3 and, in F, evidence for successful ligation via Gibson Assembly kit. Note Frag-4 data in Figure 41.

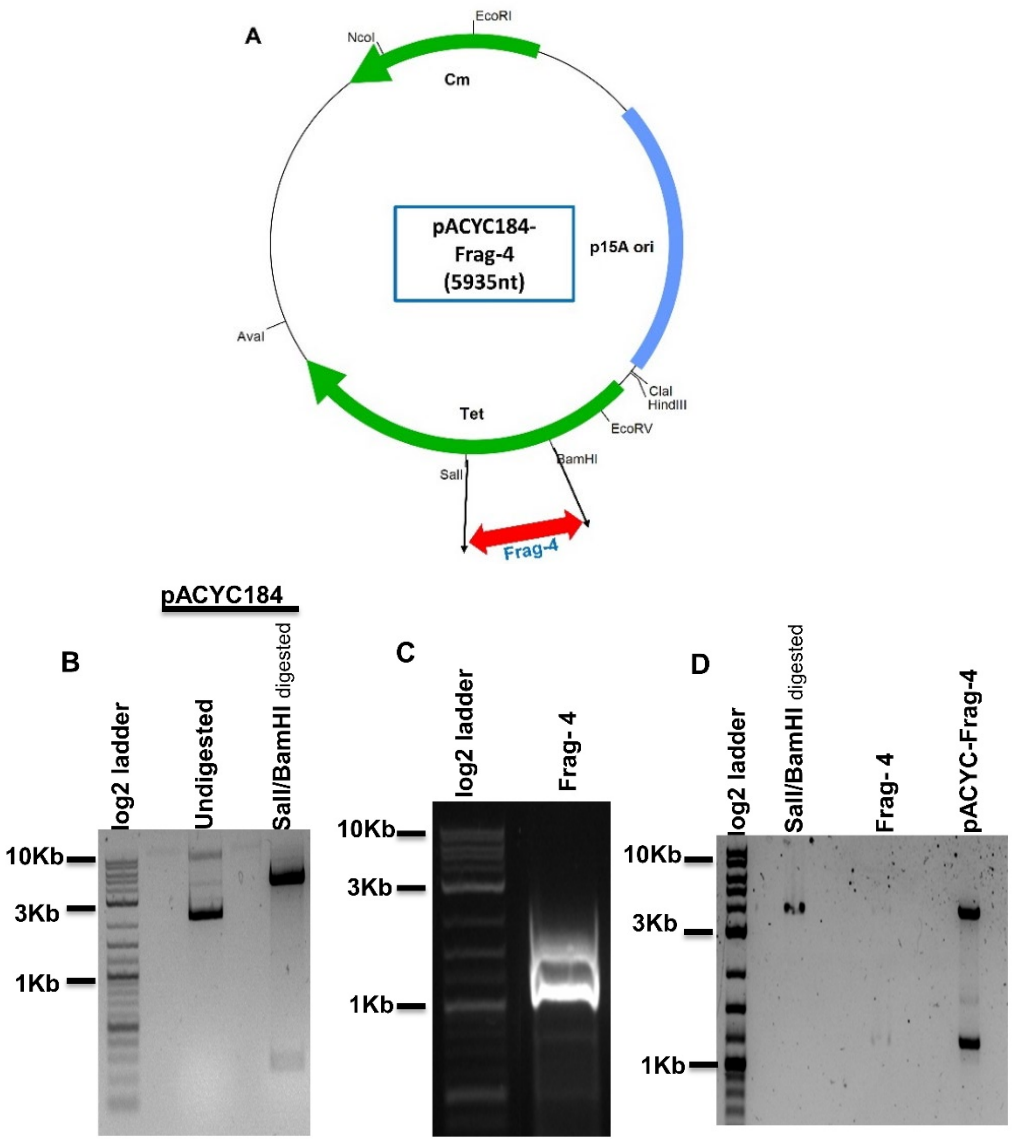


Figure 41 Sub cloning Frag-4 into pACYC-184

A-Schematic of pACYC184 plasmid carrying Frag-4 region. Agarose gel data illustrating steps in generating plasmids, B, pACYC184 digested BamHI/SalI to isolate ~4Kb vector fragment for ligation with, in C, PCR amplified Frag-4 and, in D, evidence (more evident in original file) successful ligation via Gibson Assembly kit.

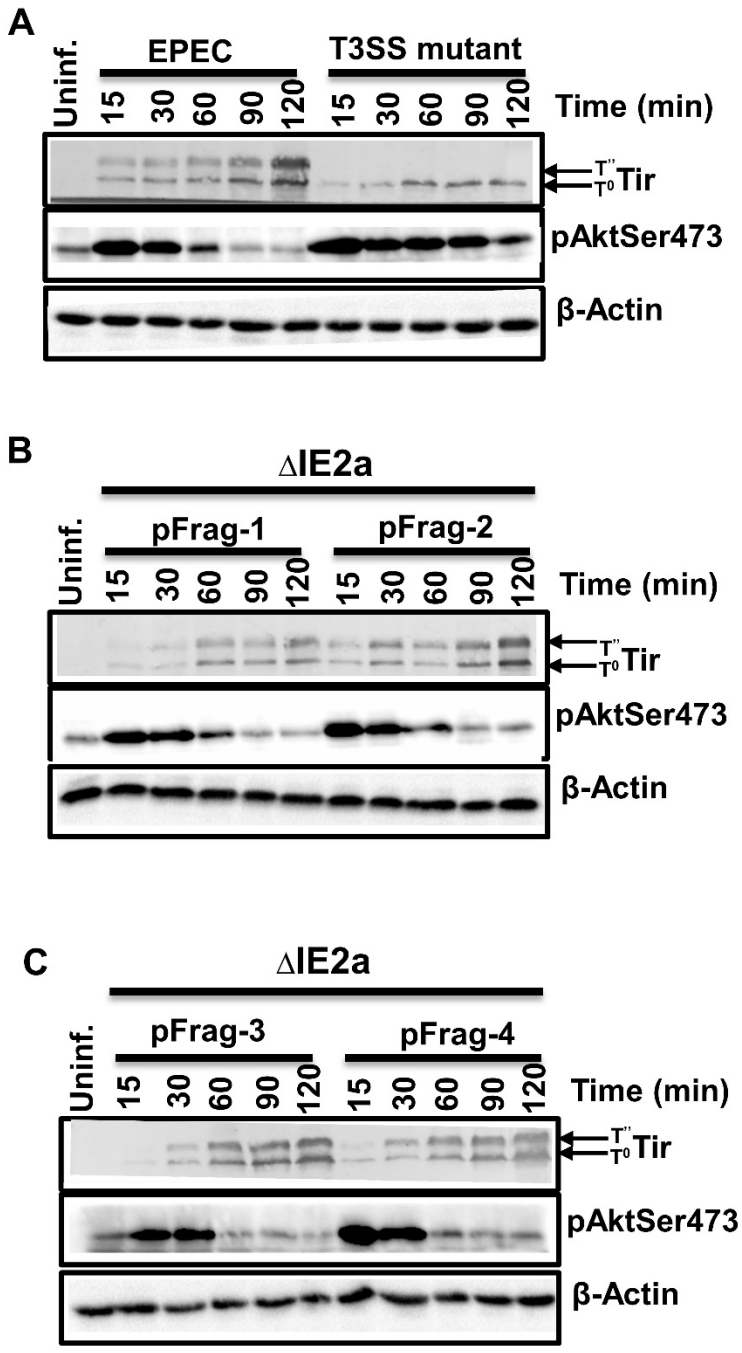


Figure 42 Restoring the ΔIE2 mutant's ability to inhibit Akt signalling is linked to 1.7Kb area of the 5.5kB Fragment B region

J774A.1 macrophage were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS), and ΔIE2a with pACYC184 plasmids (pFrag-1 to -4) carrying Frag-B sub fragments. The findings are supported by data from an additional experiment (See Supplementary Figure 9).

4.2.4 Generating ∆*Frag-B,* ∆*Frag-B/*∆*LifA-like and* ∆*Frag-B/NleS* deletion mutants in EPEC-WT

An alternative strategy was used to i) support the suggested key role of Frag-B encoded factors for EPEC to inhibit Akt signalling and ii) test whether it related to LifA and/or LifA-like protein activities. Hence, suicide vectors were generated to delete 1) the fragment B region downstream of the *lifA-like* gene (∆Frag-B), 2) ∆Frag-B plus the *lifA-like* gene (∆Frag-B/L) and 3) ∆Frag-B/L plus the *nle*/*espL* gene/pseudogene (∆Frag-B/L/N) cluster (Figure 43). Thus, oligonucleotides (Chapter 2, Table 7) were designed to PCR amplify 0.5-1Kb regions upstream and downstream of the target region for cloning into the pSuicide vector (digested SacI/Xba1). The latter involved a three-way ligation (up and downstream PCR plus vector fragments) and the Gibson recombination assembly kit (Figure 44-46) via homology provided by extensions in the oligonucleotides (Table 7; Materials & Methods). PCR data supported the generation all three required suicide vectors (Figure 44-46) (Table 4; Materials & Methods) which were introduced into SM10λpir to delete the target region from EPEC (Materials and Methods; section 2.2.8). PCR screening supported the generation of ∆Frag-B (Figure 44C), ∆Frag-B/L (Figure 45C) and ∆Frag-B/L/N (Figure 46C) mutant strains.

A standard time course infection studies was carried out with the newly generated mutants plus control strains. Importantly, probing for Tir showed the new and control ∆IE2a mutant strains had a functional T3SS and delivered as much, if not more, Tir than EPEC (Figure 47A), The T3SS mutant, as expected, expressed Tir but could not deliver it into the macrophages i.e., no T'' modified form (Figure 47A-B).

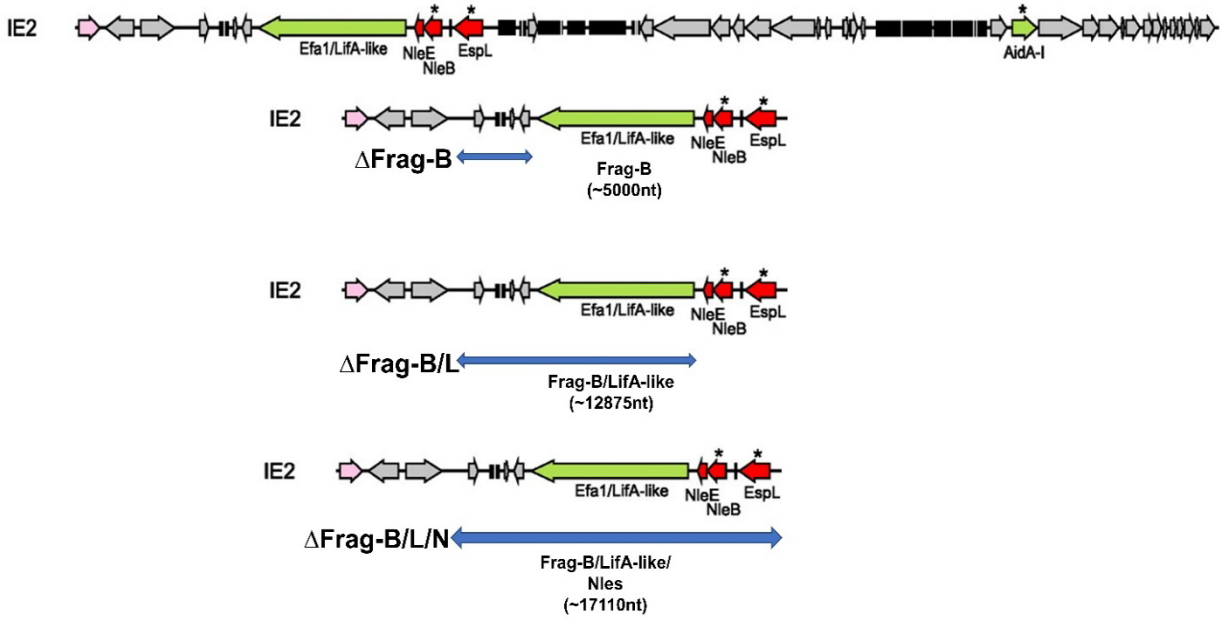


Figure 43 Schematic representation of IE2 region deletions.

Blue arrows illustrate indicated delete (Δ) regions.

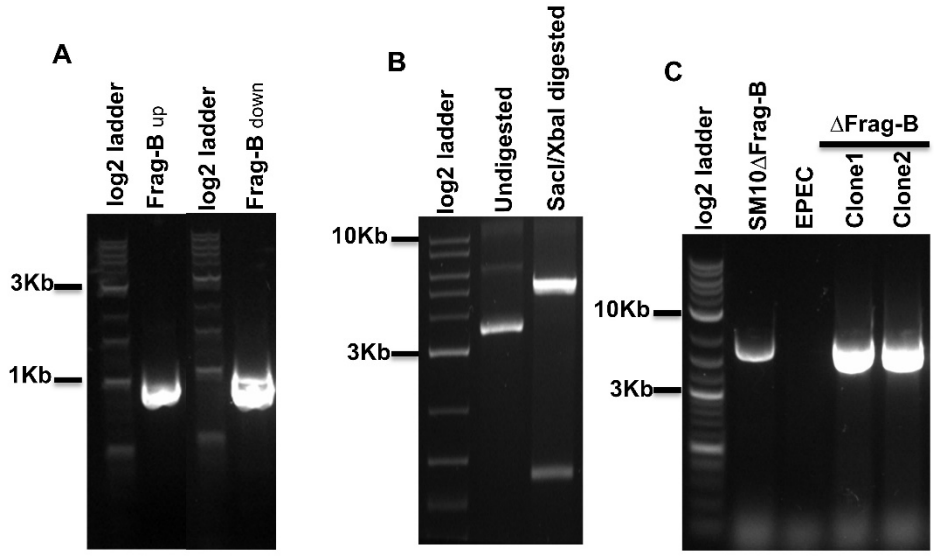


Figure 44 Generating ΔFrag-B mutant

A) PCR amplification of expected ~1kB fragments upstream and downstream of the target FragB region (see Figure 41). B) Undigested and SacI/XbaI digested pDS132 digestion before isolation of ~4Kb vector fragment for ligation with PCR fragments. C) PCR screening supports generation of EPEC ΔFrag-B mutant (clones 1 & 2). The PCR used DNA from EPEC and SM10 carrying the ΔFrag-B suicide vector.

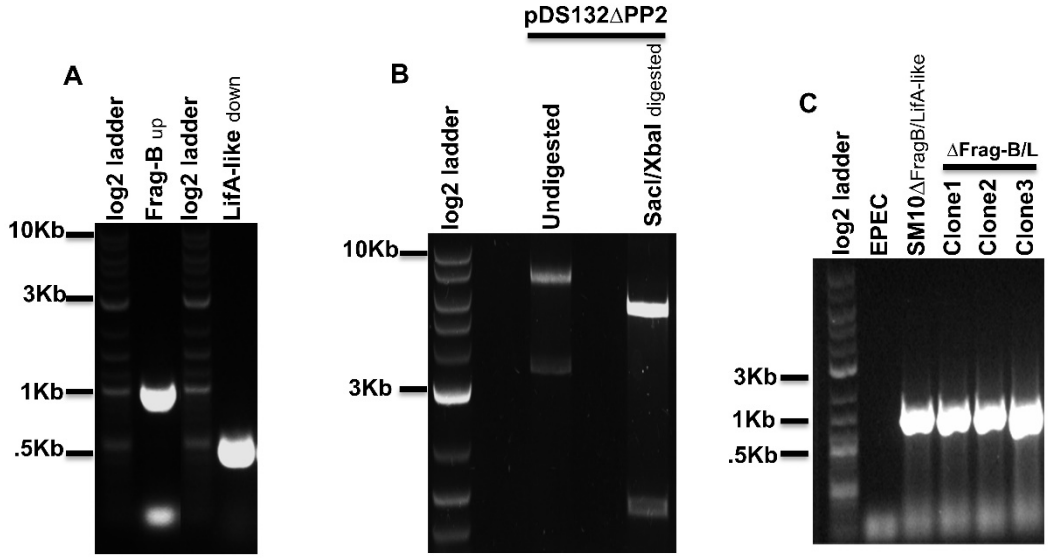


Figure 45 Generating ΔFrag-B/L mutant

A) PCR amplification of expected ~0.5-1kB fragments upstream and downstream of the target FragB region (see Figure 41). B) Undigested and SacI/XbaI digested pDS132 digestion before isolation of ~4Kb vector fragment for ligation with PCR fragments. C) PCR screening supports generation of EPEC ΔFrag-B/L mutant (clones 1-3). The PCR used DNA from EPEC and SM10 carrying the ΔFrag-B/L suicide vector.

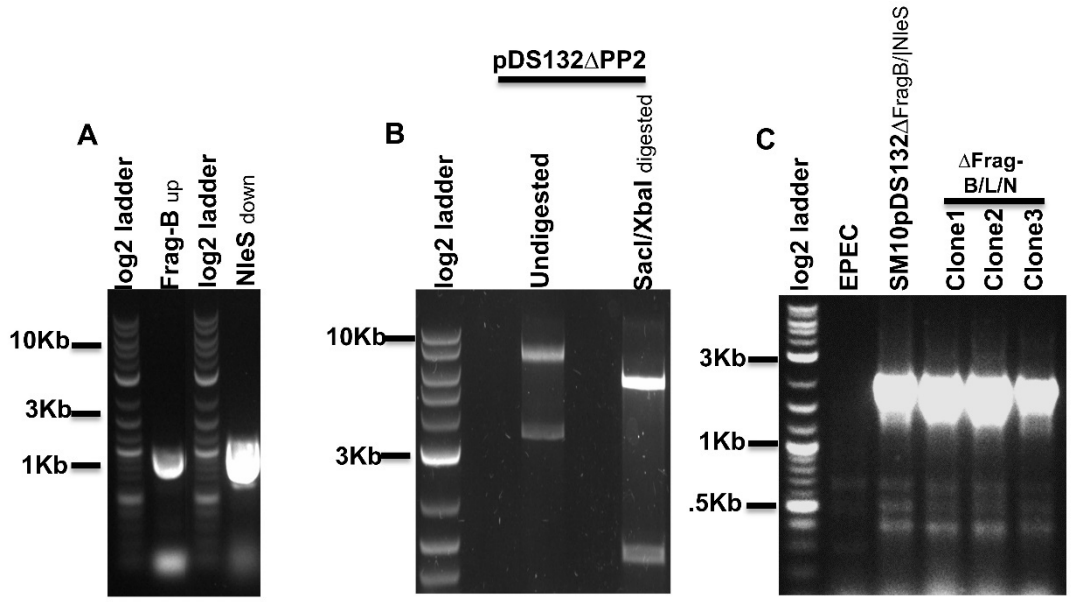


Figure 46 Generating ΔFrag-B/L/N mutant

A) PCR amplification of expected ~1kB fragments upstream and downstream of the target FragB region (see Figure 41). B) Undigested and SacI/XbaI digested pDS132 digestion before isolation of ~4Kb vector fragment for ligation with PCR fragments. C) PCR screening supports generation of EPEC ΔFrag-B/L/N mutant (clones 1-3). The PCR used DNA from EPEC and SM10 carrying the ΔFrag- B/L/N suicide vector.

Infection with the T3SS and ΔIE2a mutant strains produced sustained pAktSer473 signals, though some loss of signal was noted at later time points, not due to gel loading differences (Figure 47B). As usual, EPEC infection led to a transient pAktSer473 signal (Figure 47A) with the ΔFrag-B mutant producing, as expected, an EPEC-like profile (Figure 47B) as it encodes both LifA homologues. However, the ΔFrag-B/L/N and, to a lesser extent, the ΔFrag-B/L (Figure 47C) mutant strains behaved like the negative control strains (T3SS and ΔIE2a mutants) supporting an important role for factors on fragment B and/or 'N' regions.

The observation of a stronger, sustained pAktSer473 signal in cells infected with the ΔFrag-B/L/N, compared to ΔFrag-B/L, mutant suggested that there might be roles for factor(s) encoded on the absent 'N' region (Figure 47). Hence, another time course infection study was carried out with ΔFrag-B/L/N mutant carrying a plasmid carrying the 'N' related genes i.e., *nleE* and pseudo *nleB*/*espL*. The generation of this plasmid is described (see Materials and Methods). Western blot analysis of isolated samples supported the previous findings though the ΔFrag-B/L mutant was linked to pAktSer473 signal loss at later time points (Figure 48). However, this was noted to also occur in some infections with strains that cannot inhibit Akt signalling - including the T3SS (Figure 45 & 50), *lifA*/*lifA*-like deficient and ΔIE2a (Figure 28, 29 & 41) mutants - suggesting that is non-specific. While a sustained pAktSer473 signal was evident in cells infected with the ΔFrag-B/L/N mutant, the introduction of the 'N' region-carrying plasmid (pACYC-*nleE2*/*espL**/*nleB**) (refer suppl. Figure 10 for generation) did not provide any obvious additional capacity to inhibit Akt signalling. Collectively, this work supports the idea that a factor (or factors) encoded on the frag-1 region downstream of the *lifA*-like gene is required for LifA (encoded on IE6) to inhibit Akt signalling.

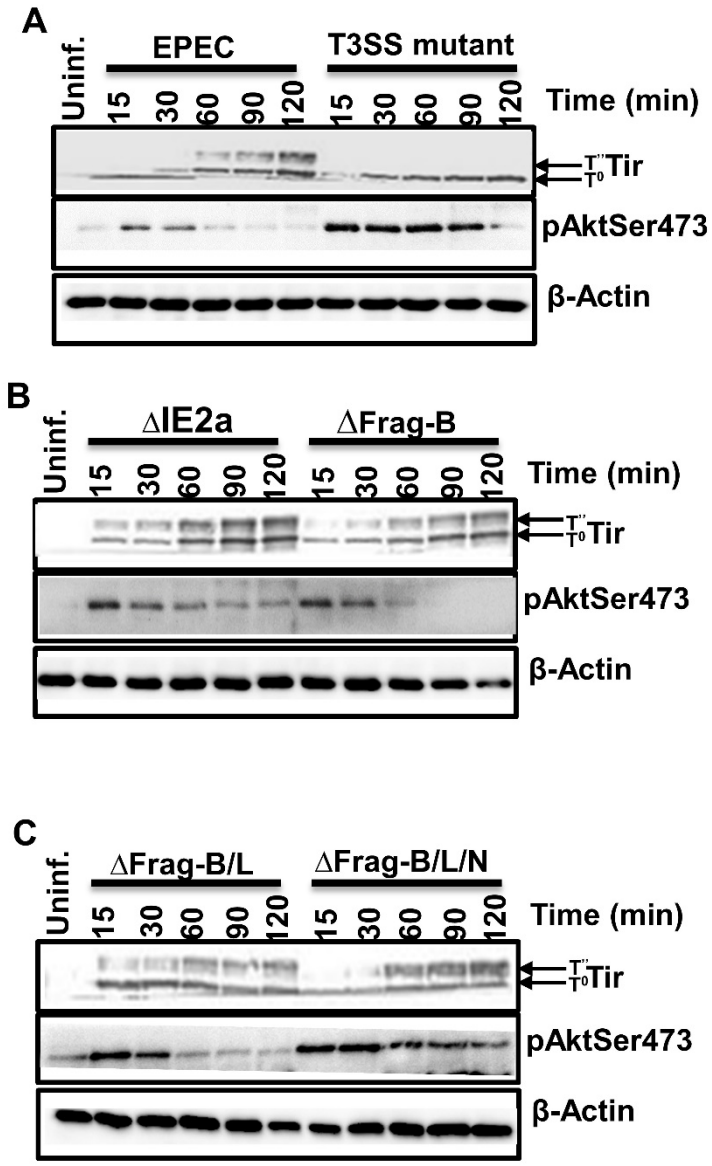


Figure 47 IE2 mutant missing Frag-B/L/N cannot interfere with Akt signalling

J774A.1 cell were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS mutant, ΔIE2, ΔFrag-B, ΔFrag-B/L and ΔFrag-B/L/N. The data is supported by findings from another experiment (see Supplementary Figure 11).

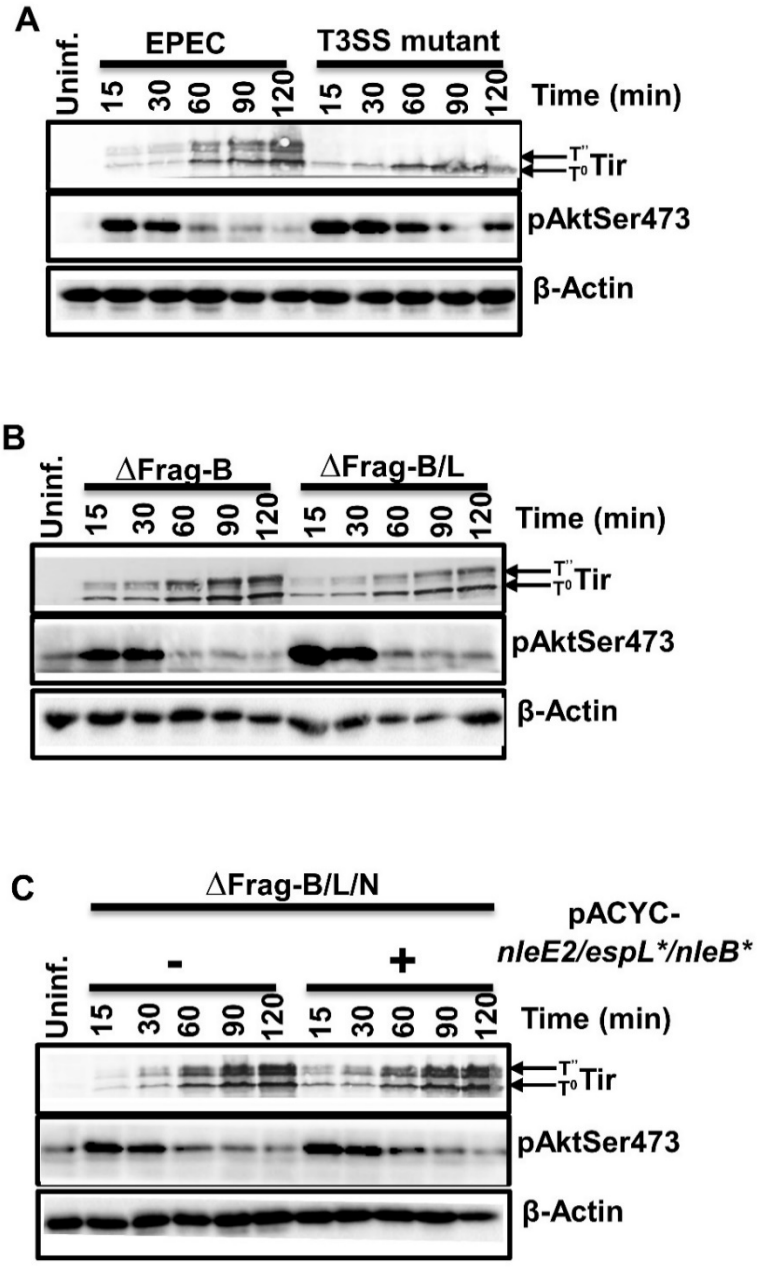


Figure 48 Akt signalling inhibitory defect of EPEC ΔFrag-B/L/N mutant is not rescued by plasmid re-introducing the 'N' region.

J774A.1 cell were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS mutant, ΔIE2a. ΔFrag-B, ΔFrag-B/*lifA*-like (ΔFrag-B/L) or ΔFrag-B/*lifA*-like/NIe region (ΔFrag-B/L/N). The ΔFrag-B/L/N carried no plasmid (-) or a plasmid (+) carrying the *nleE2*/*espL**/*nleB** gene region (* indicates pseudogene).

4.3 Discussion

The work described in this chapter not only supported earlier work (see Chapter 3) suggesting that IE2 encodes factors needed for LifA (encoded on IE6) to inhibit Akt signalling but indicates the factor is encoded on a 1.7kb fragment downstream of the *lifA*-like gene.

The studies in this chapter were started due to a conflicting result in relation in how EPEC inhibits Akt signalling. Thus, screening mutants lacking individual IE or PP islands revealed a key role for IE2-encoded factors (unlike for 15 other screened IE/PP regions including IE6; Chapter 3; Section 3.2.6) while later studies clearly revealed key, but redundant roles for the LifA (IE6 encoded) and LifA-like (IE2 encoded) proteins (see Chapter 3). As EPEC strains have been found to have undocumented genome alterations (Kenny lab, unpublished) (Cepeda-Molero *et al.*, 2017) it was important to show that the IE2 phenotype was due to absence of IE2. Hence the mutant by was remade by cloning of the deleted IE2 region – was substituted for gene encoding kanamycin resistance (Litvak *et al.*, 2017) into a suicide vector to delete IE2 from EPEC E2348/69 strain used in the Kenny lab. Importantly, this new Km-resistant strain was PCR-confirmed to lack an IE2 specific gene and failed to inhibit Akt signalling. Of note the new mutant delivered Tir into macrophages like EPEC showing it had a functional T3SS. This finding supported the idea that IE2 encodes factors needed for EPEC to inhibit Akt signalling.

Bioinformatics analysis revealed IE2 encodes at least 23 predicted proteins, 10 transposes and 1 integrase (Iguchi *et al.*, 2009). However, functional proteins can be small - for example ~10% of EPEC T3SS are only 70-100 residues (Iguchi *et al.*, 2009) - with bioinformatics analysis revealing many small Orfs (>75AA) on both strands of the IE2 region. It is also possible that the responsible factor was not a protein but a small regulatory RNA (Waters and Storz, 2009). The initial focus was on the three large (~10-13Kb) protein encoding regions as unlike to involve the two 6-9Kb pseudogene/transposon rich regions. Studies set out to clone the regions onto plasmids to examine if their introduction back into the ΔIE2 mutant would restore its ability to inhibit Akt signalling. As the target regions were large (10-13kB) it was decided to clone each region in two, overlapping, 4-5 Kb fragments onto

different antibiotic selectable vectors. The latter would enable test individually or in pairs.

Unfortunately, only 3 fragments could be cloned and only one could be successfully be introduced into the ∆IE2 mutant but amazingly it restored the mutant's ability to inhibit Akt signalling. It is possible that the failure to get the other clones reflects the fragments encoding factors harmful to the bacteria or perhaps additional attempts may have been more successful. It may be worthwhile to determine whether these fragments encode factors 'harmful' for bacterial growth when cloned onto high copy number vectors as, if so, this may provide a new research area to explore.

Studies on Fragment B not only supported it carrying factor(s) required for the ∆IE2 mutant to inhibit Akt signalling but located the responsible factor(s) to the 5' most 1.7Kb area of the ~5.5Kb Fragment B region downstream of the *lifA*-like gene. Consistent with this, the other 3 larger sub cloned fragments – all carry the 1.7Kb – also restored the ∆IE2 mutant's ability to inhibit Akt signalling. This emphasises the role for factor(s) in the shared 1.7kb region. The 1.7Kb region has the largest *Orf* downstream of the LifA-like/Nle encoding region (see Figure 37A) but bioinformatics analyses revealed the Orf was truncated during cloning process. The whole gene (encodes 611 residue *Orf*; E2348C_1074) would have been cloned intact on Fragment A. This fragment B *Orf* lacks sequence for the first 72 residues but an available start (methionine) codon may enable expression of a 537-residue protein. Surprisingly, bioinformatics analysis revealed an in-frame fusion with the 5' end of the *tet* gene that could, in theory, allow the production of a 620-residue hybrid protein. The IE2 611 residue protein for its predicted cellular location (https://www.psort.org/psortb/results.pl) indicates a cytoplasmic location with the protein lacking an N-terminal sec pathway signal sequence. It should be noted that the *tet* gene product produces an inner membrane protein (Allard and Bertrand, 1992) and thus its N-terminus has a signal sequence to direct it and, presumably, the 620-residue fusion protein to the sec pathway. Interestingly, a previous bioinformatics interrogation of EPEC proteins using a program to identify proteins carrying a T3SS secretion signal (Goldberg *et al.*, 2016) identified known EPEC effectors and predicted many more but only two were encoded on IE2: C_1079 and C_1074 encoding the LifA-like and 611 residue proteins respectively (B.

Kenny, unpublished). Given that T3SS secretion, signals are generally N-terminal and thus presumably absent from the truncated 611 C_1074 protein, this question whether it is a T3SS substrate.

Studies to determine whether the responsible factor on the 1.7Kb region relates to the Orf611 protein, another Orf or cryptic regulatory sequence were started but not completed due to COVID-19 lockdown and time constraints. As mentioned, studies were initiated to clone the complete Orf 611 gene into a vector with and without a C-terminal HA epitope tag to examine if the protein is expressed and if the cellular location i.e., cytoplasmic, membrane, periplasm, secreted or translocated. If these constructs restored the ability of the ∆IE2 mutant to inhibit Akt signalling, then it was planned to introduce a stop codon to disrupt 611 Orf production with minimal impact on other predicted *orfs* (and possible regulatory RNAs) to indicate if a key role was due to the 611 Orf or another Frag-2 factor.

The importance of the IE2 Fragment B (~6Kb region) in allowing EPEC LifA and/or LifA-like proteins to inhibit Akt signalling was investigated by deleting just this region in a strain encodes both LifA homologues (i.e., EPEC) or lacking *lifA*-like gene (i.e., strain encodes only LifA). Crucially, while both strains had a functional T3SS the strain encoding only LifA could not inhibit Akt unlike the strain encoding both homologues supporting the idea that the inhibitory function of LifA depends on this IE2 region. The findings also suggest that the inhibitory function of the LifA-like protein does not requires the Fragment B-encoded factor/s. However, it is possible that the LifA-like protein does requires Fragment B to inhibit Akt signalling but in a way that can be replaced by LifA. This possibility could be addressed by deleting Fragment B from the ∆LifA single mutant with studies to determine if the remaining LifA-like protein can still Akt signalling.

In conclusion, the work in this chapter verifies the earlier finding suggesting IE2 encodes factor(s) needed for LifA (encoded on IE6) to inhibit Akt signalling. In addition, the work located the responsible IE2 factor(s) to a ~1.7kB subregion of the ~5.5 Kb ‘Fragment B’ area immediately downstream of the *lifA*-like gene. Additional work is needed to i) confirm these findings, ii) identify the nature of the factor i.e., the large 611 residue Orf, products from one or more of many other predicted small (>77 residue) orfs or a regulatory element such as a small RNA

and iii) define the mechanism by which the factor/s enables LifA, and possibly the LifA-like protein, to inhibit Akt signalling.

Chapter 5. Investigating the mechanism(s) by which LifA homologues inhibit Akt signalling

5.1 Introduction

Bacterial effectors in EPEC and other T3SS pathogens can exhibit functional redundancy in subverting host cellular activities (Dean and Kenny, 2009). The studies described in Chapter 3 showed functional redundancy of LifA homologues in the inhibition of Akt signalling. As such, the scope of this work was broadened to examine the mechanism for the rapid loss of Akt signal. Prior to investigating the mechanism, it was necessary to review the literature on these bacterial proteins.

LifA is a very large protein (3223 residues) first identified in EPEC E2348/69 as a toxin which specifically inhibits the ability of human lymphocytes to proliferate and produce the cytokines interleukin -2 (IL-2), IL-4 and gamma interferon (Klapproth *et al.*, 2000). Cellular lysates from EPEC, but not non-pathogenic *E. coli*, led to a dose dependent inhibition of cytokine expression suggesting the inhibitory activity was due to EPEC encoded protein(s) (Klapproth *et al.*, 1995). In 1996, screening cosmids carrying EPEC fragments in non-pathogenic *E. coli* K-12 for this toxin activity identified one providing this phenotype (Klapproth *et al.*, 1996). Sequencing revealed the toxin to be encoded by a very large gene (9669bp), named LifA for Lymphocyte Inhibitory Factor A (LifA), also called Lymphostatin as inhibits lymphocyte proliferation (Klapproth *et al.*, 2000) At the same time, studies with an EHEC O111:H- strain identified a gene involved in its adherence to CHO cells with the gene product named Efa-1 for EHEC factor for adherence-1. Efa1 and LifA are virtually identical (99.9% gene sequence homologue) (Nicholls *et al.*, 2000b) and, hereafter, called *lifA* for simplicity. LifA appears to be almost exclusive to A/E pathogens but homologues have been found in Chlamydia species (Klapproth *et al.*, 2000; Nicholls *et al.*, 2000b). The *lifA* gene is chromosomally located, sometimes linked to the LEE region, with described roles for its gene product as a lymphostatin, an adhesin (involved in tissue colonization) and regulatory as influences LEE protein expression (Nicholls *et al.*, 2000b; Badea *et al.*, 2003; Klapproth *et al.*, 2005; Deacon *et al.*, 2010; Cepeda-Molero *et al.*, 2017).

A serotype specific LifA truncation (*efa1'*) has been found in many EHEC O157:H7 strains resulting in two separate *orf's*, Z4332 and Z4333 (Hayashi *et al.*, 2001; Perna *et al.*, 2001). Z4332 encodes a 433 residue protein 99.9% identical to the N-terminal LifA region while Z4333 encodes a region that 100% matches residues 437-711 with

no sequence for the C-terminal domain (Deng *et al.*, 2012) (Figure 49). PCR screening has found full length *lifA* genes in most A/E pathogens with strains which lacking an intact *lifA* gene encoding a homologue, called ToxB, on a plasmid (Badea *et al.*, 2003). The *toxB* plasmid is carried by most EHEC and some EPEC strains. ToxB is encoded on slightly smaller gene than *lifA* (9505 versus 9669bp) with the proteins sharing ~29% identity, ~62% similarity (Cassady-Cain *et al.*, 2017). ToxB is thought to be a T3SS protein and has recently been shown to also have lymphostatin activity in addition to roles in promoting strain adherence and altering LEE protein expression levels (Stevens *et al.*, 2002; Klapproth, 2010a; Cassady-Cain *et al.*, 2017). Studies have reported roles for the *efa1'* and *toxB* genes in EHEC adherence *in vitro*, but not *in vivo* models, and in altering LEE protein expression levels (Stevens *et al.*, 2004).

Genome sequencing EPEC 2348/69 revealed *lifA* on IE6 with a homologue, *lifA*-like, on IE2 (Iguchi *et al.*, 2009; Deng *et al.*, 2012). The LifA-like protein is smaller than LifA (2624 versus 3223 residues) and shares only ~30% identity to LifA. Proteomic studies identified both LifA homologues as T3SS substrates with evidence provided for T3SS-dependent delivery of LifA, but not the LifA-like protein, into mammalian cells (Deng *et al.*, 2012). Notably these studies only examined delivery of N-terminal regions, which carry features (T3SS signal sequence and chaperone binding site) needed for the delivery process (Deng *et al.*, 2012).

Studies on the LifA homologues have reveal sequence similarity (~38%) to the N-terminal domain of large clostridial toxins (LCTs) including toxin A and B from *Clostridium difficle*. Indeed, all but the C-terminally truncated LifA variants have motifs predictive of them having glucosyltransferase-, cysteine protease and/or aminotransferase activities (Klapproth, 2010b; Deng *et al.*, 2012)(Figure 50). The C-terminally truncated LifA, if expressed, would only have the putative glucosyltransferase motif (Figure 50). A shared motif (DXD) is reported to be key for glycohydrolase-glycosyltransferase activity (Busch *et al.*, 1998) crucial for the clostridial cytotoxin to inhibit, via glycosylation, the activity of small Rho GTPases such as Ras, Rac, Rho and Cdc42 (Nicholls *et al.*, 2000b).

The DXD motif (Figure 50) is essential for sugar binding by LCTs (Klapproth *et al.*, 2000). The LCTs are glycosyltransferases that bind mostly uridine diphosphate-glucose (UDP-Glc)(Schirmer and Aktories, 2004). However, toxin A binds uridine

diphosphate-N-acetylglucosamine uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) (Schirmer and Aktories, 2004). Disruption of glycosyltransferase and cysteine protease motifs in *C. rodentium.*

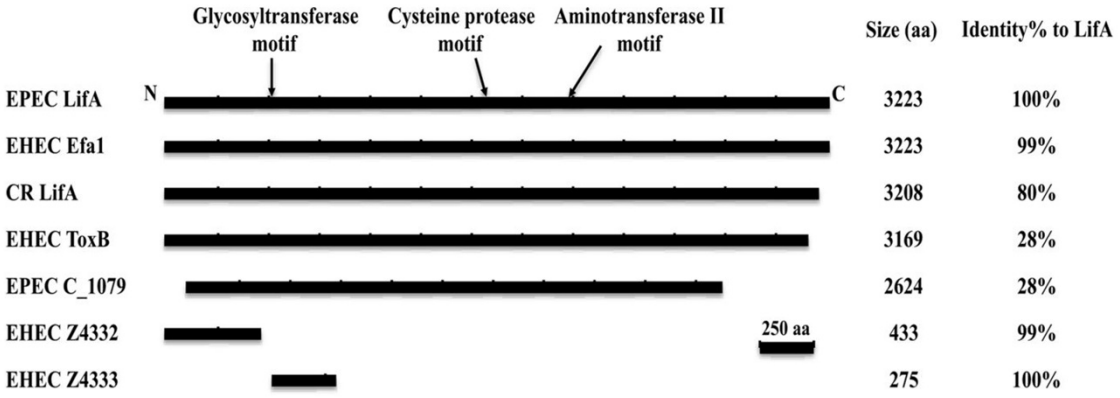


Figure 49 Schematic of different LifA homologs

Comparison of LifA homologs showing size (amino acids), percentage identity to EPEC LifA and location of motifs lined to protein glycosyltransferase, cysteine protease and aminotransferase activity. Figure taken from Deng *et al.,* (Deng *et al.*, 2012)

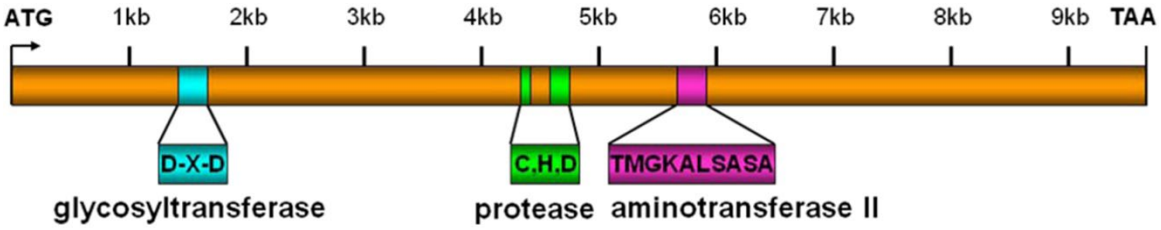


Figure 50 Schematic showing location of predicted motifs within *lifA/efa1*

The *lifA*/*efa-1* gene encodes a 3223 amino acid protein in many EPEC, EHEC and C. *rodentium* strains and indicated is the location of motifs linked to glycosyltransferase (DXD), cysteine protease (CHD) and/or aminotransferase (TMGKALSASA) activities. Figure taken from Klapproth *et al.,* (Klapproth, 2010b).

LifA inhibits bacterial colonization of the intestine and the T3SS-dependent induction of hyperplasia in mice (Klapproth *et al.*, 2005). However, substitution of DXD for triple alanine (AAA) in EHEC LifA did not affect bacterial colonization in calves or epithelial cell models (Deacon *et al.*, 2010). Recently, recombinant LifA (r*lifA*) was shown to bind UDP-GlcNAc, but not UPD-Glc, with a DXD to AAA substituted rLifA losing its ability to bind UDP-GlcNac and to inhibit T cell proliferation (Cassady-Cain *et al.*, 2016). This work suggests that LifA can act as a glycosyltransferase, similar to LCTs, but its *in-vivo* target is yet to be defined (Cassady-Cain *et al.*, 2016; Bease, 2020).

The CHD motif is found LifA is a cysteine protease associated with pathogenesis of some gram-negative bacteria. This motif is in YopT-like cysteine protease superfamily, and refers to three residues (cysteine, histidine aspartic acid i.e., CHD) critical for protease activity (Shao *et al.*, 2002). YopT is a *Yersinia* effector protein that cleaves host Rho GTPases resulting in the actin cytoskeleton degradation (Shao *et al.*, 2002). Substituting the LifA CHD residues to alanine residues had no impact on bacteria colonisation of calves but was crucial for lymphostatin activity. However, it was later noted that altered LifA protein was truncated and lacked the final 100 amino acids (Deacon *et al.*, 2010). The CHD motif is found in all but the C-terminally truncated LifA homologues and might be involved in autoproteolysis (Shao *et al.*, 2002). Indeed, recent studies support this idea (Bease, 2020). The potential role of the aminotransferase activity has not been assessed.

The objective of the studies undertaken for this chapter was to provide mechanistic insight on how LifA enables EPEC to inhibit Akt signalling in a T3SS dependent manner.

5.2 Results

5.2.1 Plasmid expression of LifA

Having shown that the EPEC T3SS dependent inhibition of Akt signalling depends on both LifA homologues, through redundant activities, studies explored the inhibitory mechanism. Studies focused on LifA as research tools were available for this - but not the LifA-like protein including LifA-expressing plasmids and antibodies (Cassady-Cain *et al.*, 2016). The *lifA* gene (9663bp) had been cloned into the pRham vector where its expression can be repressed or induced by adding repressor (0.5% glucose) and inducer (0.2% L-rhamnose) sugars respectively (Cassady-Cain *et al.*, 2016). The pRham-*lifA* (p*lifA*) plasmid encodes a C-terminally His-tagged LifA variant with the purified LifA-His protein retaining lymphostatin activity (Cassady-Cain *et al.*, 2016). In additional pRham-*lifA* plasmids were available that express LifA-His variants carrying the substitutions that disrupt the DXD (p*lifA*-ΔDXD) and cysteine protease (p*lifA*-ΔCHD) motifs (Cassady-Cain *et al.*, 2016).

As the activation-associated phosphorylation of Akt was recently reported to be controlled by glycosylation (Owaga *et al.*, 2015), the initial studies focused on p*lifA* and the ΔDXD variant (p*lifA*-ΔDXD) that lacks *in vitro* glucosyltransferase activity (Cassady-Cain *et al.*, 2016). Thus, both plasmids were introduced, by electroporation, into the Δ*lifA*/*lifA*-like double mutant (Cepeda-Molero *et al.*, 2017) with plasmid introduction supported by PCR analysis (Figure 51). Sequencing a small (~450bp) PCR-amplified fragment encompassing the region encoding the DXD motif confirmed introduction of the native and ΔDXD *lifA* variants respectively (not shown). The availability of anti-LifA antibodies allowed studies to investigate if the plasmids restored expression of a full-length LifA protein (~365 kDa). Thus, the strains were grown in the standard EPEC infection media (DMEM; contains 0.45% glucose) and, as a control, Lysogenic Broth (LB; lacks glucose) with media containing or not the repressor (0.5% glucose) and/or inducer (0.2% L-rhamnose) sugars (Giacalone *et al.*, 2006).

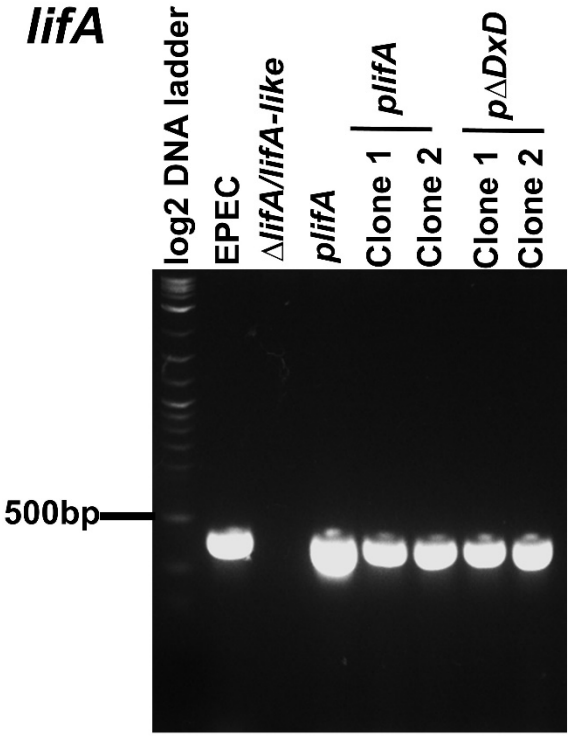


Figure 51 Introduction of plifA plasmid into the lifA-double mutant

Purified p*lifA* plasmid or total bacterial DNA - from indicated strains (released by boiling in water) - were used as templates in a PCR reaction with specific primers for amplifying a 488bp internal *lifA* fragment encompassing the sequence encoding the DXD (residues 557-559) motif. The reaction products were separated on a 1% agarose gel, alongside 2 log DNA markers, and visualised using gel green strain (see Materials and Methods). Strains used were EPEC, *lifA*-deficient double mutant (*lifA*/*lifA*-like) and 2 clones that potentially carried the plasmid encoding LifA (p*lifA*) or the LifA_ΔDXD (pΔDXD) variants. The position of 500bp DNA marker band is indicated.

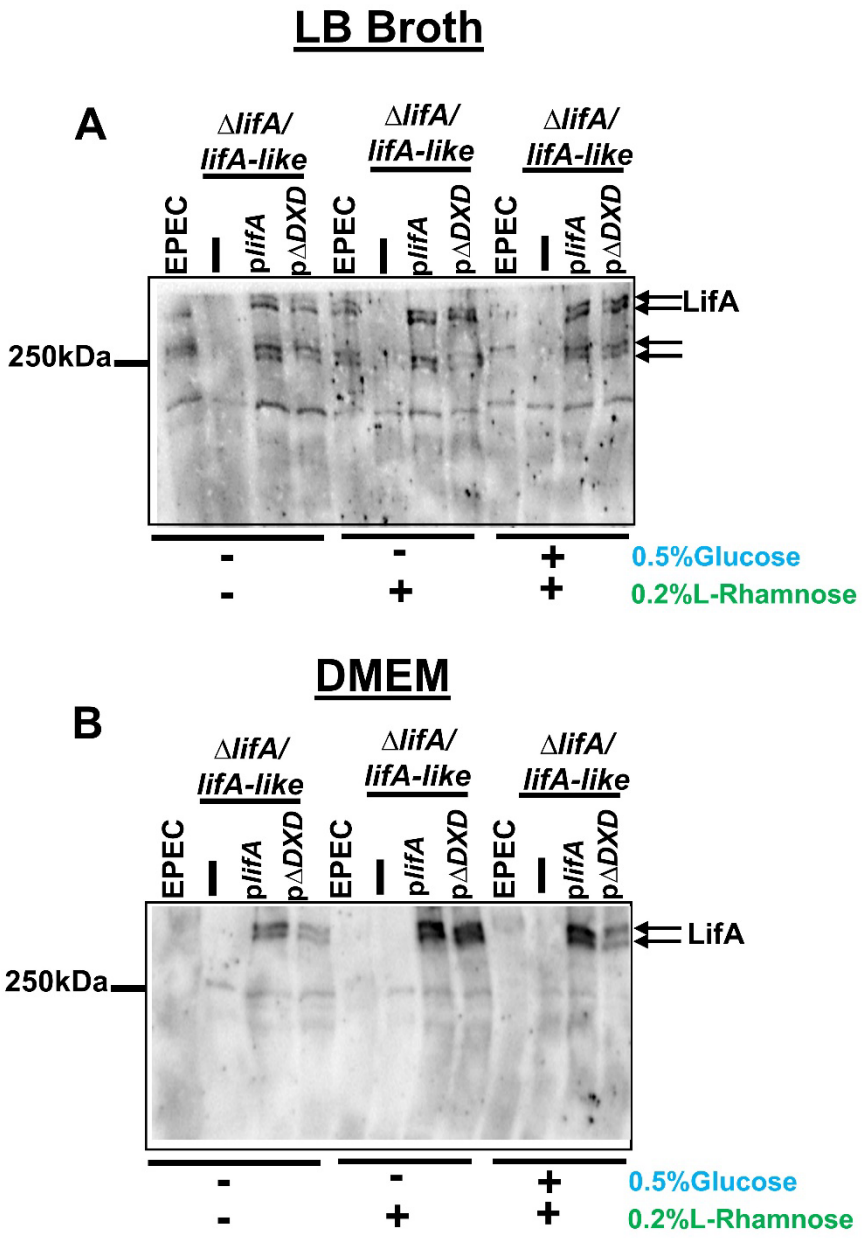


Figure 52 Anti-LifA antibodies detect LifA and a non-specific band

Indicated bacterial strains were grown in A) LB or B) DMEM in the absence (-) or presence (+) of added glucose (0.5%) or L-rhamnose (0.2%). Glucose represses plasmid expression of LifA while L-rhamnose induces expression (Giacalone *et al.*, 2006). Following a 2h growth period, total bacterial extracts were isolated and resolved on 6% SDS-PA gels before transferring to nitrocellulose and probing with anti-LifA antibodies. The position of the 250kDa molecular size marker and LifA-specific bands are indicated. Strains used were EPEC, the LifA deficient double mutant (Δ*lifA*/*lifA*-like) carrying no plasmid (-) or plasmids encoding the His-tagged LifA (p*lifA*) or LifA-ΔDXD (pΔDXD) variant. The ΔDXD variant lacks the DXD motif (residues changed to alanine residues) needed for its *in vitro* detected O-glycosylation activity (Cassady-Cain *et al.*, 2016).

Following a 2-hour incubation - time of a standard macrophage infection - total cellular extracts were isolated for Western blot analyses using the anti-LifA antibodies. The blots for LB grown cells revealed a non-specific band (<250kDa) - in extract from the LifA-deficient double mutant - and four LifA-specific bands (doublets of ~250kDa and >250KDa; Figure 52A). Surprisingly, these bands were detected independent of added repressor or inducer sugars (Figure 52A). Probing the samples from DMEM grown cells revealed a similarly sized non-specific band but only two LifA specific bands (doublet >250KDa). A LifA signal in EPEC was difficult to detect suggesting its normal expression level is very low in DMEM relative to LB (Figure 52B versus 52A). Again, plasmid encoded LifA was detected independent of one or both sugars though a higher LifA signal was seen for cells grown in DMEM containing the inducer sugar (Figure 52B). Attempts to detect the plasmid expressed LifA-His fusion protein using commercial anti-His antibodies were unsuccessful (not shown) indicating expression is below the detection level of this antibody.

This work confirms the successful introduction of the p*lifA* plasmid into the LifA deficient double mutant linked to the appearance of a single non-specific protein band and multiple (>250 kDa) LifA-specific bands. LifA could be detected in LB grown EPEC, but not the double mutant, with bands sizes like those of the plasmid expressed form. The latter suggests that the plasmid expresses a full length LifA_His fusion proteins but this could not be verified by the failure to detect the C-terminal His tag. Notably, bacterial growth in LB (versus DMEM) was linked to a higher LifA levels within EPEC with plasmid expression linked as two additional LifA-specific bands. Finally, plasmid expression of LifA was unexpectedly not inhibited by the repressor sugar but adding the inducer sugar to DMEM appeared to increase expression levels.

5.2.2 LifA expression does not require the CesT chaperone

Earlier work (Chapter 3; section 3.3.2) supported a previously suggested (Amin, 2017) key role for the multi-substrate chaperone, CesT, in EPEC's ability to inhibit Akt signalling. T3SS chaperones promote the delivery of substrate effectors by either increasing their stability within bacteria, preventing inhibitory interactions with other bacterial proteins and/or promoting efficient delivery to the T3SS export machinery (Thomas *et al.*, 2005). LifA has been shown to be an adhesin, a lymphostatin,

regulates T3SS functionality and is a T3SS secreted protein that appears to be delivered into infected host cells (Nicholls *et al.*, 2000b; Badea *et al.*, 2003; Klapproth *et al.*, 2005; Deacon *et al.*, 2010; Deng *et al.*, 2012; Cepeda-Molero *et al.*, 2017). However, it should be noted that the LifA delivery studies relate to an N-terminal region and not the full-length (365kDa) protein (Deng *et al.*, 2012).

The availability of antibodies to detect LifA provided the chance to examine the relationship between CesT and the LifA expression, secretion and/or delivery events. Initial studies examined LifA expression and secretion levels by infecting DMEM with EPEC and mutants lacking i) a functional T3SS, ii) both LifA homologues or iii) the CesT chaperone (see Materials and Methods). Following a 6 hour infection, total cellular and secreted proteins were isolated and resolved on 15% or 8% SDS-PA gels to visualised proteins by Coomassie blue staining and detect LifA by Western blot analysis (see Materials and Methods; Section 2.3.3) This work revealed typical cellular protein profiles but a few differences were evident in the Δ*lifA*/*lifA*-like double and Δ*core* (lacks 7 LEE-encoded proteins - 2 chaperones [CesT; CesF], 3 effectors [EspH, Map, Tir] and Intimin surface protein) mutant profiles (Figure 53A). Examining the secreted protein profiles revealed a typical profile with EPEC (Cepeda-Molero *et al.*, 2017) (Figure 53B) i.e., secretion of the 3 translocator proteins (EspA, EspB, EspD) and EspC autotransporter. As expected, the T3SS mutant did not secrete the translocators but, surprisingly, EspC levels were unusually low for this mutant (Figure 53B)(Kenny *et al.*, 1997a). The LifA-deficient double and CesT-deficient mutant strains secreted translocator and EspC proteins (Figure 53B) but there was no observable LifA (~365 kDa) band (see Supplementary Figure 12 & 13).

Western blot analysis of the bacterial extracts unexpectedly revealed more non-specific LifA bands than the previous shorter, 2hr growth period (Figure 53C versus Figure 52) with several strain-specific differences (Figure 53C). Importantly, the analysis revealed the LifA specific doublet (>250kDa) - as these bands were absent in the *lifA* double mutant extracts - but apparent in those from EPEC, the T3SS and CesT-deficient mutant strains (Figure 53C). The CesT-negative nature of the Δ*cesT* and Δ*core* mutant strains was experimentally supported in subsequent studies (see below). The findings are supported in additional studies (see Supplementary Figure 12 & 13) and indicates that LifA expression and/or stability in EPEC does not require CesT function.

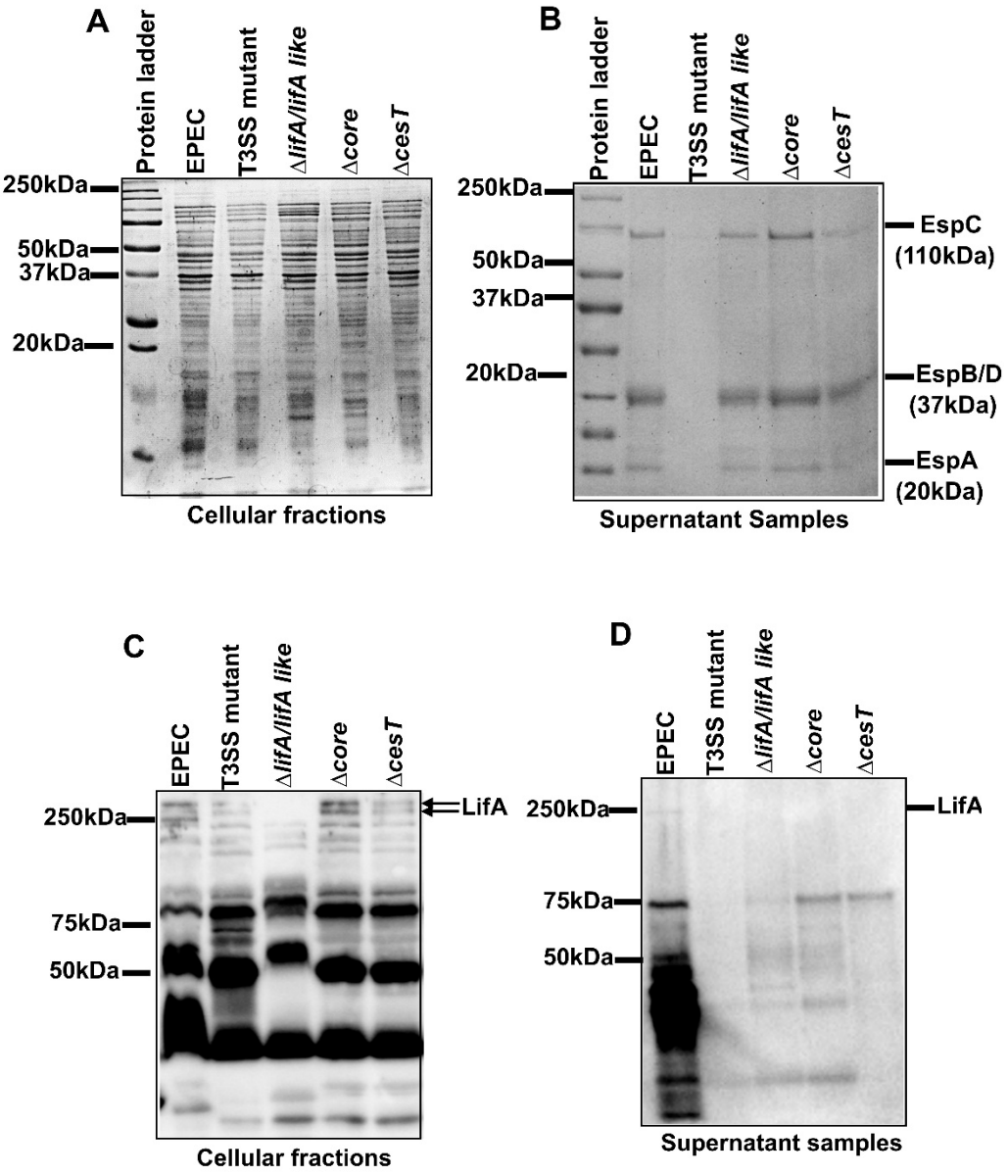


Figure 53 CesT-independent LifA expression

Indicated strains were grown in DMEM for 6 hours before isolating total cellular and secreted (supernatant) proteins (see Materials and Methods). The samples were resolved on **(A&B)** 15% SDS-PA gels and proteins visualised by Commassie blue staining or on **(C&D)** 8% SDS-PA gels for western blot detection of LifA. Positions of LifA, the secreted translocators (EspA, EspB, EspD) and EspC autotransporter proteins are indicated alongside some of molecular mass marker protein. Strains used were EPEC and mutants lacking a functional T3SS (T3SS), both LifA homologues (Δ*lifA*/*lifA*-like*)* and CesT (Δ*cesT* and Δ*core*). The Δ*core* mutant lacks the LEE region encoding 2 chaperones (CesT, CesF), 3 effectors (EspH, Map, Tir) and Intimin surface protein. These findings are supported by additional experiments (see Supplementary Figure 12 & 13).

Probing the secreted protein samples only revealed non-specific bands except in EPEC - where many bands were detected but not reproducibly (Supplementary Figure 12 & 13) - suggesting it relates to accidental loading of a cellular extract. Numerous attempts to detect LifA in the extracellular media were inconclusive linked to low signals i.e., at or below detection levels, poor gel resolution, protein transfer issues and/or the harsh concentration - chemical (TCA) - the secreted protein (See Materials & Methods). While LifA cellular levels were unaltered following loss of a functional T3SS or CesT protein, the obtained data hinted at LifA being secreted in a CesT dependent manner. LifA also functions as a surface adhesin (Nicholls *et al.*, 2000a) and thus CesT may direct a subpopulation of LifA to the T3SS.

5.2.3 LifA detection in the macrophage infection model

Studies next investigated whether the LifA antibodies would specifically detect LifA following a standard infection protocol in the J774A.1/pAkt assay model. Hence, macrophages were infected with LifA-positive and -deficient EPEC strains (pre-activated in the presence or absence of glucose for 2 hours) for the indicated times in media with or without added repressor/inducer sugars. Western blot analysis of the isolated total cellular extracts with the anti-LifA antibodies revealed inducer-related increases in the plasmid-encoded LifA protein (doublet >250kDa) as no corresponding signal were in the extract from uninfected cells or cells infected with the LifA-deficient double mutant. LifA detection in samples from cells infected with EPEC or the T3SS mutant was inconsistent but a prominent non-specific band was seen in all extracts, including non-infected cells (Figure 54). This work reveals that the anti-LifA antibodies can readily detect plasmid encoded LifA following the standard macrophage infection model (with levels increased by adding the inducer sugar) but detection of LifA in EPEC is more difficult, at least in this experiment, to detect.

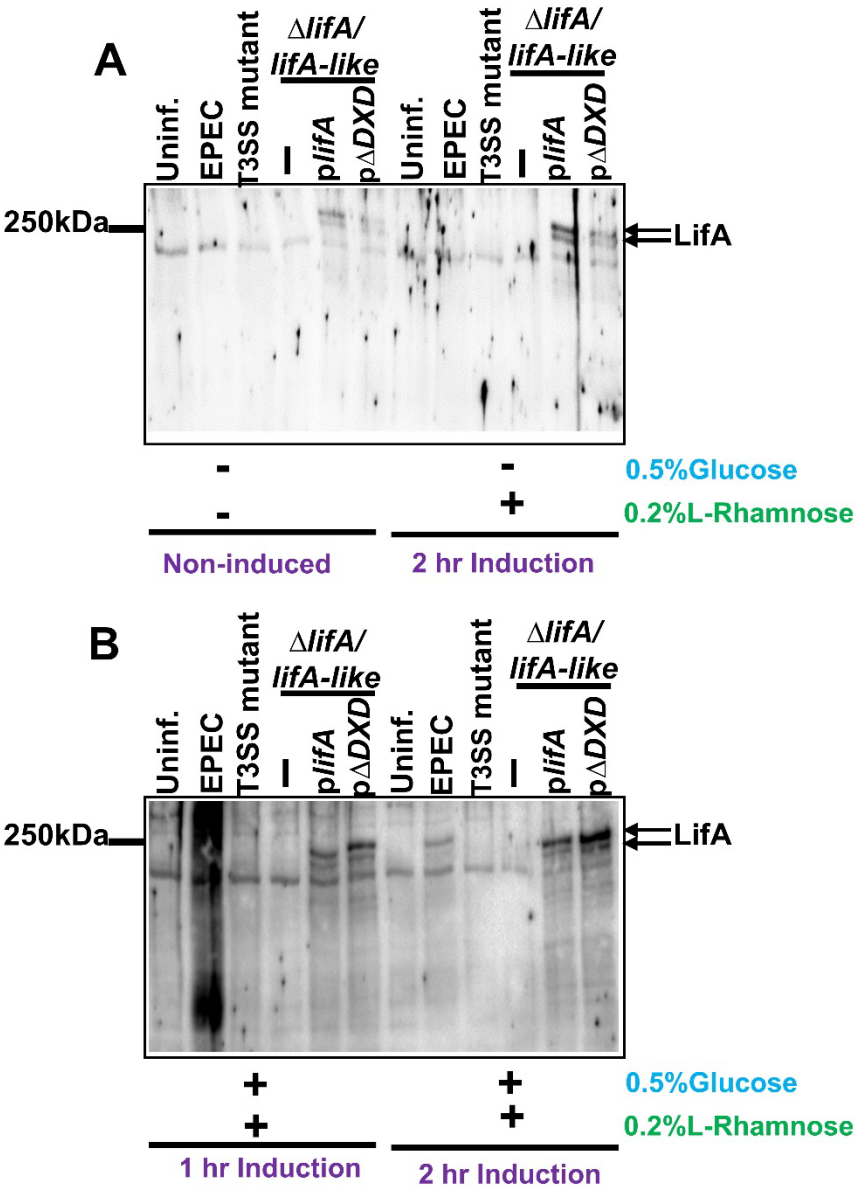


Figure 54 LifA detection in macrophage infection model

Indicated strains were preactivated in DMEM prior to a 2h infection of J774A.1 macrophage (MOI 200:1) in the presence (+) or absence (-) of added repressor (0.5% glucose) or inducer (0.2% L-rhamnose) sugars for indicated periods. The non-adherent bacteria were washed away before isolating remaining proteins in sample buffer for resolving on 6% SDS-PA gels and transferring to nitrocellulose for Western blot analysis probing for LifA Shown is the position of the LifA-specific bands and 250kDa molecular size marker protein. Strains used were EPEC and mutants lacking a functional T3SS (T3SS) or LifA homologues (Δ*lifA*/*lifA*-like). The Δ*lifA*/*lifA*-like carried no plasmid (-) or plasmids encoding LifA (p*lifA*) or the LifA-ΔDXD (p*ΔDXD*) variant with Uninfec. Indicating extracts from uninfected (Uninf.) macrophages.

5.2.4 T3SS-dependent LifA localisation to the host membrane

The next studies explored whether LifA is delivered into macrophage by examining if it could be isolated with host 'cytoplasmic' (saponin detergent-released) or host 'membrane' (Triton X100-detergent soluble) proteins (see Materials & Methods; section 2.3.2). These studies used a standard (2h) and extended (4h) infection period; latter to allow more LifA delivery which may improve detection by Western blot analysis. Probing the isolated cytoplasmic fractions for Tir confirmed that all strains, expect the T3SS mutant, had a functional T3SS (Figure 55A). Examining the same samples for LifA revealed a single, non-specific, band evident in uninfected cell samples (Figure 55A). Probing the corresponding membrane protein fractions revealed a non-specific (<250kDa) and a LifA-specific band (>250kDa) - latter as in sample from cells infected with EPEC but not uninfected or Δ*lifA/lifA*-like double mutant infected cells (Figure 55B). Importantly, the LifA signal was not detected in samples taken from cells infected with the T3SS mutant (Figure 55B). Probing for Tir confirmed that all strains, except the T3SS mutant, had a functional T3SS (Figure 55B). Notably, plasmid-expressing LifA in the *lifA*-double mutant was linked to a greater anti-LifA and anti-Tir signals (Figure 55B). This work reveals the T3SS-dependent association of LifA with host membrane proteins and linked plasmid expression of LifA with increased Tir delivery levels.

5.2.5 CesT-dependent localisation to the host membrane

Investigations next examined whether LifA localisation with host membrane proteins was dependent on CesT given its apparent key role for EPEC to inhibit Akt signalling (Chapter 3)(Amin, 2017). Hence, J774 macrophages were left uninfected or infected for 2 hours with the CesT-deficient and control strains before isolating host cytoplasmic and membrane fractions as before. However, only the membrane-related samples were resolved (6% SDS-PA gels) and processing for Western blot analysis. Probing for Tir supported strain genotype, as the T3SS mutant was unable to deliver Tir while the Δ*cesT* mutant delivered only low levels of Tir into the host membrane. Probing for LifA support CesT-dependent localisation of LifA with host membrane though there may be some T3SS-independent localisation (Figure 56A-B).

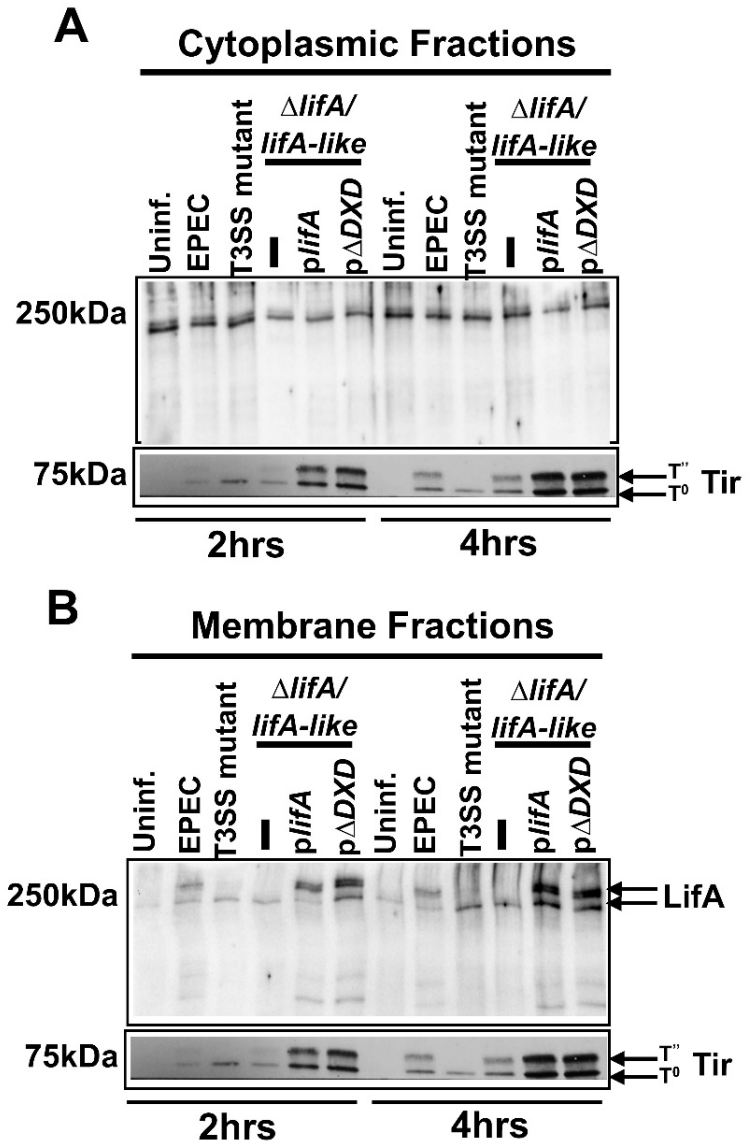


Figure 55 T3SS-dependent LifA localisation with host membrane proteins

Indicated strains were pre-grown for 2h in DMEM containing additional glucose (0.5%) prior to 2 or 4 hr infections of J774A.1 macrophages (MOI 200:1) in presence of the inducer sugar (0.2% L-rhamnose). Post-infection, the host cytoplasmic fraction (A) and membrane fractions (B) were isolated (see Materials and Methods) with samples resolved on 6% SDS-PA gels for Western blot analysis probing for LifA and Tir. The position of Tir - unmodified (T^o) and kinase modified (T'') Tir forms – and LifA proteins are indicated. Also shown are the position of two molecular weight marker proteins. Strains used were EPEC and mutants lacking a functional T3SS (T3SS) or both LifA homologues (Δ*lifA*/*lifA*-like). The Δ*lifA*/*lifA*-like mutant carried no plasmid (-) or plasmids encoding LifA (*plifA*) or the LifA-ΔDXD (*pΔDXD*) variant with Uninfec. Indicating samples from uninfected (Uninf.) macrophages.

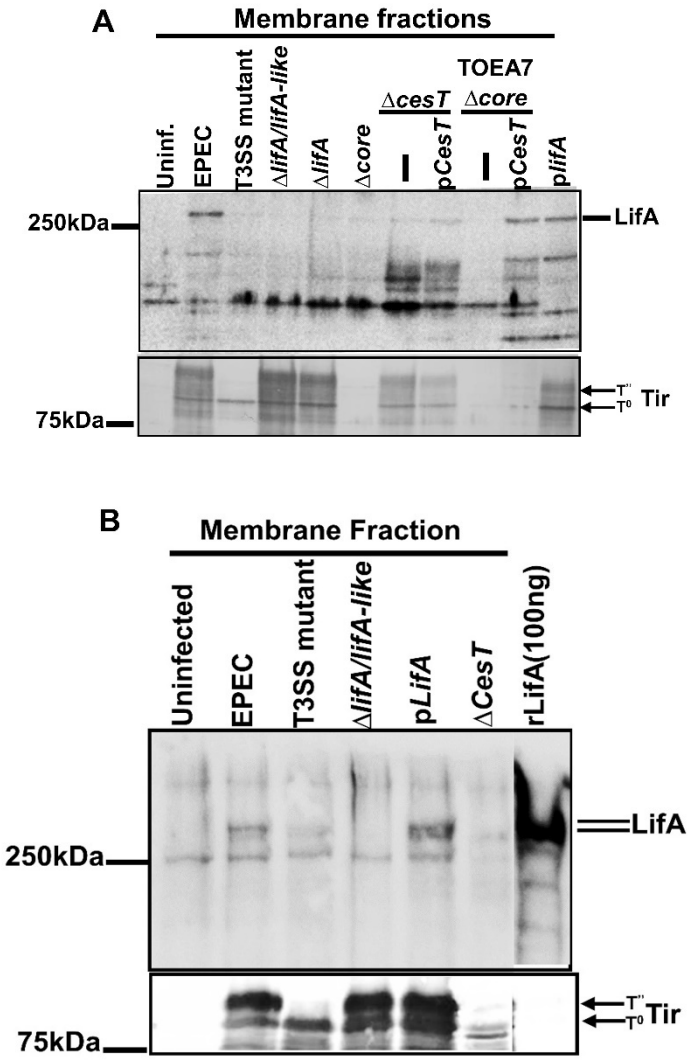


Figure 56 CesT-dependent localisation of LifA with host membrane proteins

Indicated strains were pre-grown for 2h in DMEM (contains additional 0.5% glucose) prior to a 2h infection of J774A.1 macrophages (MOI 200:1) in the presence of 0.2% L-rhamnose (promotes plasmid expression of LifA). Post-infection, the host cytoplasmic and membrane proteins were isolated with the membrane-related samples resolved on a 3-8% gradient SDS-PA gels before processing for Western blot analysis to probe for LifA and Tir. The position of Tir - unmodified (T^o) and kinase modified (T'') Tir forms – and LifA proteins are indicated alongside the position of two molecular weight marker proteins. Strains used were EPEC and strains lacking a functional T3SS (T3SS mutant), LifA (Δ*lifA*), both Lif*A* homologues (Δ*lifA*/*lifA*-like) or CesT i.e., Δ*cesT* and TOEA7Δ*core* mutants; latter lacks 14 of 17 known non-LEE effectors (not NleJ, EspC or LifA homologues) plus LEE-encoded chaperones (CesT, CesF), effectors (EspH, Map, Tir) and Intimin surface protein. Some strains carried plasmid encoding CesT (p*cesT*) or LifA (p*lifA*) with plasmid minus controls strain indicated (-). Note the last lane (labelled p*lifA*) relates to extracts from cells infected with the Δ*lifA*/*lifA*-like mutant carrying the p*lifA* plasmid.

To support this finding and improve band resolution, the experiment was repeated with additional controls before separating the membrane fractions on commercial gradient (3-8%) SDS-PA gels and processing for Western blot analysis. Probing for Tir supported strain genotype by confirming the CesT-deficient Δ*core* and TOEA7Δ*core* mutants lacked Tir with reduced and no Tir delivery by the Δ*cesT* (Abe *et al.*, 1999) and T3SS mutants, respectively (Figure 56A-B). Probing for LifA revealed several non-specific bands - i.e., in samples from the uninfected and *lifA* single/double mutant infected cells – with a prominent LifA-specific band (>250kDa) in samples from cells infected with EPEC (Figure 56 A&B). Importantly, this LifA-specific band was not evident in samples from cells infected with the T3SS or CesT-deficient mutant strains (Figure 56) i.e., Δ*cesT*, Δ*core* or TOEA7Δ*core* mutants. The Δ*core* mutant lacks 7 LEE genes - encoding 2 chaperones (CesT; CesF), 3 effectors (EspH, Map, Tir) and the Intimin surface protein - while TOEA7Δ*core* also lacks all known Nle effectors except NleJ, EspC and the LifA homologues (Amin, 2017). Importantly, re-introducing CesT (on a plasmid, p*cesT*) into the TOEA7Δ*core* mutant restored LifA detection to a level seen in the EPEC-infected cells. However, there was a less noticeable impact of introducing p*cesT* into the Δ*cesT* mutant (Figure 56) but probing for Tir revealed little additional Tir delivery suggesting that there was little or no plasmid expression of CesT (Figure 56). Collectively, this work suggests that LifA can associated with or, like Tir, insert into the host membrane in a manner dependent on EPEC having a functional T3SS and expressing the multi-substrate chaperone, CesT.

5.2.6 DXD motif-independent inhibition of Akt signalling by LifA

Studies have shown that mammalian cells can regulate Akt activity by glycosylating the Ser473 and Thr308 residues, which prevents phosphorylation-mediated activation of the kinase (Owaga *et al.*, 2015). As LifA has *in vitro* demonstrated glycosyltransferase activity that depends on the DXD motif (Cassady-Cain *et al.*, 2016), time course infection studies were carried out with the *lifA* double mutant carrying no plasmids or a plasmid encoding LifA (p*lifA*) or the ΔDxD (pΔDxD) variant (Cassady-Cain *et al.*, 2016). Western blot analysis of isolated total cellular extract samples confirmed sustained infection-induced pAktSer473 signals in cells infected by the T3SS or the *lifA* double mutant strains (Figure 57A-B). Probing for Tir confirmed EPEC and the *lifA* double, but not T3SS mutant had a functional T3SS (Figure 57C).

In contrast, cells infected with EPEC or the plasmid carrying *lifA* double mutant strains had transient pAktSer473 signal profiles (Figure 57C). Plasmid expression of LifA or ∆DxD variants was linked to a faster loss in pAktSer473 signal (Figure 57C). These results were supported by data from additional experiments (Supplementary Figure 14-16). The presence of the DXD to AAA substitution was supported by sequencing of PCR amplified DNA isolated from the strain isolated following the infection period (not shown). Probing the samples for Akt phosphorylation on Thr 308 (pAktThr308) showed similar profiles to the pAktSer473 signal (not shown). This work shows that EPEC requires a functional T3SS to prevent sustained phosphorylation of Akt (on Ser473 and Thr308 residues) in a manner dependent on LifA but not the DXD motif required for LifA's *in-vitro* demonstrated glycosyltransferase activity.

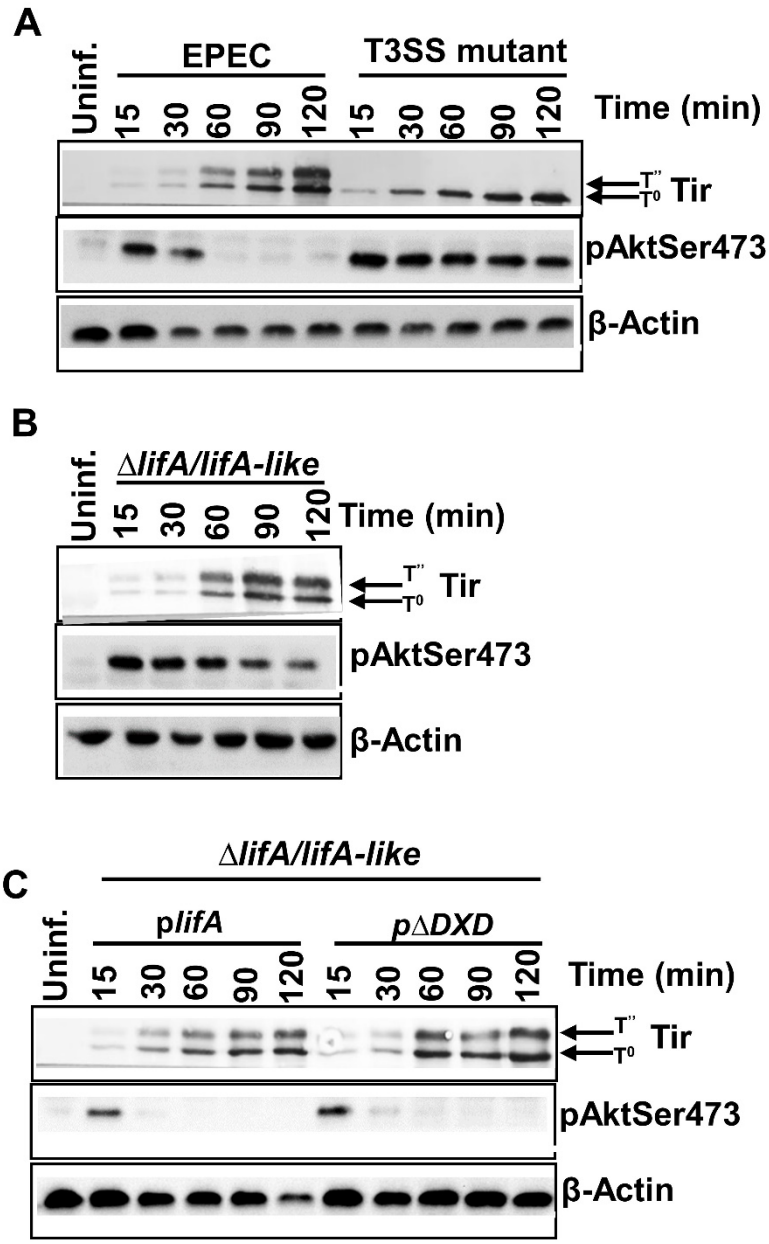


Figure 57 Loss of pAktSer473 signal does not require LifA DXD motif

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and strains lacking both LifA homologues (Δ*lifA*/*lifA*-like) and, when, indicated plasmids encoding LifA (p*lifA*) or the LifA-ΔDXD (pΔDXD) variant. The DXD is linked to LifA glycosyltransferase activity, The DXD motif is needed for glycosylation of sugar (See Supplementary Figures 14-16).

5.2.7 CHD motif-independent inhibition of Akt signalling by LifA.

Having ruled out a role for the DxD motif, studies examined the impact of disrupting cysteine protease associated CHD motif function and recently shown to be needed for auto-cleavage of host internalised, purifed LifA (Bease, 2020).Thus, another time course infection study was carried out with the *lifA* double mutant without or plasmids encoding LifA (p*lifA*) or varaints lacking a functional DXD (p∆DxD) or CHD (p∆CHD) motif. Western blot analysis probing for pAktSer473 revealed, as before, sustained infection-induced signals for cells infected with the T3SS and ∆*lifA*-double mutant strains (Figure 58A&B). However, infection with all plasmid-carrying ∆*lifA*-double mutant straines was linked to EPEC-like profile i.e. loss of pAktSer473 signal by 30 to 60 minute post-infection (Figure 58C). Probing for host actin showed loss of pAktSer473 signal was not due to gel loading or Western blot transfer issues (Figure 58A-C). These results were supported by data from an additional experiment (Supplementary Figure 17). The strains carrying plasmids encoding the ∆DXD and ∆CHD variants were supported by sequencing. PCR-amplified fragments from bacterial DNA extracted at the end of macrophage infection period (not shown). Together, these studies indicate that LifA's T3SS-dependent ability to inhibit Akt signalling does not require it to have intact DXD or CHD motifs that have been linked to LifA-mediated glycosyltransferase and cysteine protease activities respectively.

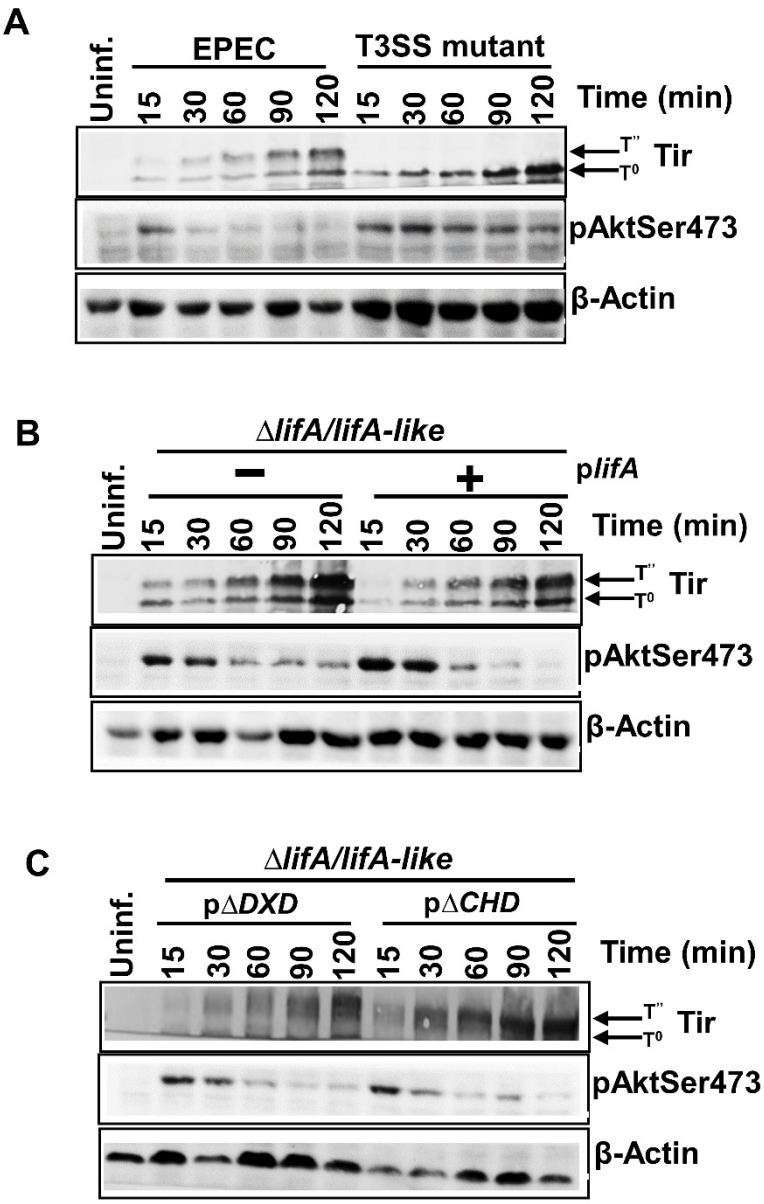


Figure 58 Loss of pAktSer473 signal does not require LifA CHD motif.

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and strains lacking both LifA homologues (Δ*lifA*/*lifA*-like). The Δ*lifA*/*lifA*-like mutant had no plasmid (-) or a plasmid (+) encoding LifA (p*lifA*), LifA-ΔDXD (pΔDXD) or LifA-ΔCHD (pΔCHD) variant. The DXD and CHD motifs are linked to glycosyltransferase and cysteine protease activities, respectively. These findings are supported by an additional experiment (see Supplementary Figure 17).

5.2.8 LifA inhibition of Akt signalling does not require other Nle effector activity

Previous work (Amin, 2017) suggested EPEC inhibition of Akt signalling does not require the functions of LEE or most of the known Nle effectors but the involvement of the LifA homologues, NleJ and EspC was not examined. Having now identified the reponsible effectors as the LifA homologues, studies examined whether the LifA inhibitory activity required other Nle effectors, including NleJ, EspC and LifA-like homologue. Hence, the LifA-encoding plasmid was introduced in EPEC0 as it lacks all known effectors, except EspB which is needed for the effector delivery process (Cepeda-Molero *et al.*, 2017). The p*lifA* plasmid was also introduced in EPEC2 - from which EPEC0 was generated (Cepeda-Molero *et al.*, 2017) - as it encodes Tir as a marker of T3SS functionality and also EspZ which prevent T3SS-dependent pAkt signal loss due to another mechanims i.e. cytotoxic death (Amin, 2017).

A standard time course infection study was carried out with isolated total cellular extracts processes for Western blot probing of Tir, pAktSer473 and actin. The Tir signal supported strain genotype as EPEC0, unlike EPEC2, lacks Tir with all Tir positive strains, except the T3SS mutant, having a functional T3SS (Figure 59). Probing for pAktSer473 revealed a sustained signal in cells infected with the T3SS-defective mutant and as expected, the *lifA*-deficient EPEC2 and EPEC0 strains. Unexpectedly, introduction of the *lifA*-expressing plasmid (p*lifA*) into EPEC2, but not EPEC0, was linked to loss of pAktSer473 signal though not as dramatic as for EPEC-infected cells (Figure 59A-C). Examining host actin levels showed that pAkt signal loss was not due to loading or Western transfer issues (Figure 59A-C). This work suggests that LifA's ability to interfere with Akt signalling does not require Nle effectors but may depend on LEE factors encoded by EPEC2 but not EPEC0 i.e., Tir and/or EspZ.

As a Tir-expressing plasmid (p*tir*) was available it was introduced in EPEC0 carrying the p*lifA* plasmid and used in another time course infection study. Probing for Tir (Figure 60A-C) provided data that supported the previous findings and confirmed that p*tir* was introduced into EPEC0 illustrating the strain has a functional T3SS (Figure 60D).

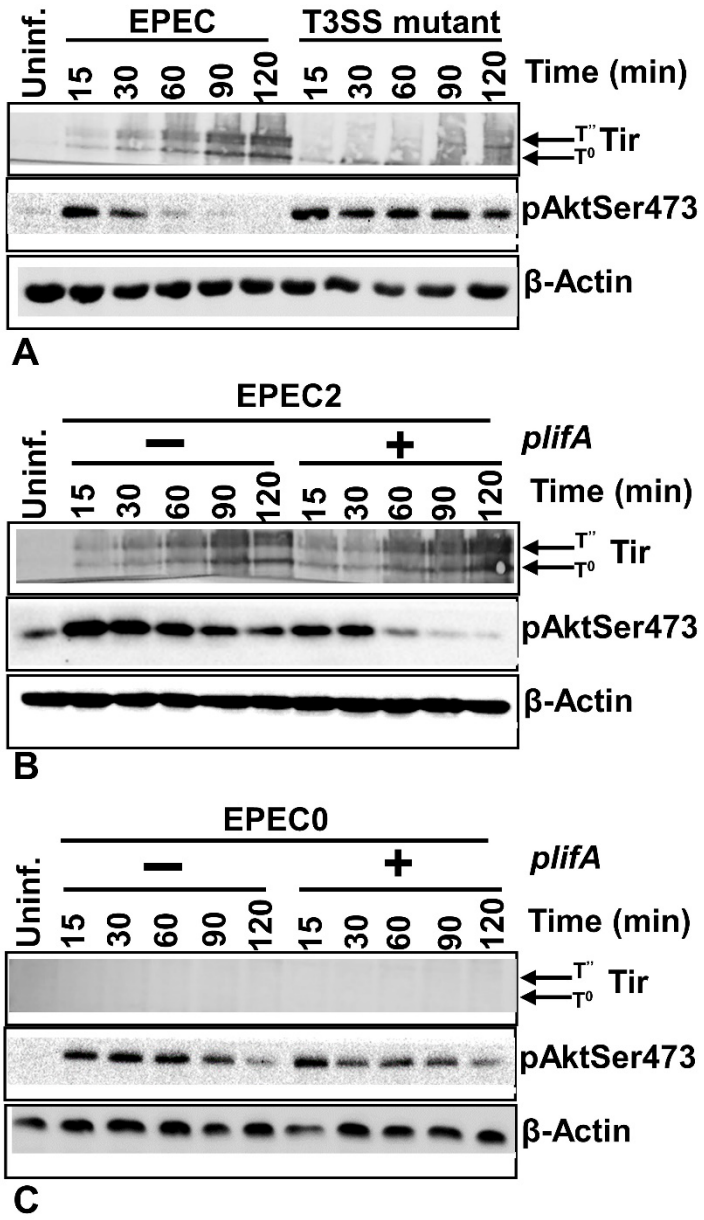


Figure 59 LifA dependent loss of pAkt signal does not require other NIe effectors

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant, EPEC2 (lacks all known effectors except EspB – needed for effector delivery process – Tir and EspZ) and EPEC0 (like EPEC2 but also lacks Tir and EspZ effectors). The EPEC0 and EPEC2 mutants either carried no plasmid (-) or a plasmid (+) encoding LifA (p*lifA*).

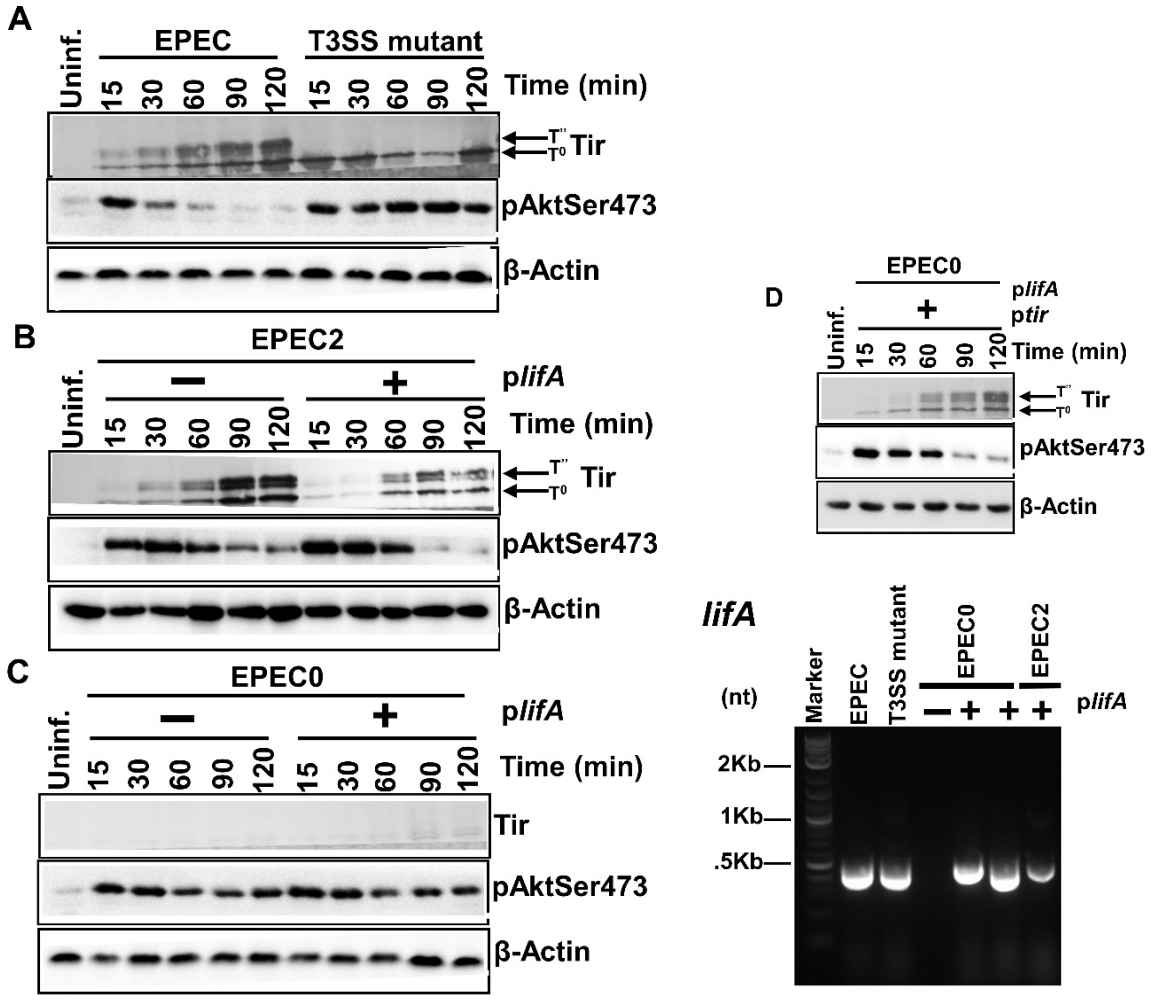


Figure 60 Possible role for Tir in the LifA driven loss of pAktSer473 signal.

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) to probe for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant, EPEC2 (lacks all known effectors except EspB – needed for effector delivery process – Tir and EspZ) and EPEC0 (like EPEC2 but also lacks Tir and EspZ effectors). The EPEC0 and EPEC2 mutants had no plasmid (-) or a plasmid (+) encoding LifA (p*lifA*) or Tir (p*tir*).

Probing the samples for pAktSer473 data (Figure 60A-C) reproduced the previous data (Figure 59) and revealed that only EPEC0 which carried the Tir and LifA plasmids had regained some ability to interfere with Akt signalling (Figure 60D). Probing for actin revealed that the loss of pAktSer473 signal was not due to gel loading or Western blot transfer issues (Figure 60). Collectively, these experiments indicate a role for Tir in the LifA dependent loss of pAkt signal despite previous studies ruling out a role for Tir alone (*tir* mutant)(Amin, 2017) or with most non-LEE-encoded (TOEA7Δ*core*/p*cesT*) effectors (Chapter 3).

5.2.9 pAkt signal loss is linked to O-GlcNAcylation of Akt

As mentioned, mammalian cells can regulate Akt function through O-GlcNAcylation of serine 473 and threonine 307 residues which inhibits phosphorylation-associated activation (Owaga *et al.*, 2015). O-GlcNAcylation involves the addition of a single N-acetylglucosamine (O-GlcNac) to serine or threonine residues. While LifA can bind GlcNAc and has DXD motif-dependent glycosyltransferase activity (Cassady-Cain *et al.*, 2016), earlier studies ruled out a need for the DXD motif in LifA-dependent inhibition of Akt signalling (Chapter 3). Nevertheless, it was still possible that the LifA inhibitory mechanism involved O-GlcNAcylation.

To investigate this hypothesis, Akt was immunoprecipitated from J774A.1 macrophage following infections with EPEC or the T3SS mutant with samples probed, by Western blot analysis, for pAktSer473 and O-GlcNac modified forms. Hence, J774A.1 macrophage were infected for 2 hours before isolating Triton X100 soluble fractions (contains host cytoplasm and membrane proteins plus EPEC delivered effectors) with a sample taken (Input) as a reference. The remaining solution was used for overnight immunoprecipitation with anti-Akt antibodies coupled to magnetic beads (see Materials and Methods). The following day the beads were isolated, and a sample of the post-immunoprecipitation solution was taken (‘Output’) to determine immunoprecipitation efficiency. The immunoprecipitate was washed several times before resuspending in sample buffer (see Materials and Methods; Section 2.3.5) and examined by Western blot analysis for pAktSer473, Tir, actin and O-GlcNac-modified proteins.

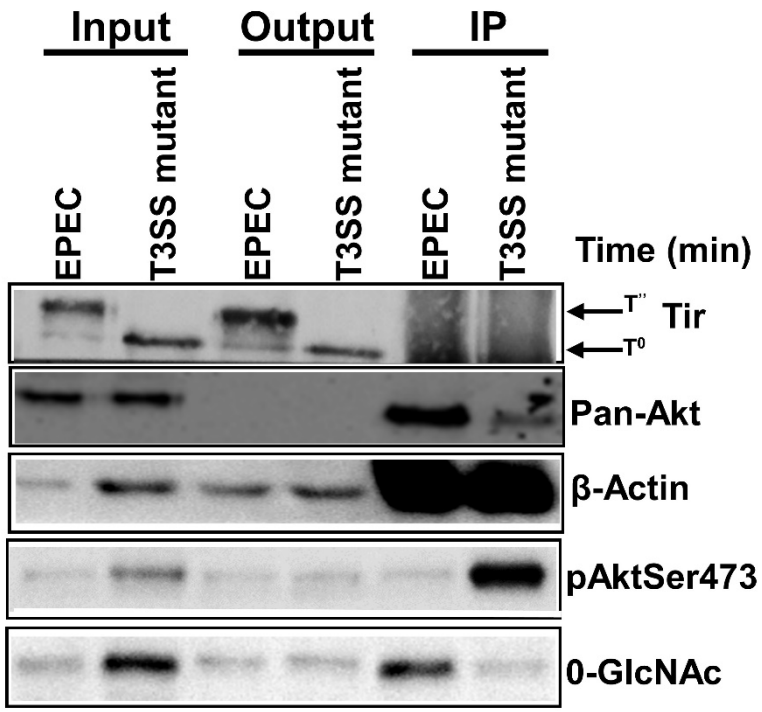


Figure 61 EPEC inhibition of Akt signalling is linked to Akt O-GlcNAcylation

J774A.1 macrophage were infected with, pre-activated, EPEC or T3SS mutant strains (MOI 200:1) for 2 hours. Following the removal of non-adherent bacteria, the J774A.1 macrophage were extracted in a 1% Triton-X 100 solution with removal (centrifugation) on the insoluble fraction leaving a solution containing host cytoplasm and membrane proteins plus T3SS-delivered substrates. A reference sample (Input) was removed before adding anti-Akt antibodies pre-coupled to magnetic beads for overnight incubations to 'capture' Akt. The following day the beads were isolated, and a sample of the remaining solution (Output) was taken. The beads (immunoprecipitate) were washed several times before these, and other samples, were resolved on 6% SDS-PA gels and processed for Western blot analyses to probe for Tir (T3SS functionality marker), total Akt (Pan-Akt), pAktSer473, β-actin (loading control) and O-GlcNAc modified proteins. Arrows indicate the position of unmodified (T^0) and host kinase-modified (T'') Tir forms. The findings are supported by data from an additional experiment (see Supplementary Figure 18).

Probing for Tir confirmed strain genotype as the host kinase modified form (T'') was detected in Input and Output samples from extracts of EPEC, but not T3SS mutant infected cells (Figure 61). Examining the samples for total Akt (with pan-Akt antibodies) revealed similar levels in the input samples with no signal in the output samples showed a very high immunoprecipitation efficiency (Figure 61). Importantly, both immunoprecipiate samples contained Akt, but not Tir, supporting specific Akt isolation (Figure 61). Probing for actin revealed similar signals in the input and output samples supporting similar gel loading with the strong 'actin' signal in the immunoprecipitate an artefact i.e detecting antibody using to immunoprecipitate Akt. Crucially, probing for pAktSer473 supported T3SS mutant dependent loss of pAktSer473 signal - most evident in the immunoprecipitation samples - with background and strong signals in samples isolated from EPEC and T3SS mutant infected cells respectively (Figure 61). Surprisingly, studies with a monoclonal antibody that detects proteins containing O-linked N-acetylglucosamine (O-GlcNAc) residues revealed an infection-associate signal in the input sample of T3SS infected cells and immunoprecipiate from EPEC infected cells (Figure 61).

While this finding was suggestive of a sample mix-up, it was confirmed through experimental repeats using non-infected cells (Supplementary Figure 18) and changing other variables (see below). Hence this work links EPEC's T3SS dependent ability to inhibt Akt signalling to O-GlcNAcylation of the kinase.

5.2.10 O-GlcNAcylation of Akt by EPEC depends on LifA

To further investigate the predicted link between infection-associated O-GlcNac modification of Akt and LifA protein function, immunoprecipitation experiments were repeated to include additional controls i.e. uninfected cells and cells infected with the LifA-deficient (∆*lifA/lifA*-like) double mutant. Probing the isolated input, output and immunoprecipitate samples for Tir confirmed that EPEC and the *lifA*-deficient double mutant, unlike the T3SS, mutant had a functional T3SS (Figure 62). As expected, Tir was not detected in the uninfected cell or any immunoprecipitate samples (Figure 62). Probing for pAktSer473 illustrated that EPEC, but not the the T3SS and *lifA*-deficient

double mutant inhibited Akt signalling with the resultant mutant-asociated pAktSer473 evident in the immunoprecipiate but not output samples (Figure 62). Probing for O-GlcNAc modified proteins revealed signals in the input samples from only the T3SS and *lifA*-deficient double mutant indfected cells and one immunoprecipitate which was surpsingly from the EPEC infected cells (Figure 62).

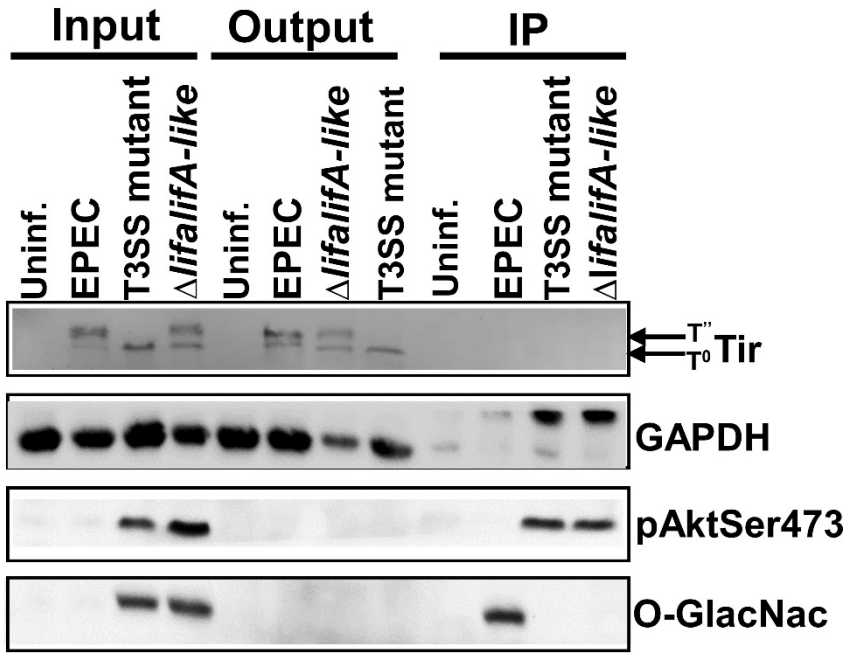


Figure 62 LifA inhibition of Akt signalling is linked to O-GlcNAcylation of Akt

J774A.1 macrophages were infected with, pre-activated, EPEC strains (MOI 200:1) for 2 hours. Following the removal of non-adherent bacteria, the J774A.1 macrophages were extracted in a 1% Triton-X 100 solution with removal (centrifugation) on the insoluble fraction leaving a solution containing host cytoplasm and membrane proteins plus T3SS-delivered substrates. A reference sample (Input) was removed before adding anti-Akt antibodies pre-coupled to magnetic beads for overnight incubations to 'capture' Akt. The following day the beads were isolated, and a sample of the remaining solution (Output) was taken. The beads (immunoprecipitate) were washed several times before these, and other samples, were resolved on 6% SDS-PA gels and processed for Western blot analyses to probe for Tir (T3SS functionality marker), total Akt (Pan-Akt), pAktSer473, β-actin (loading control) and O-GlcNAc modified proteins. Arrows indicate the position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC and mutants lacking a functional T3SS (T3SS) or both LifA homologues (Δ*lifA*/*lifA*-like). These findings were supported by data from an additional experiment (see Supplementary Figure 19).

Similar input and output samples loading were supported by probing for the host protein, GAPDH (Figure 62). Unfortunately, probing for total Akt did not generate a signal but Akt immunoprecipitation is illustrated by detecting phosphorylated Akt in input and immunoprecipitate but not output samples from cells infected with the T3SS and *lifA*-double mutants (Figure 62). Absence of Tir and GAPDH proteins from the immunoprecipitate samples supports the specific isolation of Akt (Figure 62). These findings were supported by data from an additional experiment (see Supplementary Figure 19). This work shows that EPEC infection not only leads to the T3SS-independent phosphorylation of Akt on Serine 473 but also the O-GlcNAcylation of protein(s) sharing a similar molecular mass to Akt. Crucially, the analysis shows that the EPEC LifA protein can, in a T3SS-dependent manner, inhibit Akt signalling linked to O-GlcNAc-modification of Akt. Moreover, these findings suggest that EPEC infection also leads to the GlcNAc-modification of Akt-sized protein(s) with this modified form 'lost' in a T3SS/LifA-dependent manner and, for extracts from T3SS mutant-infected cells, during the overnight immunoprecipitation period.

5.3 Discussion

The studies in this chapter provide important insights on the mechanism by which LifA inhibits Akt signalling by revealing a CesT-dependent co-localisation of LifA with host membrane proteins and LifA-dependent O-GlcNAc modification of Akt. Crucial to these studies were the availability of research reagent (provided Prof. Mark Stevens group, Edinburgh University), i.e. plasmids encoding LifA variants, antibodies to detect LifA and purified recombinant LifA::His protein

The provided p*lifA* plasmids encoded a LifA::His tagged variant whose expression is tightly repressed in presence of 0.5% glucose and induced by another sugar, 0.2% L-rhamnose (Cassady-Cain *et al.*, 2016). Surprisingly, studies with the *lifA*-double mutant carrying p*lifA* revealed constitutive expression of LifA in standard growth (LB; lacks glucose or L-rhamnose) or mammalian infection (DMEM; contains 0.45% glucose but not L-rhamnose) media with little impact of adding 0.5% glucose. Interestingly, LifA expression from the EPEC chromosome was difficult to detect when grown in DMEM (induces T3SS expression) than LB suggesting that LifA expression may be downregulated when the T3SS is expressed. However, it appeared that adding L-rhamnose increases expression of the plasmid-encoded, but not chromosomal-encoded LifA. Crucially, the LifA antibodies revealed bands in EPEC which were absent from the LifA-deficient single or double mutant unless they carried p*lifA*. While plasmid encoded LifA had a C-terminal His tag this could not be detected with anti-His antibodies (probably below detection sensitivity level), so studies could not provide further support for p*lifA* expressing a full-length (3223-residue) protein. It was noted that strain grown DMEM led to a LifA doublet (both >250kDa) suggestive of possible modification or cleavage-forms with a further double (<250kDa) when grown in LB suggestive of further processing/cleavage within the bacteria. It should be noted that LifA has been shown to have autoproteolytic activity, linked to ~225 and 140kDa bands, but this process required host factors (Bease, 2020).

EPEC was shown to inhibit Akt signalling in a CesT dependent manner (Chapter 3)(Amin, 2017) so the availability of LifA-specific antibodies allowed studies to examine if the multi-substrate chaperone impacted to LifA expression, secretion or delivery into host cells. This work revealed that LifA expression does not require CesT

with studies on LifA secretion inconclusive due to very low secretion levels and/or technical issues though there were hints of CesT-dependent release. Previous studies have reported LifA to be a T3SS substrate (Deng *et al.*, 2012; Bease, 2020).

One function of LifA is as an adhesin and indeed the cell binding capacity of non-pathogenic *E. coli* was dramatically increased when it carried plifA (not shown). The latter suggests LifA is transferred to the cell surface in a T3SS independent manner with, perhaps, CesT acting to direct a subpopulation to the T3SS and hence CesT absence would have little impact on LifA cellular levels. Most importantly, the work clearly revealed LifA, following EPEC infection of macrophages, co-localised with host membrane proteins in a CesT and T3SS-dependent manner. LifA is not predicted to have transmembrane domains (Luo and Donnenberg, 2011; Bease, 2020) suggesting that it is a membrane-associated rather than membrane inserted. Given LifA's inhibitory activity is T3SS-dependent this suggests LifA is first transferred into the cytoplasm and then associates with host membranes. Further studies should examine if LifA associates with the plasma or other membrane compartments especially given the recent report that Akt is activated at the endosome membrane (Thapa *et al.*, 2020). Such studies could use published protocols and/or commercial kits to isolate distinct subcellular compartments for western blot probing for LifA. Moreover, studies with proteases and/or reagents (including high salt or pH solutions) that extract membrane-associated proteins could be used to determine if LifA is on the cell surface or if LifA is only a membrane-associated protein (Kenny and Finlay, 1995). Moreover, the *anti-LifA* antibodies might be able to, in epifluorescent and/or confocal microscopy studies, specifically detect a T3SS-dependent LifA signal on or within infected host cells.

Notably studies with LifA variants lacking motifs required for glycosyltransferase and cysteine protease activities detected in infection and/or *in vitro* studies (Bease, 2020) were not required for LifA to inhibit Akt signalling. Sequencing of DNA from bacteria isolated at the end of the infection experiment confirmed the presence of variants with disrupted DXD and CHD motifs required for LifA glycosyltransferase and cysteine protease activity respectively. This finding highlights the multifunctionality of LifA, a common feature of T3SS effectors (Cassady-Cain *et al.*, 2016). Informatively, studies with effector deficient strains suggested that LifA's inhibitory activity does not require other known Nle effectors and supported functional redundancy with the LifA-like protein. However, these studies surprisingly indicated a possible role for Tir despite

Tir being previously shown not to be required, individually or with most known effectors, for EPEC to inhibit Akt signalling (Amin, 2017). These studies involved effector-deficient strains (EPEC2 and EPEC0) and so it is possible that this Tir role is only evident in specific genetic backgrounds - akin to studies identify roles for both LifA homologues in the attaching and effacing phenotype (Cepeda-Molero *et al.*, 2017).

The most exciting and surprising findings came from studies examining the possibility that EPEC infection leads to O-GlcNAc modification of Akt as this was reported to be a host mechanism for regulating Akt activity (Owaga *et al.*, 2015; Zhuang *et al.*, 2017). Indeed, immunoprecipitation studies (with anti-Akt antibodies) confirmed a T3SS- and LifA-dependent loss of the infection-induced pAktSer473 signal linked to O-GlcNAc modification of Akt. Surprisingly, infection by the T3SS and LifA-deficient mutants was linked to O-GlcNAc modification of an Akt-sized band/s which was 'lost' during the immunoprecipitation process thereby preventing studies to determine if the protein is indeed Akt. Finding ways to preventing loss of the O-GlcNAc modified protein (by, for example, screening inhibitors of protein proteases or deglycosylation activity) would enable studies to determine its linkage to Akt. Notably, preliminary studies report EPEC infection also leads to transient O-GlcNAc modification of this Akt-sized band (not shown). Further studies should also determine if O-GlcNAc modification of this Akt-sized band is EPEC-specific i.e., can it be induced by non-pathogenic *E. coli*, other pathogens, or even bacterial-sized beads. Examining this novel O-GlcNAc modification event could open a new research area.

In conclusion, the findings presented in this chapter suggest that CesT targets a subpopulation of LifA to the T3SS for transfer to infected host cells where its association with a host membrane compartment is linked to O-GlcNAc modification of Akt - an event that inhibits kinase activity. Interestingly, this LifA inhibitory activity is independent of motifs needed for its known glucosyltransferase and cysteine protease functions.

Chapter 6. Final discussion

Chapter 6 Final Discussion

The research described in this thesis not only achieved the main objectives of identifying the effectors enabling EPEC to inhibit Akt signalling and to provide mechanistic insights but also revealed roles for other EPEC-encoded factors and novel infection-induced modifications of host protein/s.

To define the responsible effector(s) with multiple approaches were undertaken, in parallel, with a focus on the 4 T3SS substrates (NleJ, EspC, LifA, LifA-like protein) whose contributions to the inhibitory process had not yet been examined (Amin, 2017). Studies with an available gene knock mutant (Δ*espC*) argued against a key role for EspC (O. Amin, unpublished) while use of an available suicide vector (Klapproth *et al.*, 2000) to disrupt *lifA* (Δ*lifA::Km*) allowed studies arguing that LifA was also not critical. However, it was possible that the remaining intact N-terminal-encoding region (~2000 residues) could be expressed and delivered into host cells where it may have functions needed to inhibit Akt signalling but subsequent studies revealed that this was not the case. Work to knockout all 4 T3SS substrate-encoding genes - individually and collectively - was not needed due to another approach identifying the responsible effector.

A second possible approach was promoted (but not used) by generating data supporting the idea of the Akt inhibitory process requiring the LEE-encoded multi-substrate chaperone, CesT. Hence, this opened the possibility of isolating CesT-interacting proteins (from EPEC lacking all CesT and T3SS substrates know not to be needed for the inhibitory process) to identify candidate effectors by mass spectrometry for further exploration. Future study should examine predicted CesT-LifA and, when antibodies available, CesT-LifA-like interactions by immunoprecipitating from EPEC a plasmid expressed CesT::HA fusion protein - retains known chaperone functions - with anti-HA antibodies and determining if LifA (and/or LifA-like) coimmunoprecipitate.

A third approach was to screen available genome sequenced A/E pathogens strains (have defined but distinct Nle effector repertoires) to determine if the inhibitory process is unique to EPEC or shared by one or more strains to perhaps indicate candidate effector/s to explore. As mentioned, roles for all EPEC but 4 T3SS substrates had been discounted with interestingly the available A/E pathogens all encoding EspC and LifA-type homologues. The screening program was consistent with the examined

second EPEC strain (B171), rabbit-EPEC and *Citrobacter rodentium* all possessing a T3SS-dependent capacity to inhibit Akt signalling. Interestingly, the examined EHEC O157:H7 strain appeared to inhibit Akt signalling via additive T3SS-dependent and -independent mechanisms. The subsequent discovery that EPEC uses the LifA homologues to inhibit Akt provides a possible explanation. Hence, most EHEC strains lack an intact *lifA* gene - have an *orf2* variant (*efa1'*) that encode the N-terminal 433 residue and a central 275 residue region respectively - but encode another homologue, ToxB, on a plasmid (Tatsuno *et al.*, 2001). ToxB shares 30% identity (48% similarity) with EPEC LifA with its expression linked, like LifA, to lymphostatin-like activity and promoting bacterial adherence (Burland *et al.*, 1998; Stevens *et al.*, 2002; Klapproth, 2010a; Cassady-Cain *et al.*, 2017). It is predicted that the *efa1'* gene product might produce a T3SS substrate that has some ability to inhibit Akt signalling with ToxB providing an additional, T3SS-independent, inhibitory mechanism. This possibility could be addressed by examining *efa1'* and *toxB* single and double mutants for their ability to inhibit Akt signalling by EHEC strains with and without a functional T3SS. In addition, studies could examine the predicted role for LifA homologues in enabling other A/E pathogens to inhibit Akt signalling and test the prediction that strains that lack LifA-homologues can't inhibit Akt signalling. If an inhibitory activity is uncovered for the *efa1'* gene products this could be useful for providing mechanistic insights as a much small protein (708 residues in total) than the 2600-3000 residue LifA, ToxB and LifA-like proteins.

The strategy that identified the responsible effectors related to screening available mutants that lacked, individually, most of the horizontally acquired DNA (PPs and IEs), as all known T3SS substrates are encoded on such elements. Indeed, the screen revealed the strain lacking IE2 (~61Kb, encodes LifA-like protein and the NleE2 effector) could not inhibit Akt signalling. As a role for NleE2 had been discounted this implicated the LifA-like protein or other IE2-encoded factors. Luckily, a bank of EPEC mutants was available that lacked (in a sequential manner) all genes for known Nle effectors including those for NleJ, EspC, LifA and the LifA-like protein (Litvak *et al.*, 2017). Crucially, the new screen revealed a critical role for the LifA-like protein but surprisingly in a redundant manner with LifA that is encoded on IE6 (~16Kb; also encodes three Nle effectors). Redundant roles were confirmed with studies using available *lifA*-deficient single and double mutants plus a newly-generated double

mutant which also illustrated the *lifA*::km gene does not produce a protein that can inhibit Akt signalling. Importantly, the inhibitory defect of the double mutants could be rescued by introducing a plasmid encoding a LifA::His tagged fusion protein reiterating redundancy.

The finding that the IE2-deleted mutant could not inhibit Akt signalling despite having an intact *lifA* gene suggested IE2 encodes factors needed for LifA to inhibit Akt signalling, or strain has unknown mutations in *lifA* or other genes needed for the LifA inhibitory process. The former hypothesis was supported by obtaining the same findings with a newly generated IE2-deficient strain leading to further studies. Attempts to rescue the mutant defect by introducing plasmids carrying 4.5Kb IE2 fragments only result in one being introduced into the mutant but it rescued the defect. Subcloning implicated a 1.7Kb fragment which has only I large *orf*'s encoding a putative 611 residue protein (E2348C_1074) that is the only IE2-encoded gene product, besides the LifA-like protein, predicted to possess a T3SS signal sequence. However, the cloning strategy resulted in a 5' gene truncation (the intact gene was to have been cloned on another targeted fragment) removing the N-terminal ~72 residues. However, expression may occur from an internal start codon to produce 537 residue variants. Surprisingly, bioinformatics analyses suggest that the truncated gene was cloned was inframe with the plasmid *tet* gene (into which it was inserted) thereby, in theory, allowing the production of a 620-residue hybrid protein. The first 21 residues of T3SS substrates carry the export signal with, when needed, chaperone bindings sites within the first 80-100 residues which would be absent from the truncated or fusion proteins. The latter questions Orf16 being a T3SS substrate that functions to enable LifA (IE6-encoded) to inhibit Akt signalling. Orf16 is predicted to be a cytoplasmic protein and thus could be required for LifA expression and/or transfer into host cells. These possibilities can be addressed by using the available anti-LifA antibodies to determine levels in bacterial extracts and the host membrane fraction. Unfortunately, there was not sufficient time to carry out this or other planned studies. The latter included C-terminally HA tagging the truncated and full length Orf611-related proteins for cellular fractionation and/or epifluorescent microscopy studies to examine their cellular location in EPEC (cytoplasm, membrane, periplasm, extracellular) and/or, in a T3SS-dependent manner, host cells. Other studies were going to interrogate a key role for

the Orf611-encoding *orf* (and not other small *orfs* or potential regulatory RNAs) by specifically preventing its production though the introduction of an internal stop codon. Lymphostatin, the 366 kDa *lifA* gene produce, inhibits lymphocyte proliferation and transcription of multiple lymphokines (Klapproth *et al.*, 1996; Klapproth *et al.*, 2000), acts as an adhesin (Torres *et al.*, 2005) and is a T3S substrate that may be delivered into host cells (Deng *et al.*, 2012). Studies on how LifA inhibits Akt signalling focused on variants lacking motifs suggestive of it having glycosylation and protease activities. One motif (DXD) is needed for LifA to bind a sugar (UDP-GlcNAc) and for lymphostatin activity suggesting LifA acts as a glycosyltransferase (Cassady-Cain *et al.*, 2016). The second motif (CHD) was recently shown to be needed for LifA processing within mammalian cells (Bease, 2020). However, LifA's roles in inhibiting Akt signalling did not require either motif whose disruption was supported by DNA sequencing. The report (Shi *et al.*, 2015) of host cells regulating Akt activity by competitive modification of Ser473 and Thr308 by a phosphate (activates) or O-GlcNAc (prevents activation-associated phosphorylation) prompted studies to determine if EPEC infection leads to O-GlcNAc modification of Akt. The resulting anti-Akt immunoprecipitation studies clearly revealed a T3SS- and LifA-dependent O-GlcNAc modification. Studies are needed to test the prediction that the sugar has been added to the Ser473/Thr308 residues by, for example mass spectrometry (Brunet *et al.*, 2003) or using cell model systems that express Akt variants lacking the Ser473/Thr308 phosphorylation substrates (Shi *et al.*, 2015). Another important aspect to examine is whether LifA directly glycosylates Akt (unlikely given DxD motif required to bind UDP-O-GlcNAc is not needed) or subverts the activity of the host enzymes that add or remove O-GlcNAc from Akt (Shi *et al.*, 2015) by, for example, using commercially available inhibitors.

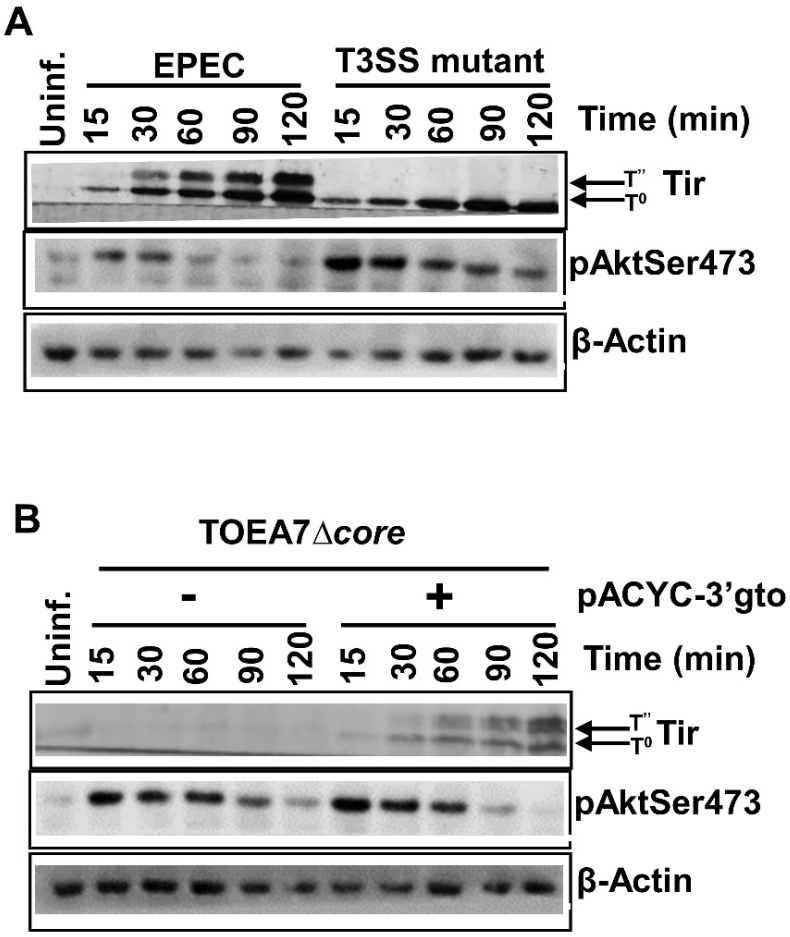
Unexpectedly the immunoprecipitation studies revealed O-GlcNAc modification of a prominent Akt-sized band in cells infected with the T3SS and LifA-deficient mutants, but not EPEC. Unfortunately, the O-GlcNAc modification was 'lost' during the immunoprecipitation process, even following a rapid (2 hr) protocol, so it identifies as Akt could not be confirmed. Future studies should investigate if the modified form be stabilised by, for example, adding inhibitors of host protease and/or deglycosylation activities. Interestingly, a recent analysis of samples from previous time-course experiments revealed similar O-GlcNAc modification and pAktSer473 profiles (not shown) linking both events. It is predicted that EPEC infection induces phosphorylation

(on Ser473 and Thr308) and O-GlcNAc modification of Akt. Investigations are needed to determine if all or only some Akt molecules are phosphorylated (active), O-GlcNAc modified (in active) or carry both modifications; noting Akt can be O-GlcNAc modified on other residues. While infection with the T3SS and LifA/LifA-like deficient mutants are linked to sustained phosphorylation (on Ser473 and Thr308) and O-GlcNAc modification, the phosphorylated-modified forms are not evident following a 2-hour EPEC infection, noting the O-GlcNAc modified form is only detected in the immunoprecipitates i.e., very low levels on, possibly, a subpopulation of Akt molecules. Previous studies linked the EPEC inhibitory process to Akt retention (in non-phosphorylated form) on host membranes (Amin, 2017). It is predicted that infection triggers a general response i.e., O-GlcNAc modification of Akt which promotes its recruitment to a particular membrane for phosphorylation-induced activation and subsequent release to phosphorylated targets in other compartments until deactivated (dephosphorylated). Additional rounds of recruitment/activation/release occur until the receptor-induced signalling cascade is shut down. It is proposed that LifA is targeted to membrane recruited, possibly O-GlcNAc modified, Akt where is prevents its kinase-mediated activation and subsequent release thereby preventing downstream Akt signalling. Further studies are needed to determine the role of the IE2-encoded factor in this process and whether the LifA-like protein shared the same, similar, or different inhibitory mechanism. For example, studies should examine if inhibition of Akt signalling by the *lifA* mutant (expresses LifA-like protein) leads to O-GlcNAc modification of Akt. It is possible the LifA-like protein uses a distinct mechanism as it is much smaller (78%) than LifA, shares only 28% identity with LifA and studies failed to support T3SS delivery, unlike LifA, into host cells (Deng *et al.*, 2012). Such studies will require the generation of plasmids that expressing the LifA-like protein and antibodies to monitor its expression and cellular location within EPEC and/or infected host cells.

In conclusion, this study clearly showed that EPEC's T3SS dependent ability to inhibit pAkt signalling depends on the LifA homologues which act in a redundant way. This inhibitory activity also appears to require EPEC to express its multi-substrate effector chaperone, CesT.

Supplementary Figures

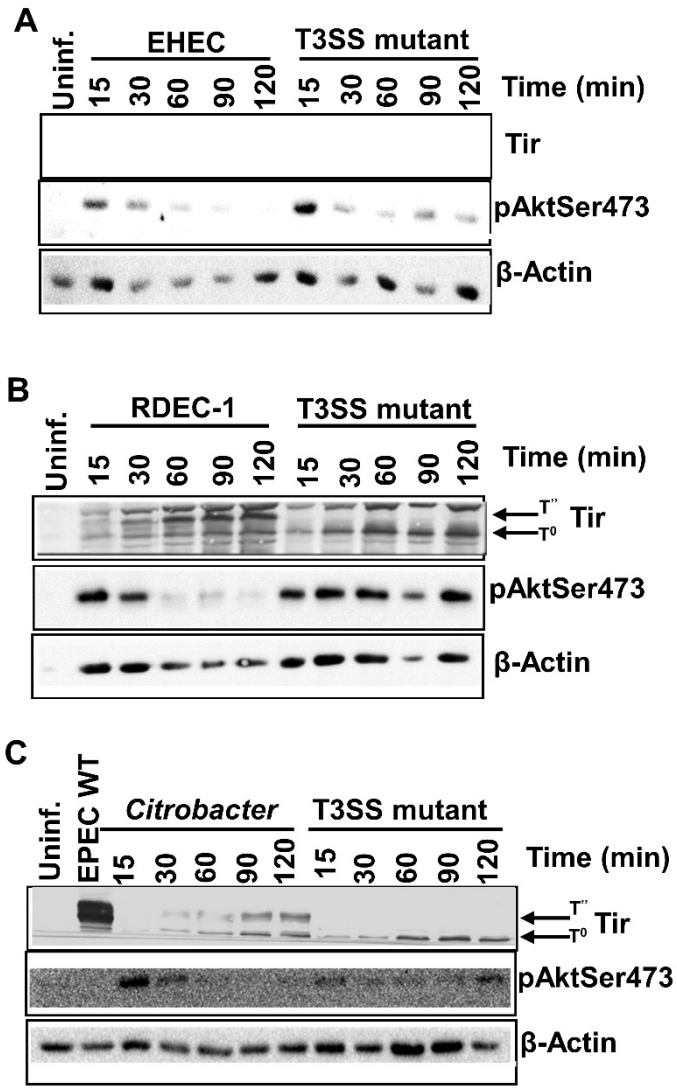
Supplementary figures



Supplementary Figure 1 TOEA7Δcore mutant inhibits Akt signalling when carrying plasmid encoding CesT and Tir

J774A.1 monolayer were left uninfected or were infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS (*cfm-14*) and *TOEA7Δcore* (lacks 14 Nle and 6 LEE proteins [3 effectors, CesT & CesT chaperones and Intimin surface protein]) mutant without (-) or with (+) a plasmid encoding CesT and Tir (pACYC-*tir/cesT*).

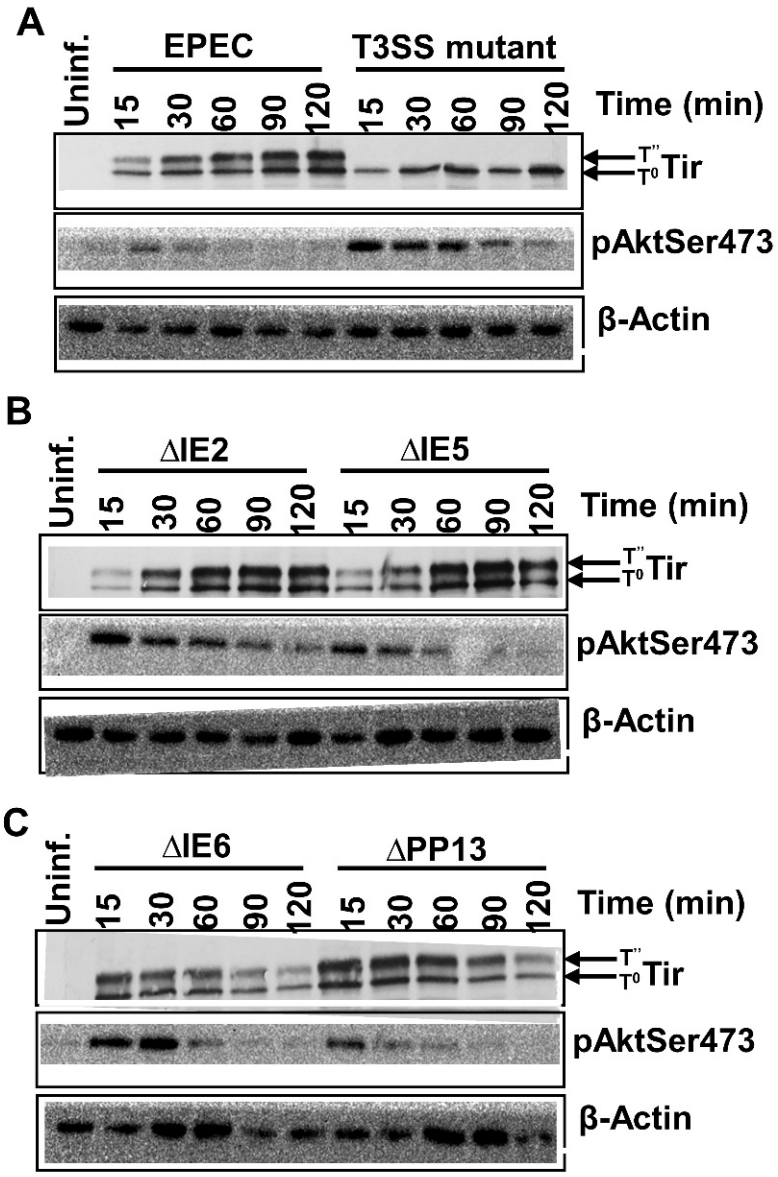
Supplementary figures



Supplementary Figure 2 Other A/E pathogens inhibit Akt signalling

J774A.1 cell were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAKTSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') EPEC Tir forms. Strains used were EPEC B171-8, *Citrobacter rodentium* (*Citrobacter*), rabbit EPEC O15:KH (RDEC-1) and enterohaemorrhagic *E. coli* 0157:H7 EDL993 (but lacks genes encoding Shiga-like toxins; EHEC) and T3SS-deficient variant of each strain except EPEC B171 (used EPEC E2348/69 T3SS mutant).

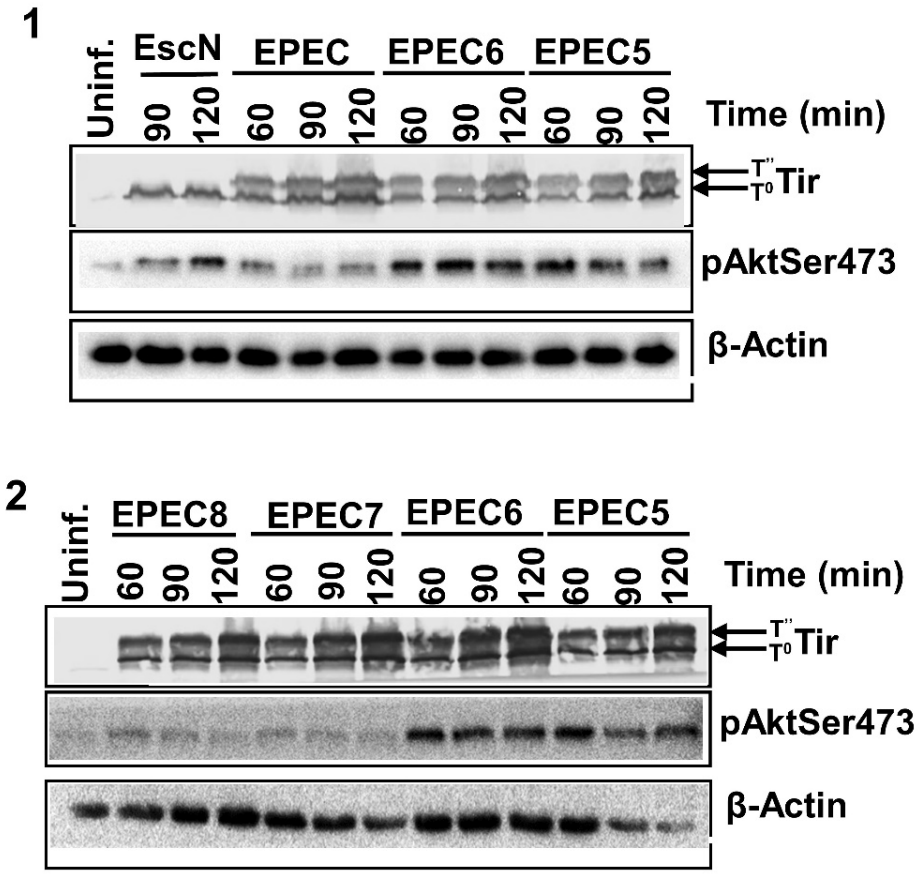
Supplementary figures



Supplementary Figure 3 Major contribution of IE2-encoded factor for EPEC to inhibit Akt signalling

J774A.1 macrophage were left uninfected or infected (MOI 200:1) with indicated pre-activated strains for 90 (not shown) and 120 minutes before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC and mutants lacking a functional T3SS (T33S) or horizontally acquired IE2 (ΔIE2), IE5 (ΔIE5), IE6 (ΔIE6) or PP13 (ΔPP13) regions (Litvak *et al.*, 2017).

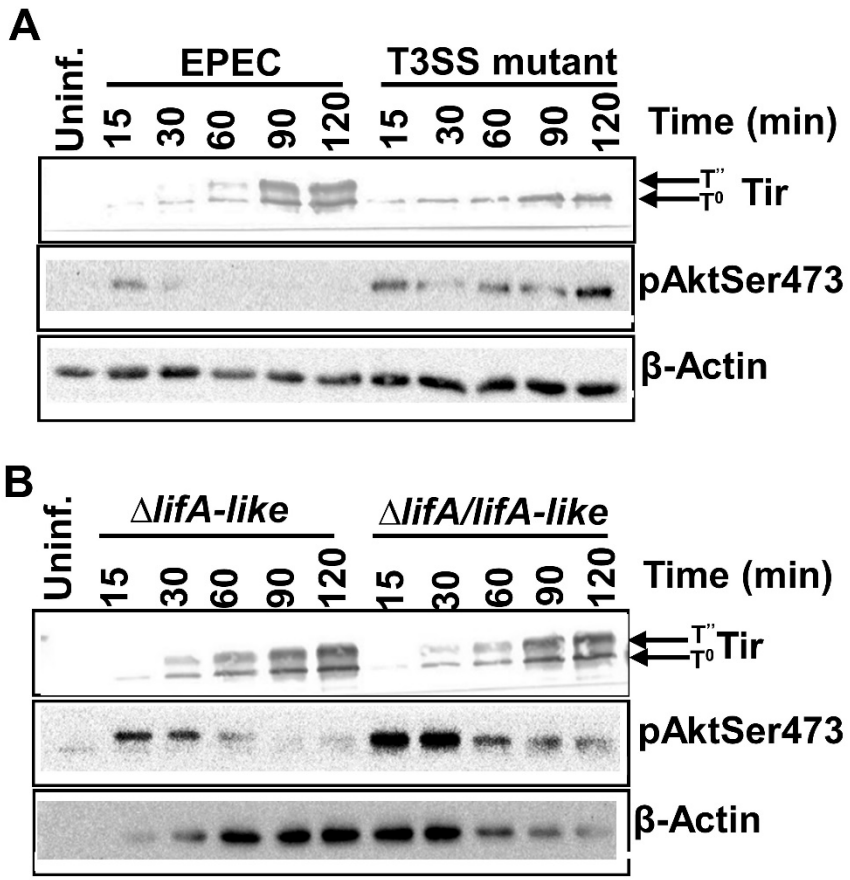
Supplementary figures



Supplementary Figure 4 EPEC6 multi-effector deficient strain cannot inhibit Akt signalling

J774A.1 macrophage were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane, and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS), EPEC8 (missing *map*, *espF*, *espH*, *espG*, *espG2*, *espC*); EPEC7 (as EPEC8 but also missing *espL, nleB1, nleE1, lifA*); EPEC6 (as EPEC7 but also missing *nleE2* and *lifA_like*); EPEC5 (as EPEC6 but also missing *nleH1 and espJ*).

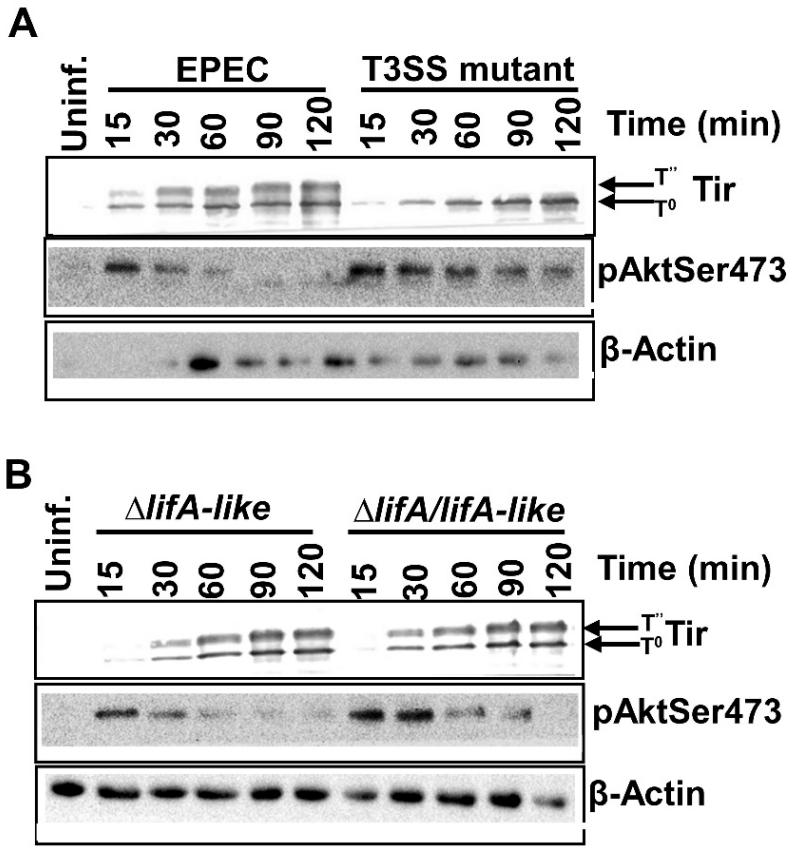
Supplementary figures



Supplementary Figure 5 Redundant role for LifA homologues in loss of pAktSer473 signal

J774A.1 cell were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS) and strains lacking LifA (Δ*lifA*), LifA-like (Δ*lifA-like*) or both (Δ*lifA*/*lifA-like*) proteins.

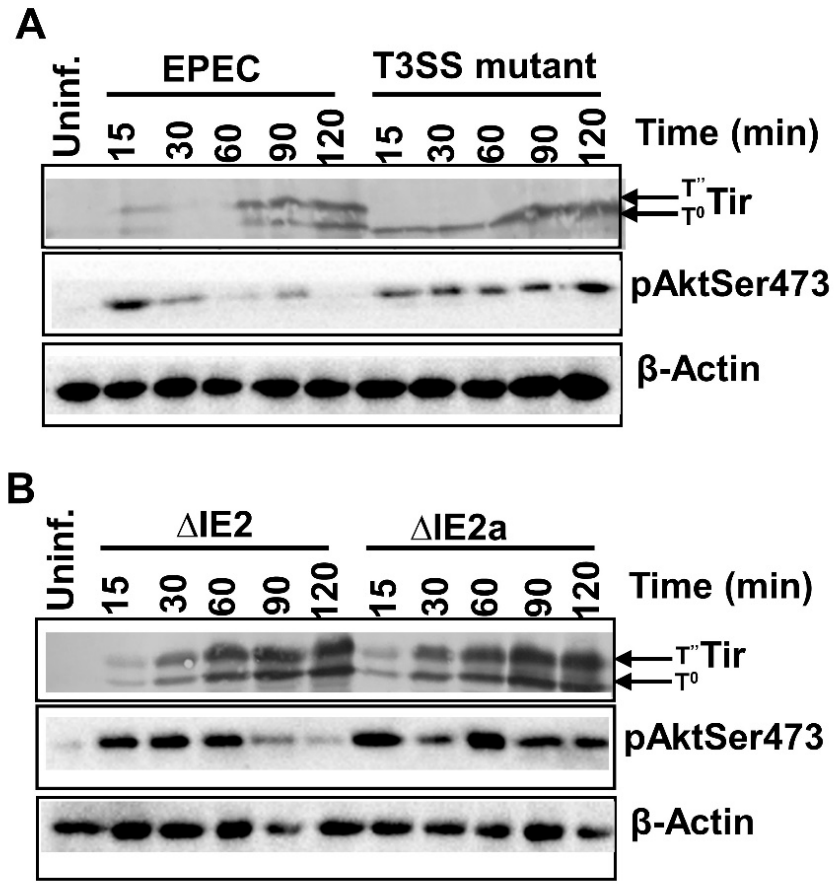
Supplementary figures



Supplementary Figure 6 Role for LifA homologues in loss of pAktSer473 signal.

J774A.1 cells were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS) and strains lacking LifA (Δ*lifA*), LifA-like (Δ*lifA-like*) or both (Δ*lifA*/*lifA-like*) proteins.

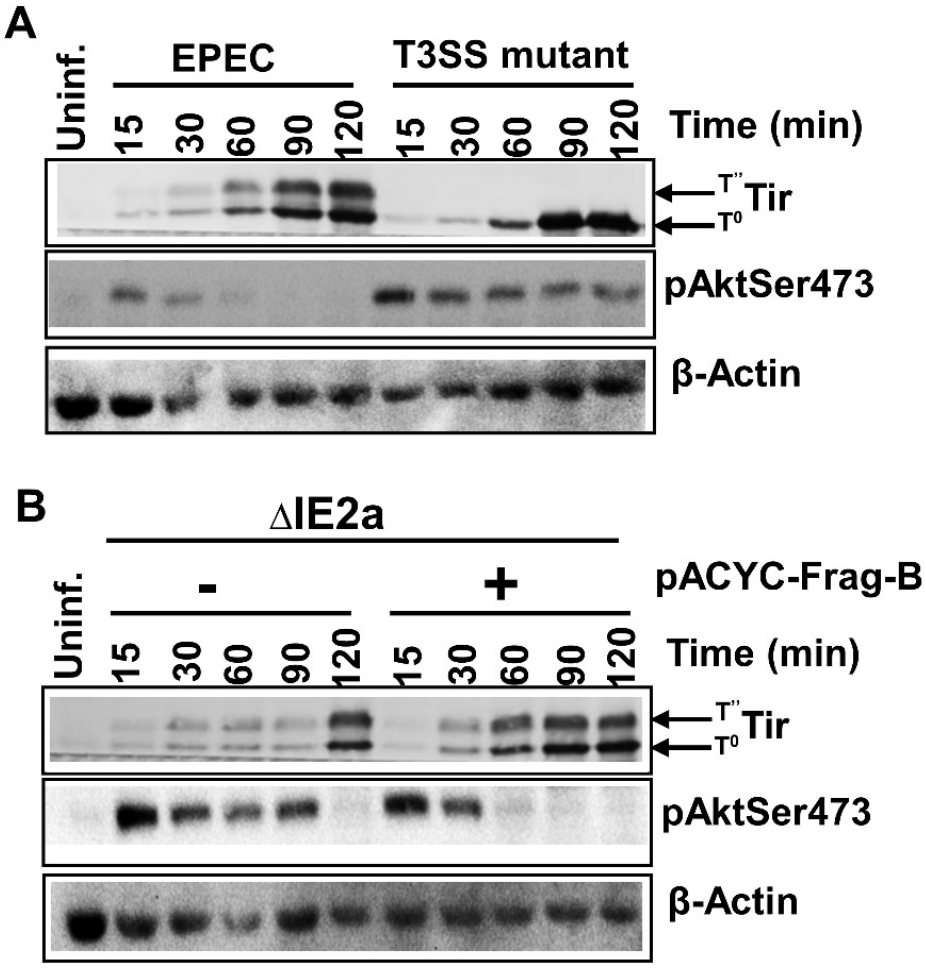
Supplementary figures



Supplementary Figure 7 IE2 mutant confirms the T3SS mutant like profile of pAktSer473 signal

J774A.1 monolayer were left uninfected or were infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS (*cfm-14*), and ΔIE2 and ΔIE2a.

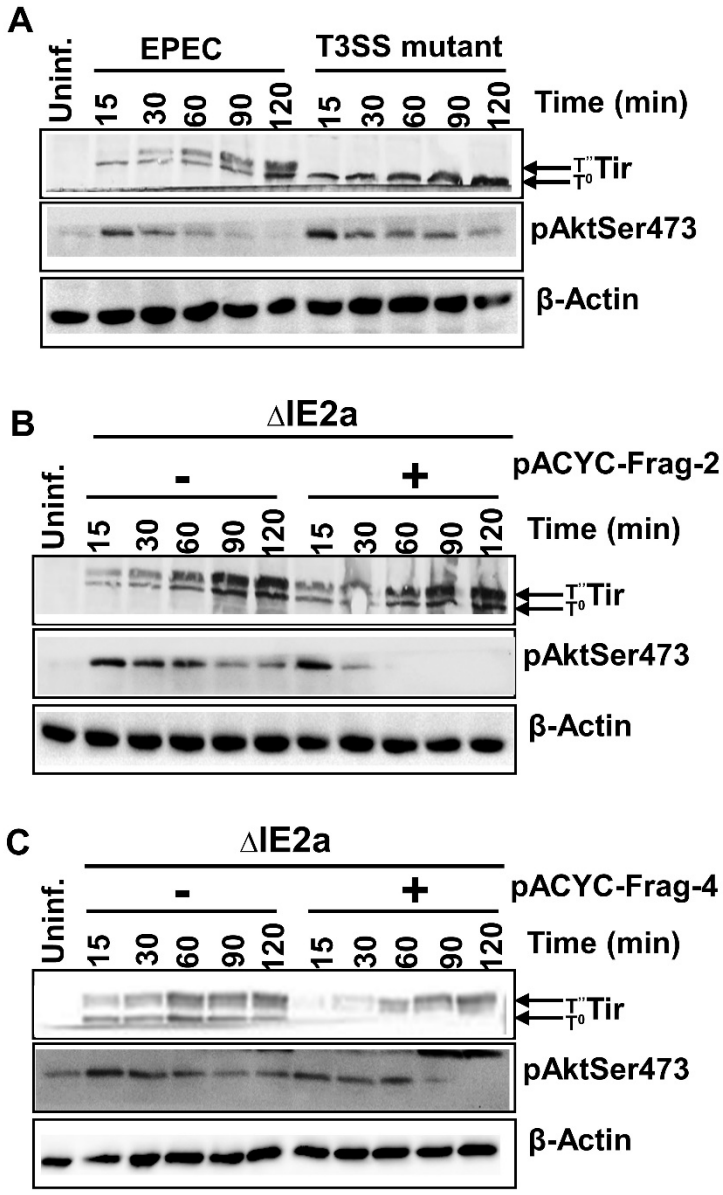
Supplementary figures



Supplementary Figure 8 Rescuing EPEC like loss of pAktSer473 signal with pACYC-Frag-B

J774A.1 cell were left uninfected or infected (MOI 200:1) with pre-activated strains for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control). Arrows indicate position on unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS mutant, ΔIE2+/-pACYC-Frag-B.

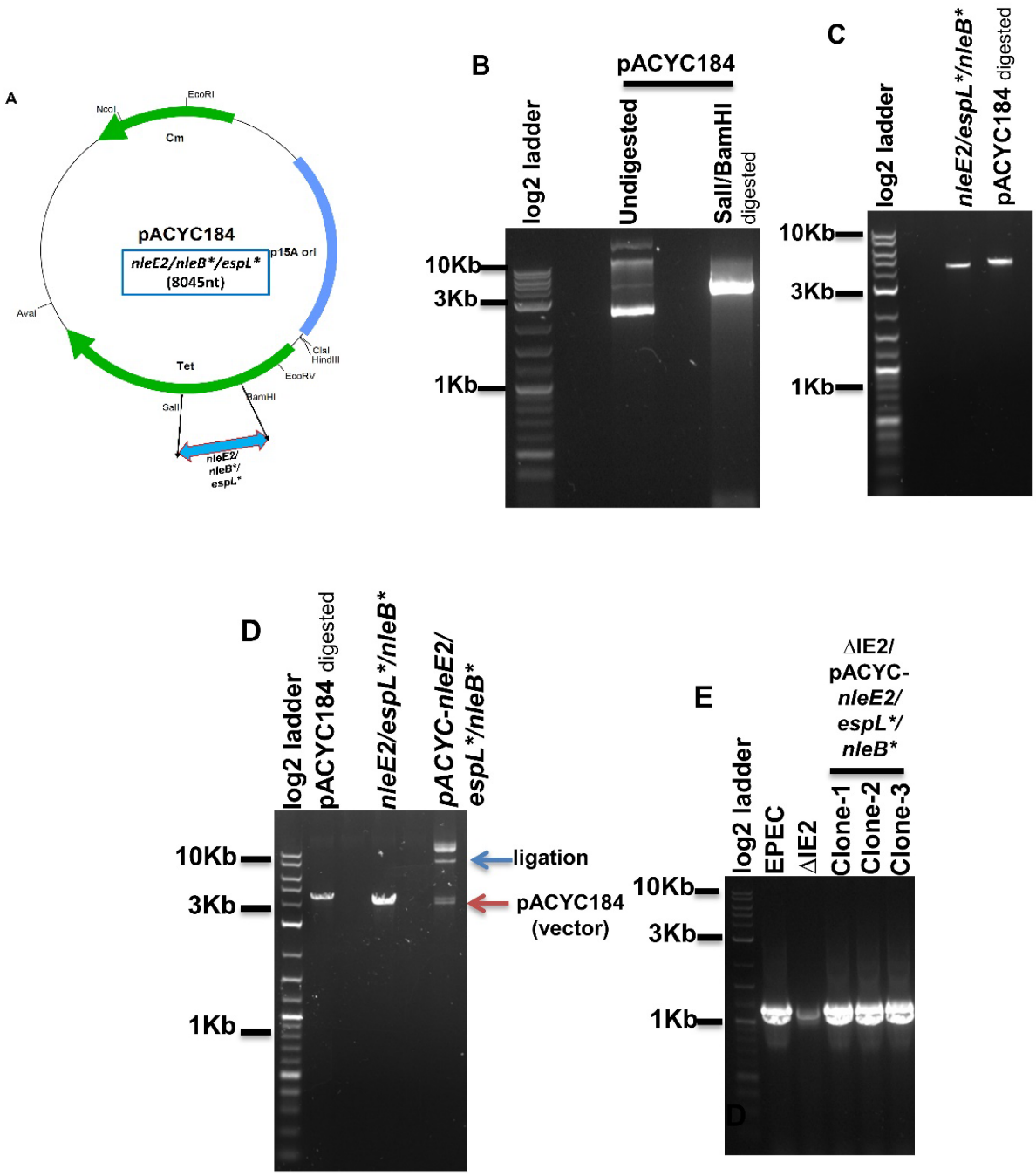
Supplementary figures



Supplementary Figure 9 Restoring ΔIE2 mutant's ability to inhibit Akt signalling

J774A.1 macrophage were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant (T3SS), and ΔIE2a with pACYC184 plasmids (pFrag-2 or pFrag-4) carrying Frag-B sub fragments.

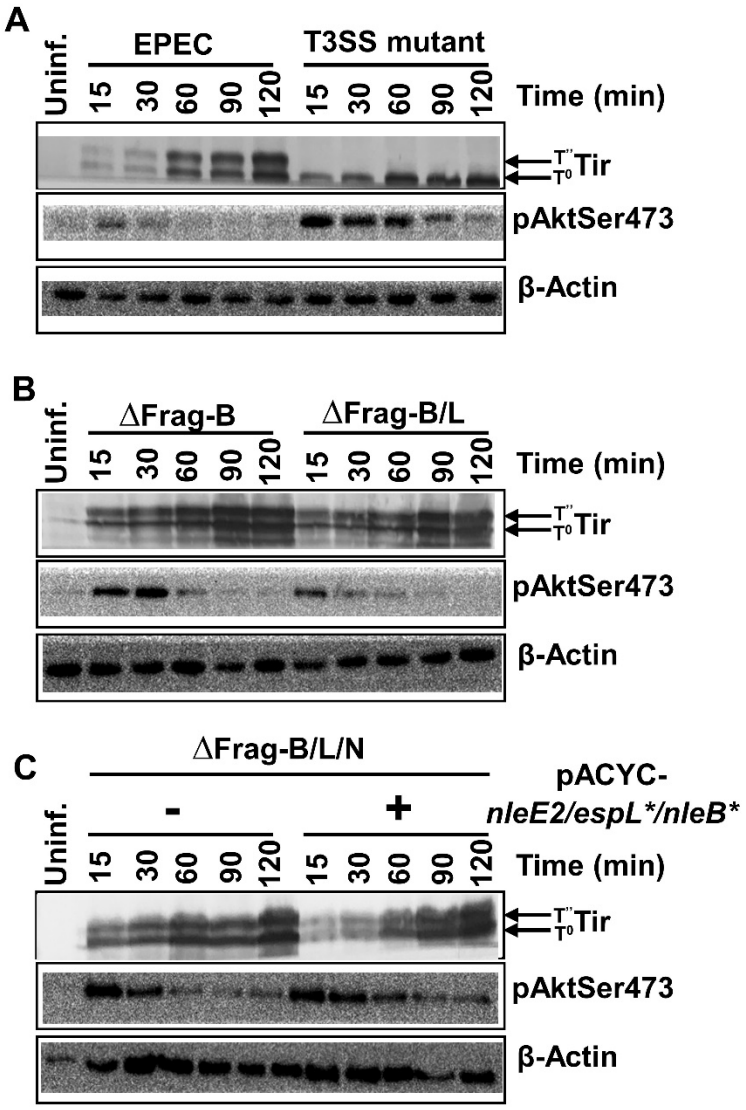
Supplementary figures



Supplementary Figure 10 Generating pACYC-*nleE2/espL*/nleB***

A-Schematic of pACYC184 plasmid carrying *nleE2*/*espL**/*nleB** genes (* indicates pseudogene). Agarose gel data illustrating some steps in generating plasmids -pACYC184 digested BamHI/SalI to isolate ~4Kb vector fragment for ligation with PCR amplified *nleE2*/*espL**/*nleB** region with, in D, evidence of successful ligation via Gibson Assembly kit. E) PCR support of pACYC184-*nleE2*/*espL**/*nleB** introduction into ΔIE2a mutant.

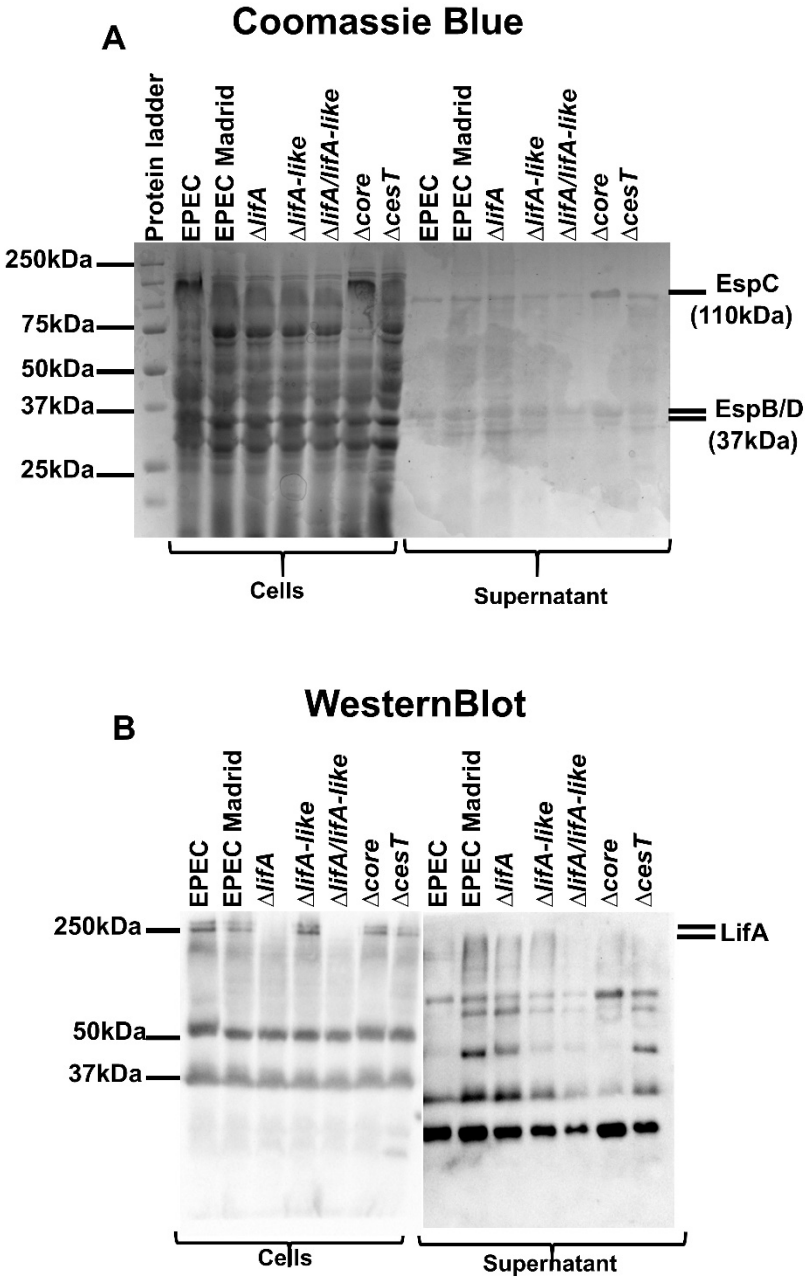
Supplementary figures



Supplementary Figure 11 *nle2/espL*/nleB region is not required for EPEC to inhibit Akt signalling**

J774A.1 cells were left uninfected or infected with pre-activated strains (MOI 200:1) for indicated times before washing away non-adherent bacteria and re-suspending remaining macrophages in sample buffer. Samples were resolved on 10% SDS-PA gels, transferred to nitrocellulose membrane and probed for Tir (T3SS functionality marker), pAktSer473 and actin (loading control) antibodies. Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, T3SS mutant, ΔIE2a, ΔFrag-B, ΔFrag-B/LifA-like (ΔFrag-B/L) or ΔFrag-B/LifA-like/Nle/Esp region (ΔFrag-B/L/N). When indicate the ΔFrag-B/L/N carried no plasmid (-) or a plasmid (+) with the *nleE2*/*espL**/*nleB** gene region (* indicates pseudogene).

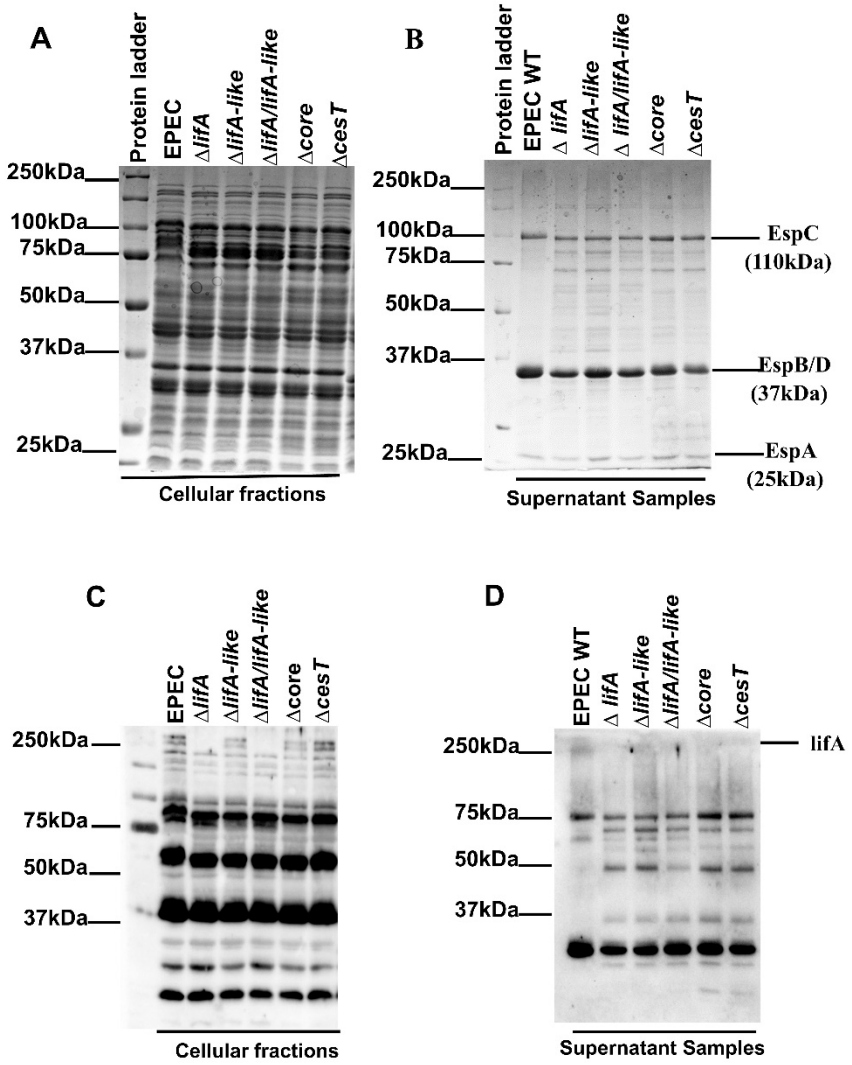
Supplementary figures



Supplementary Figure 12 CesT independent LifA expression

Indicated strains were grown in DMEM for 6 hours before isolating total cellular and secreted (supernatant) proteins (see Materials and Methods). The samples were resolved on **(A&B)** 15% SDS-PA gels and proteins visualised by Commassie blue staining or on **(C&D)** 8% SDS-PA gels for western blot detection of LifA. Positions of LifA, the secreted translocators (EspA, EspB, EspD) and EspC autotransporter proteins are indicated alongside some of molecular mass marker protein. Strains used were EPEC and mutants lacking a functional T3SS (T3SS), both LifA homologues (Δ*lifA*/*lifA*_like) and CesT (Δ*cesT* and Δ*core*). The Δ*core* mutant lacks the LEE region encoding 2 chaperones (CesT, CesF), 3 effectors (EspH, Map, Tir) and Intimin surface protein.2 chaperones (CesT, CesF), 3 effectors (EspH, Map, Tir) and Intimin surface protein.

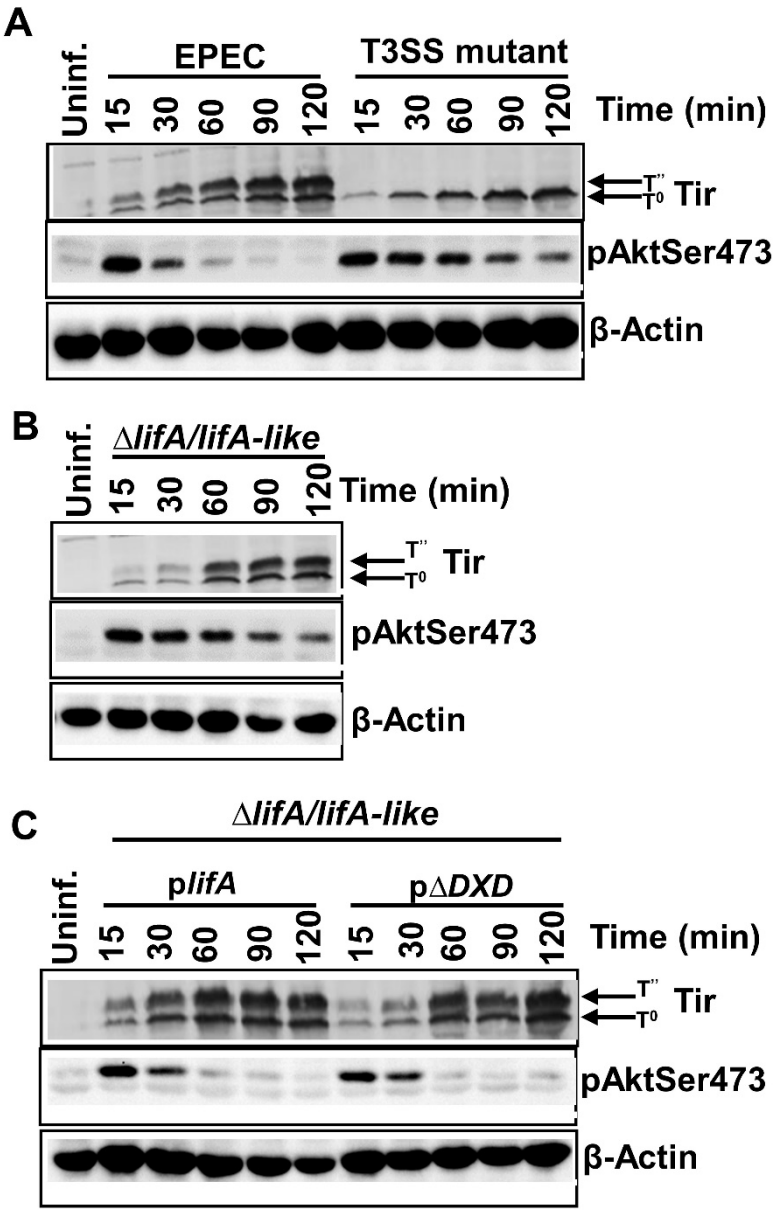
Supplementary figures



Supplementary Figure 13 CesT independent expression of LifA

Indicated strains were grown in DMEM for 6 hours before isolating total cellular and secreted (supernatant) proteins (see Materials and Methods). The samples were resolved on **(A&B)** 15% SDS-PA gels and proteins visualised by Commassie blue staining or on **(C&D)** 8% SDS-PA gels for western blot detection of LifA. Positions of LifA, the secreted translocators (EspA, EspB, EspD) and EspC autotransporter proteins are indicated alongside some of molecular mass marker protein. Strains used were EPEC and mutants lacking a functional T3SS (T3SS), both LifA homologues (Δ*lifA*/*lifA*_like) and CesT (Δ*cesT* and Δ*core*). The Δ*core* mutant lacks the LEE region encoding 2 chaperones (CesT, CesF), 3 effectors (EspH, Map, Tir) and Intimin surface protein.

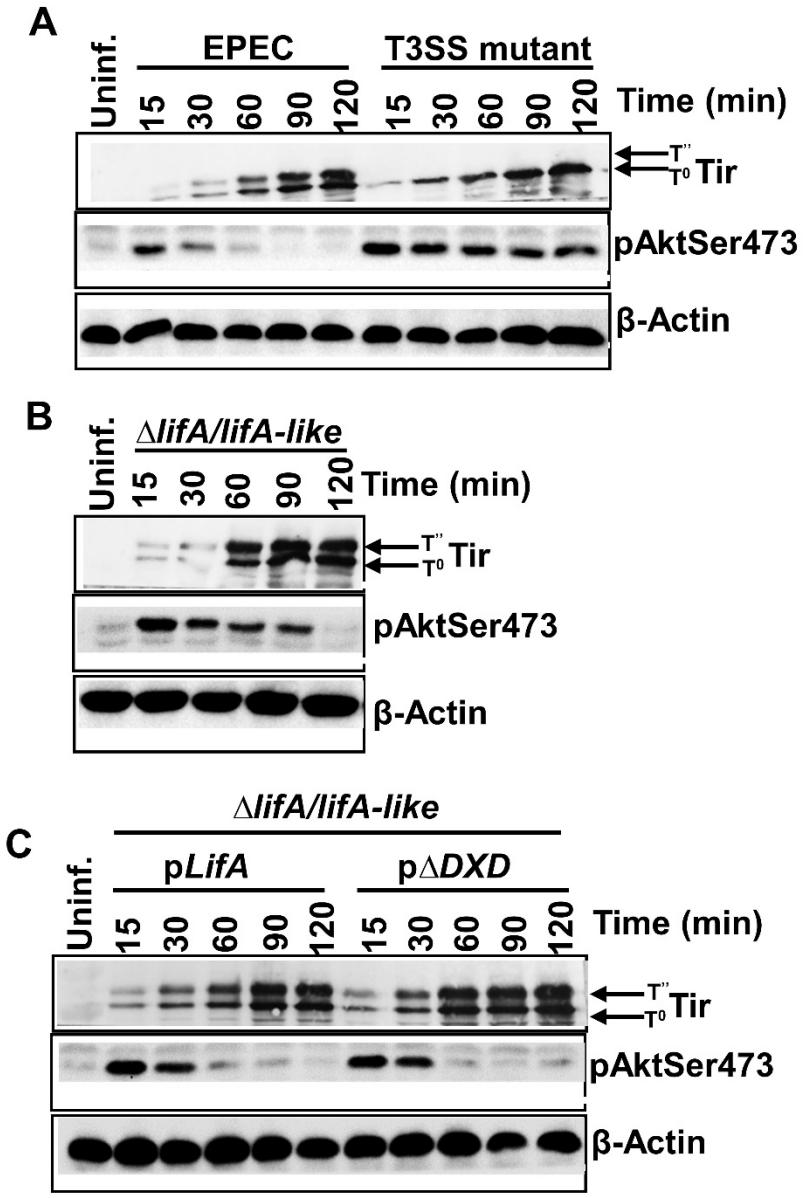
Supplementary figures



Supplementary Figure 14 Loss of pAktSer473 signal does not require LifA DXD motif

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and strains lacking both LifA homologues (ΔlifA/lifA-like) and, when, indicated plasmids encoding LifA (p*lifA*) or the LifA-ΔDXD (pΔDXD) variant.

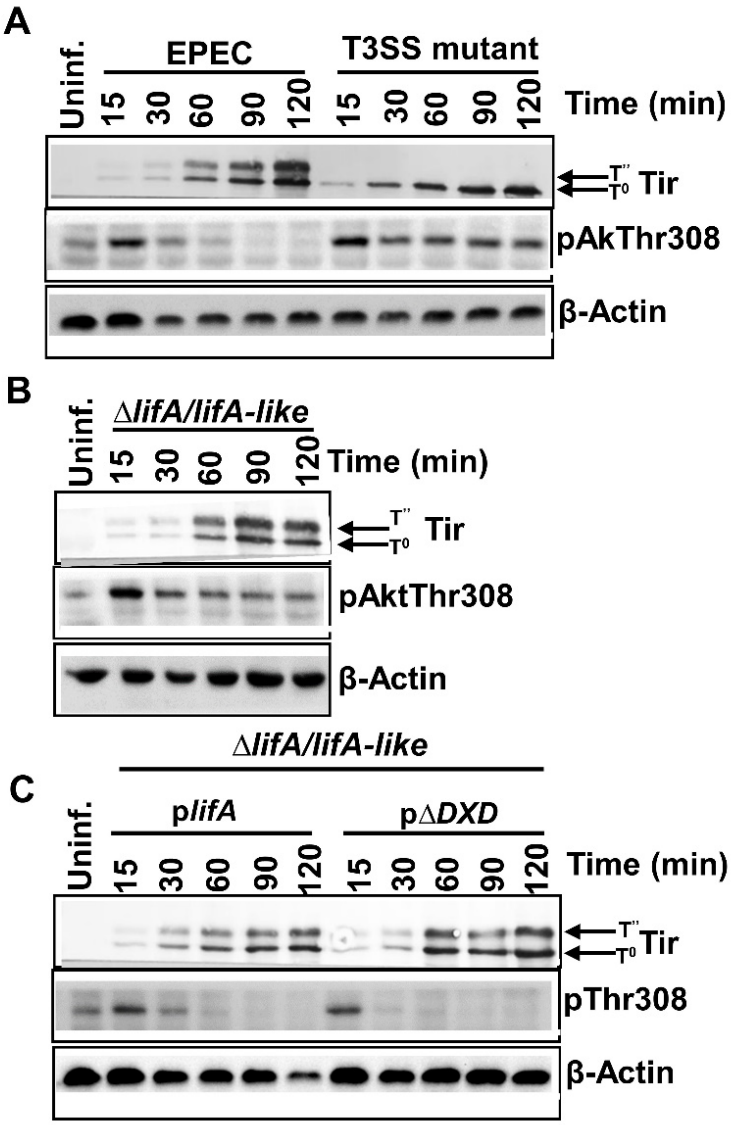
Supplementary figures



Supplementary Figure 15 Loss of pAktSer473 signal does not require LifA DXD motif.

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and strains lacking both LifA homologues (ΔlifA/lifA-like) and, when, indicated plasmids encoding LifA (p*lifA*) or the LifA-ΔDXD (pΔDXD) variant.

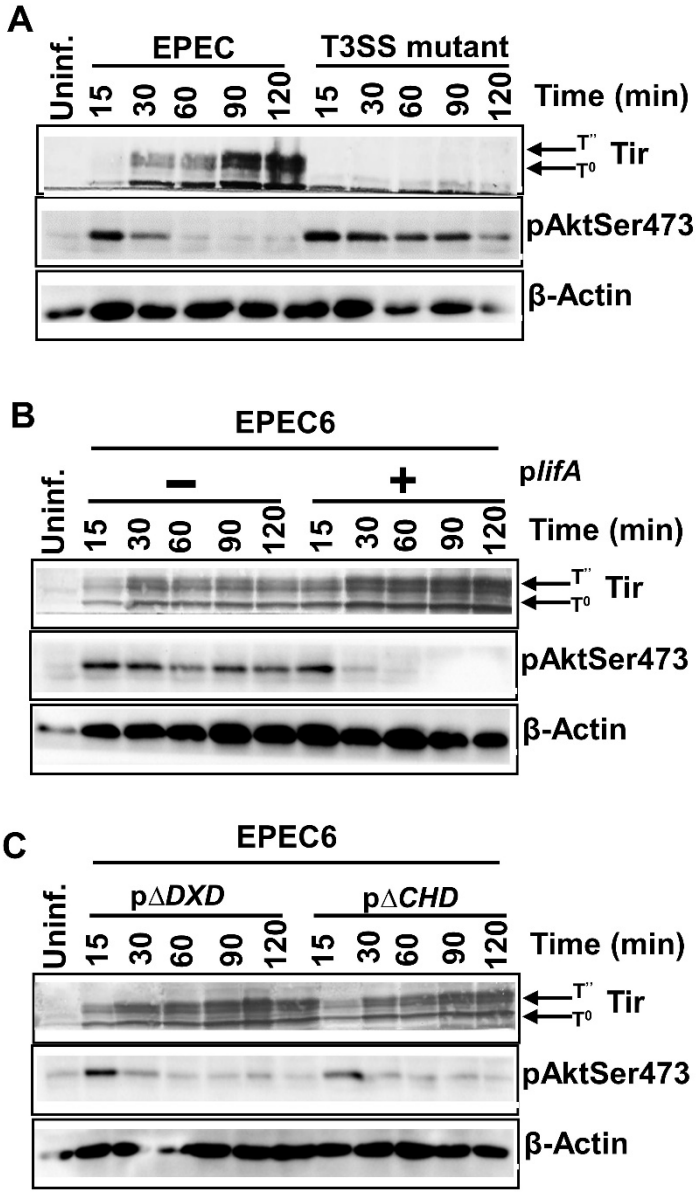
Supplementary figures



Supplementary Figure 16 Loss of pAktThr308 signal also not require LifA DXD motif

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and strains lacking both LifA homologues (Δ*lifA*/*lifA*-like) and, when, indicated plasmids encoding LifA (p*lifA*) or the LifA-ΔDXD (pΔDXD) variant.

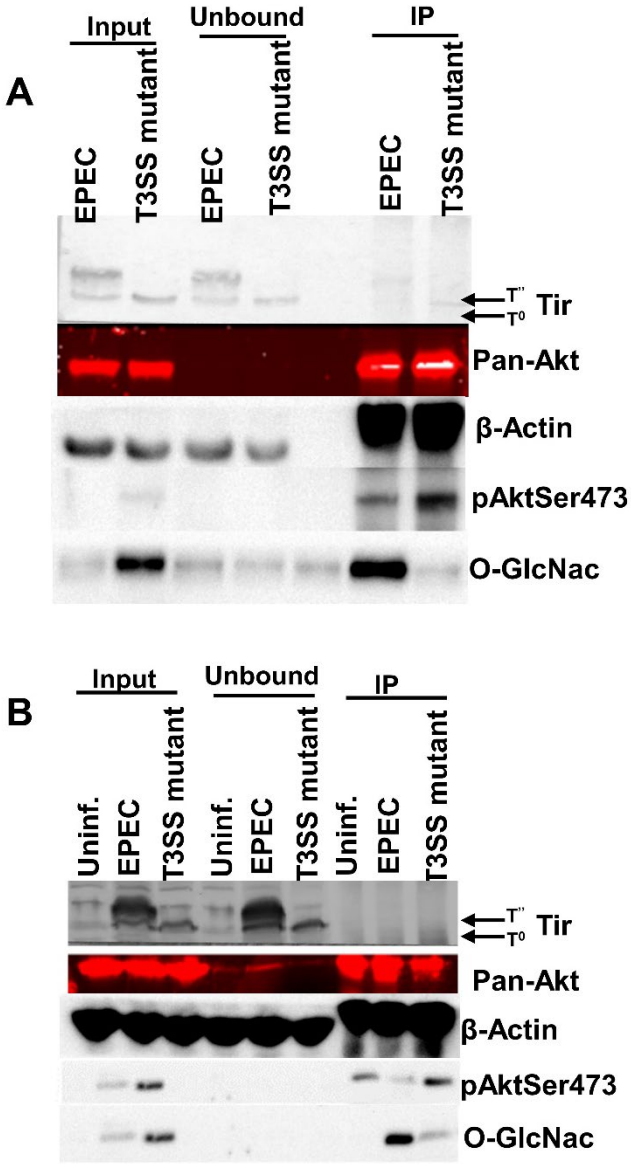
Supplementary figures



Supplementary Figure 17 Loss of pAktSer473 signal does not require LifA DXD or CHD motifs

J774A.1 macrophage were left uninfected (Uninf.) or infected with, pre-activated, strains (MOI 200:1) for indicated times. Following the removal of non-adherent bacteria, remaining proteins were isolated in sample buffer and processed for Western blot analysis (6% SDS-PA gels) and probed for Tir (T3SS functionality marker), pAktSer473 and β-actin (loading control). Arrows indicate position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC, a T3SS mutant and EPEC6 (missing 4 LEE effectors [EspF, EspG, EspH, Map] and non-LEE effectors from IE2, IE5 and IE6 including *lifA* and *lifA-like*. EPEC6 had no plasmid (-) or a plasmid (+) encoding LifA (p*lifA*), LifA-ΔDXD (pΔDXD) or LifA-ΔCHD (pΔCHD) variant. The DXD and CHD motifs are linked to glycosyltransferase and cysteine protease activities, respectively.

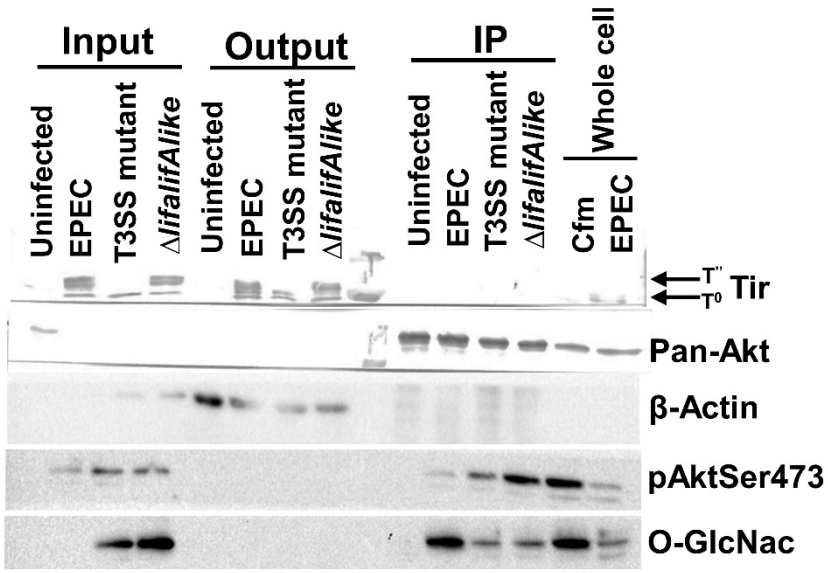
Supplementary figures



Supplementary Figure 18 EPEC inhibition of Akt signalling is linked to Akt O-GlcNAcylation.

J774A.1 macrophage were infected with, pre-activated, EPEC or T3SS mutant strains (MOI 200:1) for 2 hours. Following the removal of non-adherent bacteria, the J774A.1 macrophage were extracted in a 1% Triton-X 100 solution with removal (centrifugation) on the insoluble fraction leaving a solution containing host cytoplasm and membrane proteins plus T3SS-delivered substrates. A reference sample (Input) was removed before adding anti-Akt antibodies pre-coupled to magnetic beads for overnight incubations to 'capture' Akt. The following day the beads were isolated, and a sample of the remaining solution (Output) was taken. The beads (immunoprecipitate) were washed several times before these, and other samples, were resolved on 6% SDS-PA gels and processed for Western blot analyses to probe for Tir (T3SS functionality marker), total Akt (Pan-Akt), pAktSer473, β-actin (loading control) and O-GlcNAc modified proteins. Arrows indicate the position of unmodified (T^0) and host kinase-modified (T'') Tir forms. A & B are from independent experiments.

Supplementary figures



Supplementary Figure 19 LifA inhibition of Akt signalling is linked to O-GlcNAcylation of Akt

J774A.1 macrophages were infected with, pre-activated, EPEC strains (MOI 200:1) for 2 hours. Following the removal of non-adherent bacteria, the J774A.1 macrophages were extracted in a 1% Triton-X 100 solution with removal (centrifugation) on the insoluble fraction leaving a solution containing host cytoplasm and membrane proteins plus T3SS-delivered substrates. A reference sample (Input) was removed before adding anti-Akt antibodies pre-coupled to magnetic beads for overnight incubations to 'capture' Akt. The following day the beads were isolated and a sample of the remaining solution (Output) was taken. The beads (immunoprecipitate) were washed several times before these, and other samples, were resolved on 6% SDS-PA gels and processed for Western blot analyses to probe for Tir (T3SS functionality marker), total Akt (Pan-Akt), pAktSer473, β-actin (loading control) and O-GlcNAc modified proteins. Arrows indicate the position of unmodified (T^0) and host kinase-modified (T'') Tir forms. Strains used were EPEC and mutants lacking a functional T3SS (T3SS) or both LifA homologues (Δ*lifA*/*lifA*-like).

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