Investigating how enteropathogenic *Escherichia coli* (EPEC) subverts AKT signalling

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Submitted for the degree of Doctor of Philosophy

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January 2017

Abstract

The phosphoinositide 3-kinase (PI3K) signalling pathway is activated in macrophages in response to many bacterial pathogens, triggering phagocytic uptake mechanisms and phosphorylation-associated activation of the serine/threonine kinase AKT. Enteropathogenic *E. coli* (EPEC) inhibits both PI3K mediated phagocytosis and AKT phosphorylation; dependent on a type 3-secretion system (T3SS) critical for delivering up to 24 known effector proteins into target cells. The efficient translocation of most EPEC effectors is dependent on the T3SS effector chaperone CesT. Although the effectors and mechanisms for inhibiting phagocytosis are well described, little is known how EPEC inhibits AKT phosphorylation. AKT activation is a multi-step process involving its recruitment to the cell membrane and phosphorylation of Thr308 by PDK1 and Ser473 by mTORC2. This activation process is supressed by inositol (such as PTEN) and protein (such as PP1 & PP2A) phosphatases. Altered AKT signalling is associated with many cancers, diabetes, cardiovascular and infectious disease, thus identifying how EPEC inhibits AKT activity could provide insight into its complex regulatory process and/or new therapeutic strategies.

Screening of bacterial strains, lacking or expressing subsets of EPEC effectors, by western blot analysis suggests that the inhibitory mechanism depends on the CesT chaperone but not the function of the 21 most studied effectors. The EPEC inhibitory mechanism was investigated through the development of a two-wave infection model, examining for T3SS dependent changes in AKT phosphorylation (Thr308 & Ser473), membrane localisation and activity of AKT associated signalling proteins (PDK1 & PTEN). This strategy revealed inhibition of AKT phosphorylation to be stable (up to 3 h) and linked to increased activity of serine/threonine protein phosphatase(s). This finding was supported by phosphatase inhibitor studies, suggesting the involvement of a host activated or bacterial delivered protein phosphatase. Thus, this study provides new insights into the requirement of the EPEC effector repertoire and suggests a novel mechanism by which EPEC inhibits AKT signalling.

Acknowledgements

First and foremost I would like to thank my supervisor Professor Brendan Kenny for his expertise, time, guidance and support throughout this PhD project. The past four years has been a challenging and rewarding experience that I have no doubt will put me in good stead for future endeavours.

To all my colleagues past and present; Lorna Young, Sabine Quitard, Aseel Al-layla and Azzeldin Madkour thank you for your help, patience and friendship over the past four years. To Nicholas Vita, Gianpiero Landolfi and Javier Abellon thank you for being there to answer my endless questions and most importantly your friendship. Wherever we end up I hope to continue to share a drink and a joke together, EU regulations permitting ;-). To all my friends and in particular everyone at #251 WR (Jamie, Vicky, Jack, Thomo, Gile and Jones) thank you for making this experience that much better.

Most of all I would like to thank my family Mum, Dad and Kris for the unwavering support, love and encouragement throughout this process. I hope I have made you proud and will continue to do so. Finally, I would like to thank Murray and more importantly Kat for your love and support these past few of years, for providing the necessary distractions and making sure I got there in the end.

This PhD was funded by the National Insitute for Health Research Newcastle Biomedical Research Centre (BRC).

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Abbreviations

ABST	Apical sodium dependent bile acid transporter
A/E lesion	Attaching and effacing lesion
ARP2/3	Actin related protein 2/3
BFP	Bundle forming pilus
BSA	Bovine serum albumin
Carb	Carbenicillin
Cmp	Chloramphenicol
CR	*Citrobacter rodentium*
CR3	Complement receptor 3
c-Raf	c-rapidly activated fibrosarcoma
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's minimal eagles medium
DNA-PK	DNA dependent protein kinase
DRA	Downregulated adenoma
E2348/69	EPEC strain E2348/69
EAF	EPEC adherence factor
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	EGF receptor
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Esc	EPEC secretion
Esp	EPEC secreted/signalling protein
FCS	Foetal calf serum
FYVE	Fab1p/YOTB/Vac1p/EEA1 domain
GFP	Green fluorescent protein
GlcNAc	N-Acetylglucosamine
GPCR	G-protein coupled receptor

GSK3	Glycogen synthase kinase 3
h	Hour
IE	Integrative element
IKK	IκB kinase kinase
ITIM	Immune receptor tyrosine based inhibition motifs
JAM	Junction adhesion molecule
Km	Kanamycin
LB	Luria broth
LDH	Lactate dehydrogenase
LEE	Locus of enterocyte effacement
Ler	LEE encoded regulator
LPS	Lipopolysaccharide
M-cells	Microfold cells
Map	Mitochondrial associated protein
MAPK	Mitogen activate protein kinase
Min	Minute
MKKK	MAP kinase kinase kinase
MKK	MAP kinase kinase
MOI	Multiplicity of infection
mTORC2	Mammalian target of rapamycin complex 2
NaCl	Sodium chloride
Nal	Nalidixic acid
NF-κB	Nuclear factor-kappa B
NHE	Sodium hydrogen (Na^+/H^+) exchanger
Nle	Non-LEE encoded
NLR	NOD-like receptor
N-WASP	Neural Wiskott-Aldrich syndrome protein
OD_{600}	Optical density 600 nm
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerised chain reaction

PDK1	Phosphoinositide-dependent kinase 1
Per	plasmid encoded regulator
PFA	Paraformaldehyde
PH	Pleckstrin homology
PHLPP	PH domain Leucine rich repeat protein phosphatase
PI	Phosphoinositide
PI3K	Phosphoinositide 3-kinase
PI[4,5]P2	Phosphatidylinositol 4,5-bisphosphate
PI[3,4]P2	Phosphatidylinositol 3,4-bisphosphate
PI[3,4,5]P3	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethane sulfonyl fluoride
PP	Prophage
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRR	Pathogen recognition receptor
PTEN	Phosphatase tensin homolog
PX	Phox homology
RPEC	Rabbit pathogenic *E. coli*
RT	Room temperature
RTK	Receptor tyrosine kinase
S6K	S6 kinase
SAM	S-adenosyl-L-methionine-dependent methyltransferase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SFK	SRC family kinase
SGLT1	Sodium glucose transporter 1
SHIP2	SH2 domain containing inositol 5-phosphatase
SH2	Src homology 2

SNX9	Sorting nexin 9
SOC	Super optimal broth with catabolite repression
TAK1	TGFβ-activated kinase 1
TBK1	TANK binding kinase 1
Tet	Tetracycline
Tir	Translocated intimin receptor
Thr	Threonine
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNFR	TNF receptor
TRADD	Tumour necrosis factor receptor type1-associated death domain protein
TRAF	TNF receptor associated factor
TSC2	Tuberous sclerosis complex 2
Tyr	Tyrosine
T3SS	Type 3-secretion system
UPEC	Uropathogenic *Escherichia coli*
WT	Wild type
ZO	Zonula occluden

Chapter 1 Introduction

1.1 General introduction

Pathogenic bacteria manipulate many key host cellular processes to promote bacterial colonisation and the establishment of a replicative niche (Grishin *et al.*, 2015). The virulence of invading bacteria is often dependent on the secretion of bacterial proteins known as effectors. These effector proteins are typically delivered into host cells via a specialised secretion system. Once in the host cell, these effectors function to modify the cellular environment through a variety of mechanisms, including the modification of eukaryotic signal transduction pathways (Cui and Shao, 2011). Indeed, one of the most significant mechanisms is the manipulation of cell signalling through modifications of the host phosphoproteome (Rogers *et al.*, 2011; Grishin *et al.*, 2015; Scholz *et al.*, 2015). How bacterial effectors function to subvert eukaryotic signalling cascades is the subject of continuing investigation. This study will focus its discussion towards the role of the enteropathogenic *Escherichia coli* (EPEC) bacteria and its capacity to subvert host cellular processes, including the activity of the eukaryotic protein kinase B, also known as AKT.

1.2 Protein kinases and cellular function

Protein kinases are important regulators of cellular function, forming one of the largest and most important of gene families in eukaryotes (Manning *et al.*, 2002). These kinase proteins play a crucial role in regulating signal transduction pathways through the modification of proteins by the addition of phosphate groups to Serine (Ser), Threonine (Thr) or Tyrosine (Tyr) residues. The phosphorylation of proteins typically serves to induce a functional change in the target protein, including a change in activity (activation/inhibition), the location and overall function (Bononi *et al.*, 2011). As such, protein kinases act to coordinate the activity of almost all cellular processes including metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and apoptosis (Manning *et al.*, 2002); including those of the phosphoinositide-3-kinase (PI3K)/AKT signalling pathway (Fayard *et al.*, 2005). The activity of protein kinases are themselves controlled through a highly regulated process, including: gene transcription, inhibitory/activating proteins, protein degradation, protein phosphorylation and cellular localisation (Endicott *et al.*, 2012). It is therefore unsurprising that the dysregulation of protein kinases are implicated in many human diseases.

The human genome encodes approximately 518 putative protein kinases, encompassing approximately 1.7% of all human genes (Manning *et al.*, 2002). Although relatively few in number, the activity of kinases is vast with approximately 30% of all cellular proteins estimated to be modified by protein kinase activity (Manning *et al.*, 2002). Crosstalk between signalling pathways adds an extra level of complexity in cell regulation, playing an important role in fine-tuning the cell response, which is often dependent on the context of cell activation (Bononi *et al.*, 2011). This is particularly apparent for mitogen activated protein kinase (MAPK) and PI3K signalling pathways, noted for their numerous interconnections (Bononi *et al.*, 2011).

Despite significant progress in characterising kinase regulatory cascades, understanding the discrete nature of the many signalling pathways communicating together cannot always be predicted by studying individual pathways alone (Krachler *et al.*, 2011). To further understand these complex kinase signalling networks it is becoming increasingly popular to examine the mechanisms by which pathogens have evolved to subvert them, such as the *Salmonella enterica*, *Shigella flexneri* and EPEC (Krachler *et al.*, 2011).

1.3 The PI3K/AKT signalling pathway

1.3.1 PI3K and phosphoinositides

Phosphatidylinositol and its phosphorylated form (phosphoinositide; PI) play a critical role in many cell signalling pathways. Often referred to as second messengers, phosphoinositides function as membrane scaffolds, recruiting specific proteins of a signalling cascade via their phosphoinositide binding domains (Pendaries *et al.*, 2006). Among the best characterised binding domains identified so far include the pleckstrin homology (PH) domain, phox homology (PX) domain and Fab1p/YOTB/Vac1p/EEA1 (FYVE) domain (Pendaries *et al.*, 2003). The importance of phosphoinositides is highlighted through their organisation and spatiotemporal control of key signalling cascades, including the PI3K/AKT signalling pathway (Vanhaesebroeck and Alessi, 2000). The structure of phosphatidylinositol consists of a diacylglycerol backbone linked to an inositol ring via a phosphate group (Figure 1). This inositol ring can be phosphorylated at free –OH groups located at positions 3, 4 and 5 in different combinations (Vanhaesebroeck and Alessi, 2000).

PI3K is a conserved family of intracellular lipid kinases that phosphorylate the 3' hydroxyl group on the inositol ring of phosphoinositides. This family of kinase are classified into three groups based on their structure and substrate specificity, known as class I, class II or class III PI3K (Liao and Hung, 2010). However, only class I PI3Ks are reported to stimulate AKT activity (Vanhaesebroeck and Alessi, 2000). The structure of class I PI3Ks is of a heterodimer composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (Shepherd *et al.*, 1998). Class I PI3Ks may be further divided into a subgroup known as class IA (p110α, p110β or p110δ) and class IB (p110γ). Although the catalytic subunits of PI3K subgroups shows significant sequence homology, class IA and class IB kinases each associate with a distinct family of adaptor proteins (p85α/β & p101 respectively), which enables their association and activation downstream of receptor tyrosine kinase (RTKs) and G-protein coupled receptors (GPCRs) respectively (Shepherd *et al.*, 1998; Vanhaesebroeck and Alessi, 2000; Yuan and Cantley, 2008). In addition, class IA PI3K are also activated downstream of cytokine receptors and integrin dependent signalling (Wymann *et al.*, 2003; Fayard *et al.*, 2005). A more detailed review of PI3K structure and function is available elsewhere (Vanhaesebroeck and Waterfield, 1999; Papakonstanti *et al.*, 2008).

In resting cells, class I PI3K are predominantly located in the cell cytosol in an inactive state. Following receptor activation, class IA PI3K are recruited to the RTKs via interaction of sequence specific phospho-tyrosine residues with the Src homology 2 (SH2) domains on p85 adaptor proteins (Figure 2A) (Foster *et al.*, 2003). This recruitment relieves p85 mediated inhibition of p110 catalytic subunit resulting in the activation of PI3K (Berenjeno and Vanhaesebroeck, 2009). In contrast, Class IB PI3K are recruited to GPCRs through p101 adaptor protein mediated interaction with G$\beta\gamma$ subunits of GPCRs (Figure 2B) (Foster *et al.*, 2003). However, it is becoming increasingly suggested that most class I PI3K subunits may in fact be activated by GPCRs, either directly or indirectly through Ras family proteins that can be activated by both RTK and GPCR (Vanhaesebroeck *et al.*, 2010). It is therefore likely that class IA PI3K may be more responsive to GPCR signalling than initially predicted. Once at the membrane, class I PI3K preferentially target PtdIns-[4,5]-bisphosphate (PI[4,5]P2), generating the lipid product PtdIns-[3,4,5]-trisphosphate (PI[3,4,5]P3) (Figure 2) (Yuan and Cantley, 2008). An increase of PtdIns-[3,4]-bisphosphate (PI[3,4]P2) is also observed at the membrane, though this is thought to occur through dephosphorylation of PI[3,4,5]P3 by 5'-inositol phosphatases (Vanhaesebroeck and Alessi, 2000). This localised increase of 3-

phosphoinositides (PI[3,4,5]P3 and PI[3,4]P2) now functions as a second messenger, activating downstream pathways that regulate cell growth, cell survival and other metabolic processes (Bononi *et al.*, 2011). This includes the extensively studied downstream PI3K effector kinase AKT; often used as a marker of PI3K activity (Vasudevan and Garraway, 2010; Bononi *et al.*, 2011).

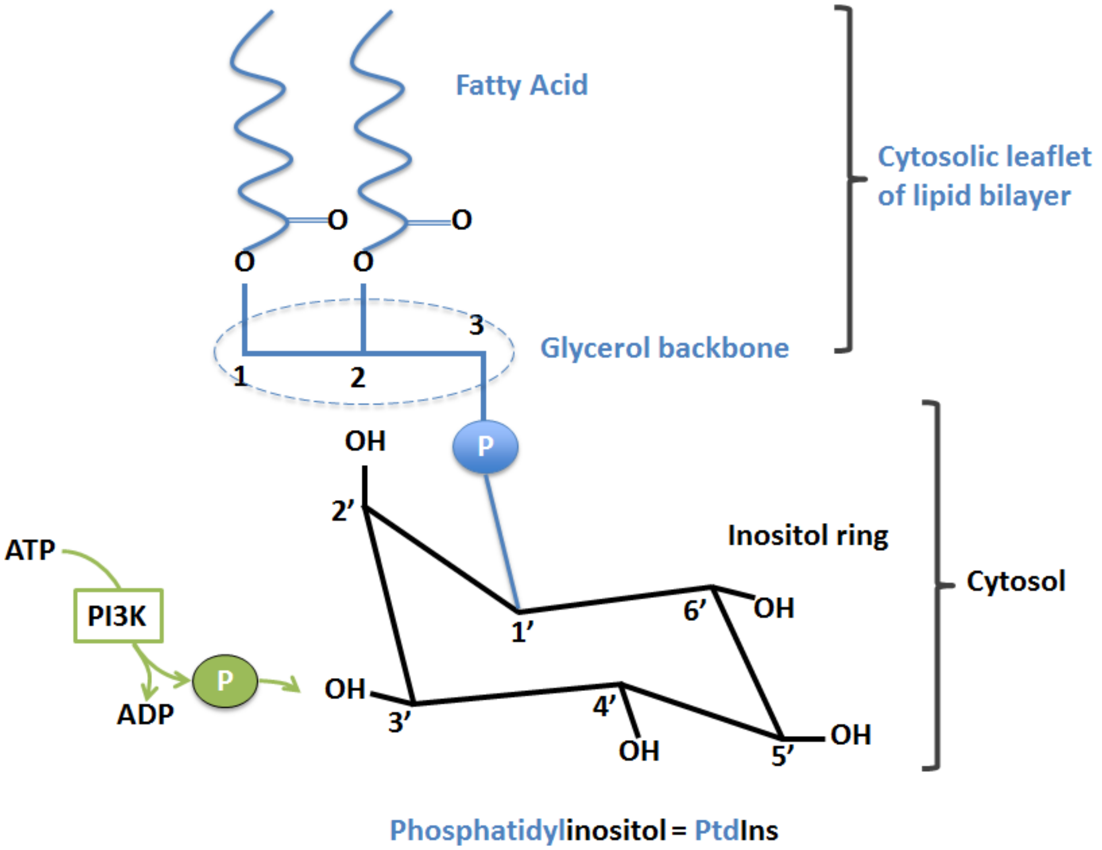


Figure 1 Simplified illustration of phosphatidylinositol.

Phosphatidylinositol is composed of two fatty acid chains embedded in the membrane lipid bilayer, attached at carbon positions 1 and 2 of a glycerol backbone. The inositol ring is attached to the glycerol backbone at carbon position 3 via a phosphate group. The free OH groups on the inositol ring may be phosphorylated, except for positions 2’ and 6’. PI3K phosphorylate the inositol group at position 3’ on the carbon ring as indicated. Adapted from Vanhaesebroeck and Alessi (Vanhaesebroeck and Alessi, 2000).

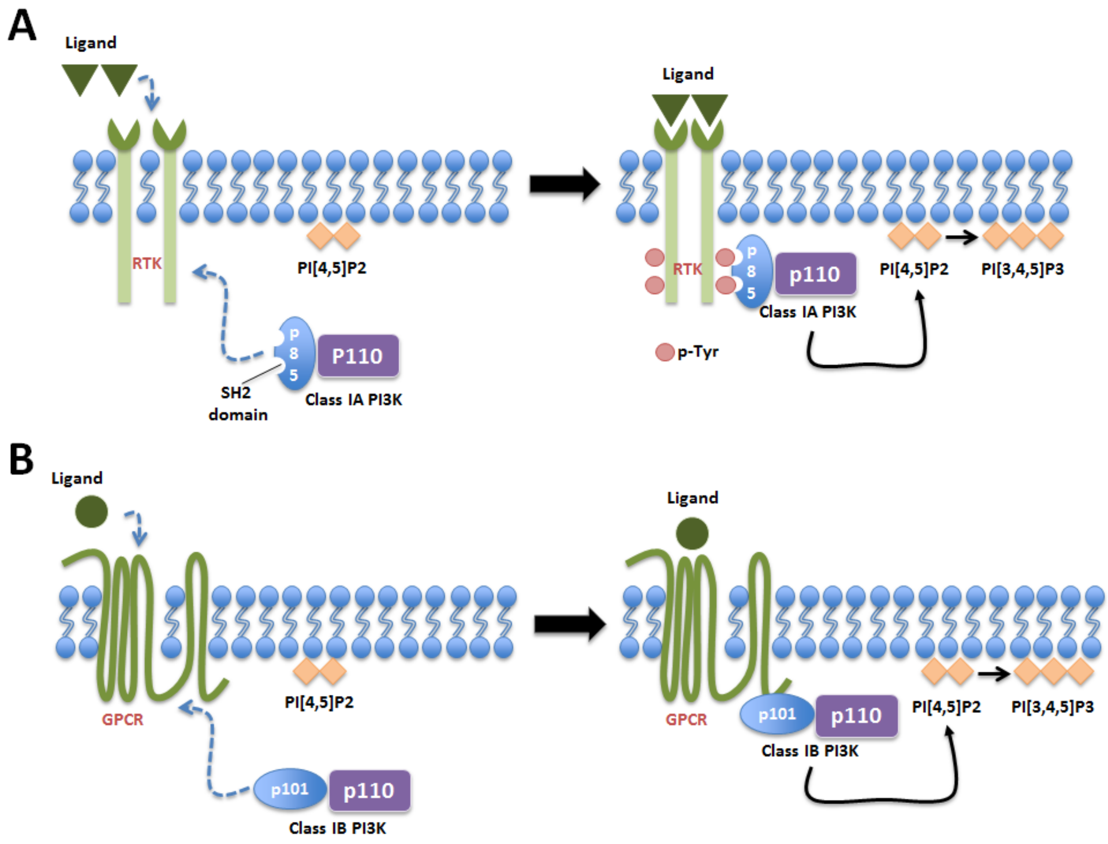


Figure 2 Receptor mediated activation of class I PI3K.

Recruitment and activation of class I PI3K is predominantly mediated through receptor tyrosine kinase (RTK) and G-protein coupled receptor (GPCR) signalling. (**A**) Class IA PI3K catalytic subunit p110 (α,β,δ) is recruited to the membrane by its adaptor protein p85. p85 contains two Src-homology 2 (SH2) domains that bind phosphorylated tyrosine residues of RTK, induced by auto-phosphorylation following receptor ligand binding. (**B**) The catalytic subunit p110γ of class IB PI3K is recruited to membrane via the adaptor protein p101, which interacts with the G$\beta\gamma$ subunits of GPCRs post ligand binding. At the membrane, class I PI3K generate lipid products PI[3]P, PI[3,4]P2 and PI[3,4,5]P3 by phosphorylating the inositol ring of phosphoinositides at the 3' carbon position. Phosphorylation of PI[4,5]P2 to PI[3,4,5]P3 is the preferred target of class I PI3K activity. Adapted from Vanhaesebroeck and Alessi (Vanhaesebroeck and Alessi, 2000).

1.3.2 AKT structure and function

The serine/threonine protein kinase AKT belongs to the AGC subgroup of protein kinases; named after its homology with the kinase catalytic domains of protein kinase A, G and C (Fayard *et al.*, 2010). AKT is reported to phosphorylate up to 130 known substrates, regulating a number of host cell processes including cell proliferation, transcription, translation, apoptosis and metabolism (lipid and glucose) (Pearce *et al.*, 2010; Bononi *et al.*, 2011). AKT is regarded as one of the most important and best understood downstream effectors of PI3K activity (Shepherd *et al.*, 1998; Bononi *et al.*, 2011). Originally identified in 1995 to be activated by insulin signalling, AKT has undergone a surge in interest for its role in cell signalling pathways and its mechanisms of regulation (Kohn *et al.*, 1995; Hers *et al.*, 2011).

AKT exists as three isoforms (known as AKT1, AKT2 & AKT3) that share approximately 80% amino acid sequence identity and are widely expressed throughout human tissues (Liao and Hung, 2010); except for AKT3 which is expressed mainly in neuronal tissues (Vanhaesebroeck and Alessi, 2000). Although AKT isoforms show overlapping functions, mouse knockout studies have suggested specific/dominant roles for each isoform: AKT1, cell survival and proliferation; AKT2, glucose homeostasis; and AKT3, brain development (Cho *et al.*, 2001; Tschopp *et al.*, 2005). Each AKT isoform possesses a highly conserved domain structure made up of an N-terminal pleckstrin homology (PH) domain, a central kinase catalytic domain and a C-terminal regulatory tail, containing the hydrophobic motif (Vanhaesebroeck and Alessi, 2000). This architecture is highly conserved amongst AGC kinase members, such as protein kinase C (PKC) and S6 kinase (S6K).

In un-stimulated cells, AKT is predominantly localised to the cell cytosol in an inactive state through an intramolecular interaction between its PH domain and its central catalytic kinase domain (Figure 3) (Calleja *et al.*, 2007). Following the stimulation of PI3K activity, AKT is recruited to the plasma membrane in a PH dependent manner, binding with similar high affinity for 3-phosphoinositides PI[3,4,5]P3 and PI[3,4]P2 (Frech *et al.*, 1997). This recruitment of AKT to the membrane induces a conformational change, leading to the disruption of the PH-kinase domain interaction resulting in the exposure of its ‘activation loop’ (Milburn *et al.*, 2003; Calleja *et al.*, 2007). This step is critical to expose the residue

Thr308 within the catalytic kinase domain for phosphorylation by the key activating kinase, phosphoinositide-dependent kinase 1(PDK1) (Calleja *et al.*, 2007).

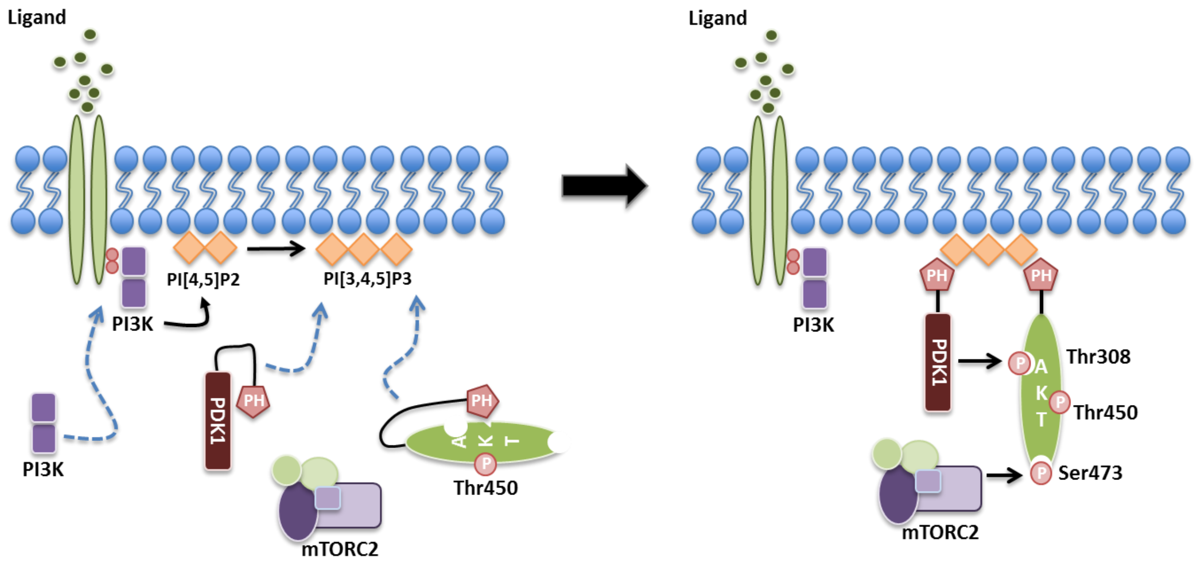


Figure 3 A model of PI3K dependent activation of AKT.

Upstream receptor agonist interaction stimulates the recruitment and activation of class I PI3K to the membrane. Once recruited, class I PI3K phosphorylates PI[4,5]P2 to PI[3,4,5]P3. The increase of PI[3,4,5]P3 stimulates the recruitment and co-localisation of AKT and PDK1 to the membrane. AKT and PDK1 interact with PI[3,4,5]P3 through binding via their respective pleckstrin homology (PH) domains. Binding of AKT to PI[3,4,5]P3 alters the conformation of AKT, exposing the catalytic kinase domain. PDK1, which is now in close proximity, can now phosphorylate and activate AKT within the activation loop at Thr308. AKT is then phosphorylated within its hydrophobic motif at Ser473, leading to its full activation, by the mammalian target of rapamycin complex 2 (mTORC2). AKT is also constitutively phosphorylated at Thr450 to stabilise newly synthesised AKT, which is dependent on mTORC2. Adapted from Hers *et al.,* (Hers *et al.*, 2011).

1.3.3 AKT activating kinase - PDK1

PDK1 is a serine/threonine kinase, consisting of a C-terminal kinase domain and an N-terminal PH domain (Vanhaesebroeck and Alessi, 2000). PDK1 is constitutively active in cells through the autophosphorylation of residue Ser241 within the kinase domain (Alessi *et al.*, 1997a; Casamayor *et al.*, 1999). Autophosphorylation of PDK1 occurs independently of PI3K activation. Localised to the plasma membrane in a PH domain dependent manner, PDK1 is named after its requirement for 3-phosphoinositides (PI[3,4,5]P3 & PI[3,4]P2) for AKT activation (Figure 3) (Alessi *et al.*, 1997b; Currie *et al.*, 1999). Binding with high affinity for PI[3,4,5]P3 and PI[3,4]P2, the PH domain of PDK1 can also bind with low affinity to PtdIns-[4,5]-bisphosphate (PI[4,5]P2) (Currie *et al.*, 1999; Komander *et al.*, 2004). Since PI[4,5]P2 is consistently present in the membrane under basal conditions, this may account for the pool of PDK1 associated with the plasma membrane in un-stimulated cells (Currie *et al.*, 1999). Indeed, whether PDK1 is further recruited to the plasma membrane in response to PI3K activation remains controversial, with conflicting studies reporting growth factor stimulated translocation or no effect at all (Currie *et al.*, 1999; Filippa *et al.*, 2000).

1.3.4 AKT phosphorylation and activation

AKT phosphorylation is critical for its activation. Co-localisation of AKT and PDK1 at the plasma membrane brings the exposed kinase domain of AKT in close proximity to PDK1, leading to the phosphorylation of residue Thr308 (Figure 3) (Alessi *et al.*, 1997b). This phosphorylation of Thr308 stabilises the activation loop of AKT into an active conformation, increasing its activity by approximately 100 fold (Alessi *et al.*, 1996; Alessi *et al.*, 1997b). To achieve maximum activity, AKT undergoes a second phosphorylation step within its hydrophobic motif at residue Ser473 (Figure 3) (Alessi *et al.*, 1996; Hers *et al.*, 2011). This second phosphorylation event is attributed to the activity of the mammalian target of rapamycin complex 2 (mTORC2), though the mechanism by which this is regulated remains to be fully defined (Sarbassov *et al.*, 2005; Pearce *et al.*, 2010; Yang *et al.*, 2015). Interestingly, studies by Yang *et al*., have proposed a mechanism by which mTORC2 activity is increased through the phosphorylation of its subunit SIN1 by AKT (phosphorylated at Thr308), thus stimulating the phosphorylation of AKT at Ser473 in a positive feedback loop (Yang *et al.*, 2015). This phosphorylation of AKT at Ser473 is reported to increase AKT activity by approximately 10 fold (Alessi *et al.*, 1996), promote phosphorylation of Thr308 (Sarbassov *et al.*, 2005) and contribute to the substrate specificity of AKT (Jacinto *et al.*, 2006). In addition, AKT is also constitutively phosphorylated at Thr450 in an mTORC2 dependent, but PI3K independent manner (Facchinetti *et al.*, 2008; Liao and Hung, 2010; Pearce *et al.*, 2010). This phosphorylation of residue Thr450 is thought to both stabilise and protect AKT from dephosphorylation at its hydrophobic motif (Hauge *et al.*, 2007). This phosphorylated and active form of AKT can then dissociate from the plasma membrane and translocate to the cytosol and nucleus. Once here, AKT can regulate a number of host cellular functions, mediated through the phosphorylation of several downstream substrates such as glycogen synthase kinase 3 (GSK3), tuberous sclerosis complex 2 (TSC2) and c-rapidly activated fibrosarcoma (c-Raf). Phosphorylation by AKT can have various effects on these proteins including inhibiting or stimulating their activity, altering subcellular localisation and/or protecting proteins from degradation (Manning and Cantley, 2007; Hers *et al.*, 2011). These substrates are implicated in many diverse functions, including glycogen metabolism, cell cycle progression/survival and ribosome biogenesis.

AKT signalling is typically short lived, terminated by the rapid dephosphorylation of AKT at Thr308 and Ser473 through the action of protein phosphatases 2A (PP2A) and PH domain leucine rich repeat phosphatase (PHLPP) respectively (Bayascas and Alessi, 2005; Brognard *et al.*, 2007; Liao and Hung, 2010). In addition, the membrane localised phosphatase and tensin homolog deleted on chromosome 10 (PTEN) can also negatively regulate the PI3K/AKT pathway by dephosphorylation of PI[3,4,5]P3 to PI[4,5]P2, thereby preventing further recruitment and activation of AKT (Hers *et al.*, 2011). A similar role has been proposed for the SH2 domain containing inositol 5-phosphatase (SHIP), inducing the dephosphorylation of PI[3,4,5]P3 to PI[3,4]P2 (Liu *et al.*, 1999b; Carver *et al.*, 2000).

1.3.5 Dysregulation of the PI3K/AKT signalling pathway

As discussed earlier, the PI3K/AKT signalling pathway plays a critical role in number of cellular processes, including cell survival, cell proliferation and metabolism. Due to its complex regulation of key cellular events it is unsurprising that altered AKT activity is linked with various human diseases including cancer, diabetes and cardiovascular disease (Hers *et al.*, 2011). The PI3K/AKT signalling pathway is one of the most frequently mutated pathways in human cancer, with AKT2 found to be the most common isoform overexpressed in 10% of pancreatic cancers and 57% of colorectal cancers (Hers *et al.*, 2011). Despite being one of the most hyperactive pathways in cancer, mutations of AKT itself are in fact extremely rare. Dysregulation of the PI3K/AKT signalling pathway therefore typically occurs through mutation or altered expression of the many components regulating AKT activity (Hers *et al.*, 2011). These include the overexpression or activating mutations of upstream receptor signalling (RTKs or GPCRs) (Rusch *et al.*, 1997; Bache *et al.*, 2004), activating mutations of PI3K (mutations of the regulatory subunit) (Lin *et al.*, 2001), and inactivating mutations of the phosphoinositide 3'-phosphatase PTEN (negatively regulated AKT activity) (Dillon and Miller, 2014). Given the central role of AKT in human cancers and other life threatening diseases, targeting of AKT is becoming an increasingly explored strategy for therapeutic treatment. However, with AKT implicated in numerous biological processes, the risk of causing off target effects using inhibitors can be a significant limitation to any potential long-term therapy (Hers *et al.*, 2011). Better dissection of the PI3K/AKT signalling cascade and its interactions with other pathways is therefore required to improve outcomes of future treatment strategies.

1.4 Pathogenic Bacteria

Pathogenic bacteria are often considered the masters of survival, evolving strategies to establish colonisation and avoid detection by the host immune system. This is achieved by targeting of host cells, either through cell attachment or invasion, before subverting cellular functions. An emerging area of study has been the ability of bacteria to subvert host cell signalling pathways that control a number of critical cellular functions, including cytoskeletal dynamics, cell death and the immune response. Much of this work has focused on defining the mechanisms employed by bacteria to disrupt signalling cascades, including MAPK and nuclear factor kappa B (NFκB) (Vallance and Finlay, 2000; Reddick and Alto, 2014). Examining the mechanisms employed by pathogenic bacteria to subvert eukaryotic cell signalling has provided critical insight into these signalling cascades and the host proteins affected (Cui and Shao, 2011). Thus, studying pathogenic bacteria has proved a unique tool to understand the complexities of eukaryotic cell signalling, which may prove useful for the identification of potential therapeutic targets for the treatment of disease (Krachler *et al.*, 2011).

1.4.1 Pathogenic bacteria can disrupt MAPK and NFκB signalling cascades

The innate immune response acts as the first line of defence against invading pathogens; triggered through recognition of pathogen associated molecular patterns (PAMPs), such as the bacterial cell surface protein lipopolysaccharide (LPS). PAMPs are detected by a collection of evolutionary conserved receptors known as pattern recognition receptors (PRR), which include toll-like receptors (TLR) and NOD-like receptors (NLR). Upon recognition of PAMPs, PRRs trigger the rapid initiation of intracellular signalling cascades including the MAPK and NFκB (Figure 4) (Takeuchi and Akira, 2010). Activation of MAPK and NFκB signalling induces the transcription of a number of pro-inflammatory genes, including cytokines and anti-microbial proteins. Furthermore, both MAPK and NFκB signalling also regulate other cellular functions including cell proliferation, cell migration and apoptosis.

The NFκB transcription factor exists as a homo or heterodimer of proteins that include RelA (p65), RelB, c-Rel, p50, p52 and RPS3 subunit, of which the p65/p50 hetrodimer is the most abundant. Under normal conditions, the NFκB complex is maintained in an inactive state in the cytosol through binding with the inhibitory subunit IκB (Figure 4). Activation of the NFκB signalling pathway induces the phosphorylation of IκB, resulting in its dissociation and degradation via the ubiquitin-proteasome system. Phosphorylation of IκB is mediated by the IκB kinase kinase (IKK) complex, which is activated downstream of PRR signalling (Figure 4). This dissociation of IκB enables the NFκB complex to translocate to the nucleus, binding promoters and enhancers that regulate gene transcription. Alternatively, activation of MAPK signalling pathway triggers a series of phosphorylation steps culminating in the activation of MAPK family proteins, including p38, JNK and ERK (Figure 4). This kinase cascade is mediated by the sequential phosphorylation of MAP kinase kinase kinase (MKKK), then MAP kinase kinase (MKK), and finally MAP kinase. Once active, MAPK proteins can then translocate to the nucleus, binding and activating transcription factors, including those of the AP-1 family (Jun & Fos). Together, induction of NFκB and MAPK signalling regulates the transcription of many pro-inflammatory genes. Bacteria that interfere with these signalling cascades typically target one or more stages in the pathway for inhibition, highlighting their importance to the bacteria for establishing an infection (Krachler *et al.*, 2011).

The capacity of bacteria to subvert host cellular processes is often dependent on its ability to translocate virulence proteins (effectors) into host cells via a delivery system. These effector proteins may target and manipulate several critical steps in cell signalling cascades, acting as inhibitors or activators of target substrates (Krachler *et al.*, 2011). One of the earliest examples of bacteria subverting host signalling was by the effector protein YopJ, delivered into host cells by the pathogenic *Yersinia* species (Palmer *et al.*, 1998; Orth *et al.*, 1999). YopJ inhibits MAPK and NFκB signalling pathways by preventing activation of regulatory kinases, MKKKs and IKK (β subunit only) respectively. The inhibitory capacity of YopJ is dependent on its acetyl transferase activity, preventing the phosphorylation of both MKK and IKK through acetylation of serine and/or threonine residues within their activation loop (Mukherjee *et al.*, 2006). By preventing their phosphorylation, YopJ acts to inhibit MKK and IKK activation, thus terminating both MAPK and NFκB signalling cascades. The advantage this confers to *Yersinia* include the initiation of host cell death by preventing cell survival and apoptosis signals that are critical to the immune response (Alto and Orth, 2012). Other notable examples of bacteria subverting MAPK and NFκB signaling include *Shigella flexneri* (IpaH9.8), enterohemorrhagic *E. coli* (EHEC; NleH1/NleH2) and EPEC (NleE) (Alto and Orth, 2012).

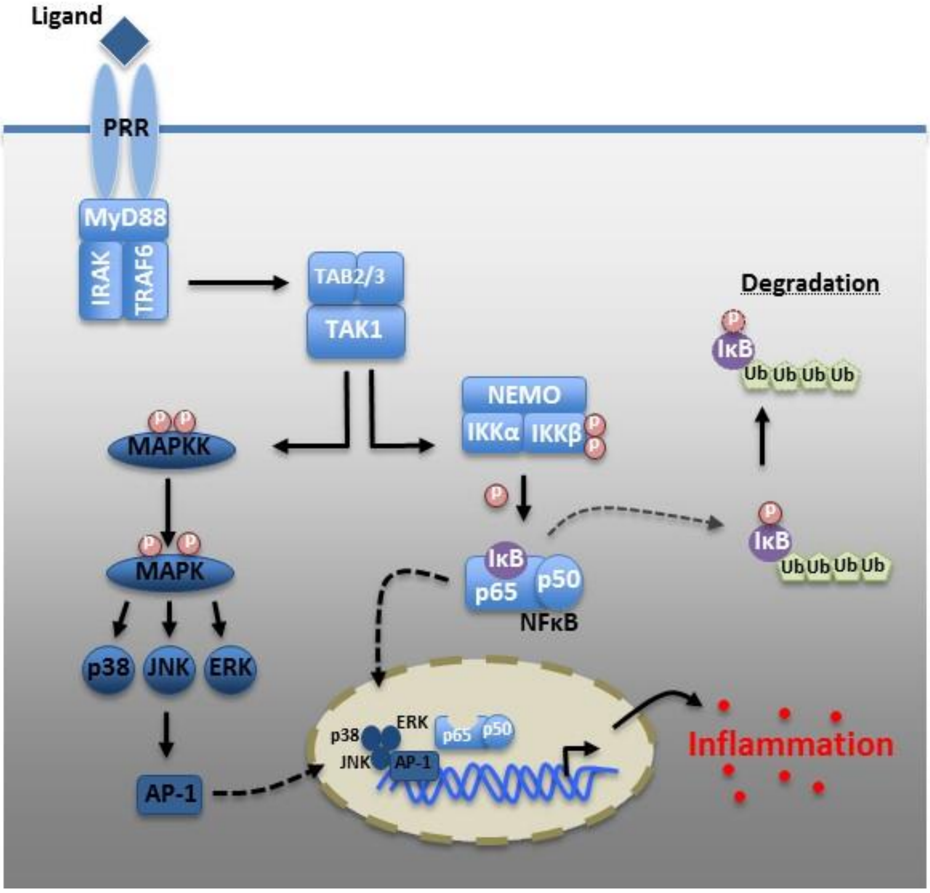


Figure 4 Simplified representation of the MAPK and NFκB inflammatory signalling cascade.

PRR ligand binding stimulates the recruitment of adaptor proteins such as MyD88. Binding of MyD88 enables the recruitment of IRAK to the receptor complex, which in turn recruits the ubiquitin ligase, TRAF6. TRAF6 then recruits TAK1 and TAB2/3, inducing their activation through close proximity, before dissociating from the receptor complex. Once active, TAK1 stimulates pro-inflammatory signalling by two distinct pathways: MAPK and NFκB. **1)** TAK1 initiates MAPK signalling through a series of sequential phosphorylation steps (MAPKKK > MAPKK > MAPK), resulting in the phosphorylation and activation of activating protein 1 (AP1) transcription factors (p38, JNK & ERK). This ultimately leads to the activation and translocation to the nucleus regulating gene expression. **2)** TAK1 activates NFκB signalling through its activation of the IKK complex, composed of subunits NEMO, IKKα and IKKβ. TAK1 phosphorylates IKK, which in turn phosphorylates the NFκB inhibitory subunit IκB, inducing its dissociation and degradation by ubiqutination. NFκB can now translocate to the nucleus and regulate expression of pro-inflammatory genes. Adapted from Krachler *et al.,* (Krachler *et al.*, 2011).

1.4.2 Pathogenic bacteria can subvert the PI3K-AKT signalling pathway

The PI3K/AKT signalling pathway is an increasingly recognised target for pathogenic bacteria, used to promote their entry into host cells and modulate host survival signals (Pizarro-Cerda and Cossart, 2004). Among these bacteria include *Shigella flexneri*, *Salmonella enterica* and uropathogenic *E. coli* (UPEC) (Wiles *et al.*, 2008). *Shigella flexneri* and *Salmonella enterica* are facultative intracellular pathogens, responsible for a number of human diseases including gastroenteritis and bacillary dysentery. Like many gram-negative bacteria, pathogenicity of *Shigella flexneri* and *Salmonella enterica* is dependent on the delivery of a plethora of effector proteins into host cells through a type 3 secretion system (T3SS); hijacking cellular systems to promote their colonisation. IpgD and SopB are two effector proteins synthesised and delivered by *Shigella flexneri* and *Salmonella enterica* respectively, reported to promote their uptake by host cells through manipulation of phosphoinositide metabolism (Pizarro-Cerda and Cossart, 2004). In addition to promoting uptake, IpgD and SopB are known to activate the pro-survival kinase AKT; however, the mechanism by which this activation occurs remains to be fully defined (Steele-Mortimer *et al.*, 2000; Pendaries *et al.*, 2006).

IpgD and SopB possess phosphoinositide phosphatase activity, which is critical for AKT activation; demonstrating preference for phosphoinositides PI[4,5]P2 and PI[3,4,5]P3/PI[3,4]P2 respectively (Steele-Mortimer *et al.*, 2000; Pendaries *et al.*, 2006). Delivery of IpgD and SopB is associated with the localised accumulation of PtdIns-[5]-monophosphate (PI[5]P) at the site of infection; though its function is not fully understood (Pendaries *et al.*, 2006). Studies by Pendaries *et al*., have suggested the increase of PI[5]P, induced by IpgD, is critical to phosphorylate AKT at Thr308 and Ser473 (Pendaries *et al.*, 2006). This phosphorylation of AKT was found to be dependent on the activation of class IA PI3K via tyrosine phosphorylation, though how PI[5]P might activate PI3K is not known (Pendaries *et al.*, 2006). Furthermore, PI[5]P may also indirectly increase AKT activity through inhibition of PP2A induced dephosphorylation of AKT at Thr308 (Ramel *et al.*, 2009). Although speculative, increased PI[5]P accumulation at the membrane may play a similar role in AKT activation, stimulated by *Salmonella* effector protein SopB (Cooper *et al.*, 2011).

In addition to the increase of the phosphoinositide PI[5]P, *Salmonella enterica* infection is associated with increased PI[3,4,5]P3 and PI[3,4]P2 at the site of invasion, coupled with the recruitment and co-localisation of AKT and PDK1 (Cooper *et al.*, 2011). In contrast to *Shigella* effector protein IpgD, the requirement of class IA PI3K activity in SopB induced AKT phosphorylation has proved controversial. Studies by Cooper *et al*., have suggested that AKT activation stimulated by SopB is both sensitive and insensitive to PI3K inhibitors LY294002 and Wortmannin respectively (Cooper *et al.*, 2011). However, reports by Roppenser *et al.*, have shown the formation 3-phosphoinositides (PI[3,4,5]P3 & PI[3,4]P2) and AKT phosphorylation is inhibited by both PI3K inhibitors (LY294002 & wortmannin) at high concentrations (Roppenser *et al.*, 2013). Discrepancies between the two studies may represent differences in cell model used; suggesting that AKT phosphorylation induced by *Salmonella enterica* may employ PI3K dependent and/or independent mechanisms depending on cell type (Cooper *et al.*, 2011; Roppenser *et al.*, 2013). This finding is supported by Roppenser *et al.,* who revealed that up to 90 kinases linked to AKT activation are influenced by *Salmonella enterica* infection (Roppenser *et al.*, 2013).

In contrast to *Shigella flexneri* and *Salmonella enterica*, active AKT is rapidly decreased following infection with the uropathogenic bacteria, UPEC. The leading cause of urinary tract infections, UPEC triggers the rapid dephosphorylation of AKT at Thr308 and Ser473 (Foxman and Brown, 2003; Wiles *et al.*, 2008). Dephosphorylation of AKT and the resulting loss of activity are dependent on UPEC secretion of the potent pore-forming toxin HlyA; used to enhance pathogenesis and promote bacterial crossing of mucosal barriers, thereby promoting colonisation (Wiles *et al.*, 2008). Although the precise mechanism through which HlyA induces the loss of AKT activity is unknown, Wiles *et al.,* have shown this rapid dephosphorylation of AKT (Thr308/Ser473) to be dependent on the activity of protein phosphatases, protein phosphatase 1 (PP1) and PP2A (Wiles *et al.*, 2008). This seemingly aberrant activation of protein phosphatases is dependent on the pore forming activity of HlyA, which is linked to the stimulation of osmotic stress (Meier *et al.*, 1998; Wiles *et al.*, 2008). Similar observations have been identified using other pore forming toxins including aerolysin and α-toxin, indicating HlyA targeting of AKT for inactivation is not unique (Wiles *et al.*, 2008). This suggests the activity of pore forming toxins may have additional major pathogenic roles other than cell lysis through modulation of host cell signalling pathways.

The enteric pathogen EPEC is also recognised to target and inhibit the PI3K/AKT signalling pathway, though how this occurs is not understood (Celli *et al.*, 2001). Examining the mechanism utilised by EPEC to subvert PI3K/AKT signalling will form the basis of this study.

The remainder of this report will focus on the role of EPEC and the effector proteins used to manipulate host cellular functions.

1.5 Enteropathogenic *E. coli* (EPEC)

EPEC is a gram-negative human pathogen of the small intestine. Identified in 1978 by Levine *et al* using the now prototypic strain E2348/69, EPEC pathogenesis contributes a significant risk to human health and disease, causing vomiting, fever and watery diarrhoea predominantly in infants and young children (Levine *et al.*, 1978; Chen and Frankel, 2005). Although outbreaks are largely rare in economically developed nations, EPEC infection remains a persistent problem to developing countries accounting for at least 20% of reported diarrheal cases, which can often prove fatal (Gomes *et al.*, 1991; Kaper *et al.*, 2004; Ochoa *et al.*, 2008). With *E. coli* infections implicated in approximately 2 million diarrhoeal related deaths per year, understanding the pathogenic mechanisms of *E. coli* strains such as EPEC has become of increasing importance (Chen and Frankel, 2005). Despite significant advances in cell biology, genetics and intestinal physiology that have improved the characterisation of EPEC pathogenesis, the mechanisms and bacterial proteins behind many of these clinical features remain largely unknown (Chen and Frankel, 2005; Dean and Kenny, 2009).

EPEC is a member of a family of diarrhoeagenic bacteria known as attaching effacing (A/E) pathogens, inducing the formation of characteristic histopathological structures termed A/E lesions (Figure 5) (Levine *et al.*, 1978). This family of A/E pathogens also include the human pathogen enterohemorrhagic *E. coli* (EHEC), the mouse pathogen *Citrobacter rodentium* (CR), and the Rabbit pathogenic *E. coli* (RPEC). Caused by intimate attachment between the host plasma membrane and bacteria, A/E lesions can be observed at ultra-structural level on the apical surface of intestinal epithelial cells, known as enterocytes. These A/E lesions are characterised by the destruction of absorptive microvilli and significant cytoskeletal changes that include the accumulation of polymerized actin beneath adherent bacteria, known as pedestals (Figure 5) (Moon *et al.*, 1983; Knutton *et al.*, 1989). These pedestal structures protrude from the apical surface of enterocytes by as much as 10 μm *in vitro*, cupping the individual bacteria (Rosenshine *et al.*, 1996b). Although the functional significance of this structure remains incompletely understood, it is thought that pedestal formation may promote EPEC's extracellular lifestyle. The ability of these bacteria to induce A/E lesion formation is encoded on the highly conserved 35.6 kb pathogenicity island termed the locus of enterocyte effacement (LEE; Figure 6), which is both necessary and sufficient for EPEC to induce a number of subversive events including the formation of A/E lesions (McDaniel and Kaper, 1997). Acquired by horizontal gene transfer, the LEE region of EPEC (E2348/69) contains 41 genes organised into 5 polycistronic operons, which encode gene regulators, chaperones, EPEC secreted/signalling proteins (Esp) and components of the type 3 secretion system (T3SS) (Chen and Frankel, 2005).

EPEC infection predominantly occurs by transmission through oral-faecal contact from contaminated hands or food (Bardiau *et al.*, 2010). Once localised to the small intestine, the pathogenicity of EPEC infection is proposed to follow four main stages: (1) initial adherence and colonisation of the small intestine; (2) translocation of EPEC effector proteins into host cells; (3) formation of attaching effacing lesions; and (4) intimate attachment of EPEC to host cell membrane leading to pedestal formation and diarrhoea (Figure 7) (Clarke *et al.*, 2003; Chen and Frankel, 2005).

A

B

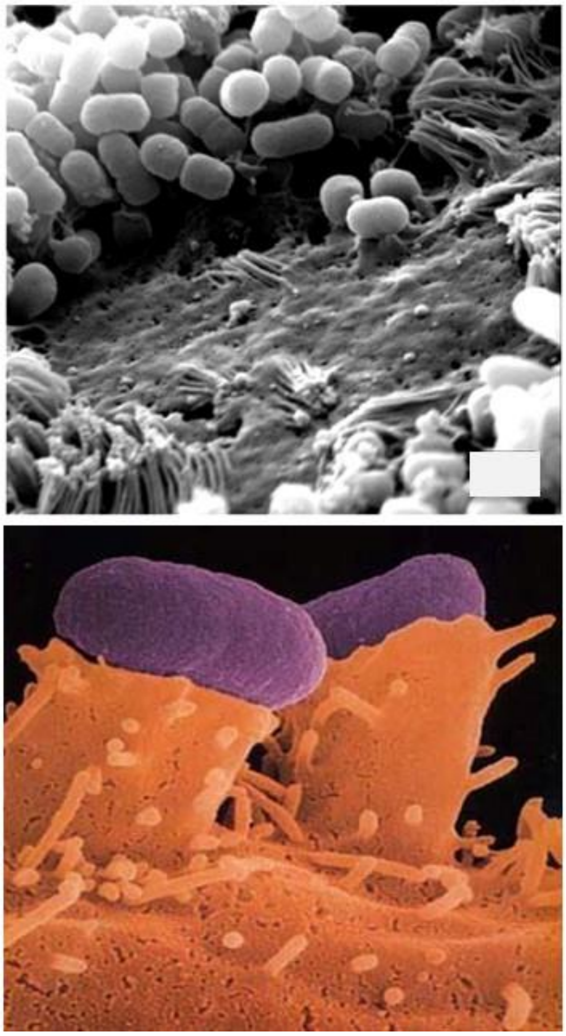


Figure 5 EPEC induced attaching effacing lesions and actin rich pedestals.

Representative images of EPEC induced infection characteristics. (**A**) The extensive effacement of microvilli on the apical surface of epithelial cells; revealed by scanning electron microscopy (SEM) after 4 h of infection with wild type EPEC, using a Caco-2 cell model. Taken from Dean *et al.,* (Dean *et al.*, 2006). (**B**) An SEM image of EPEC induced actin rich structures known as pedestals, using a HeLa cell model. These pedestal structures protrude from the apical surface, cupping individual bacteria. The image was artificially coloured to distinguish bacteria (purple) from HeLa cell (brown). Taken from Rosenshine *et al.,* (Rosenshine *et al.*, 1996b).

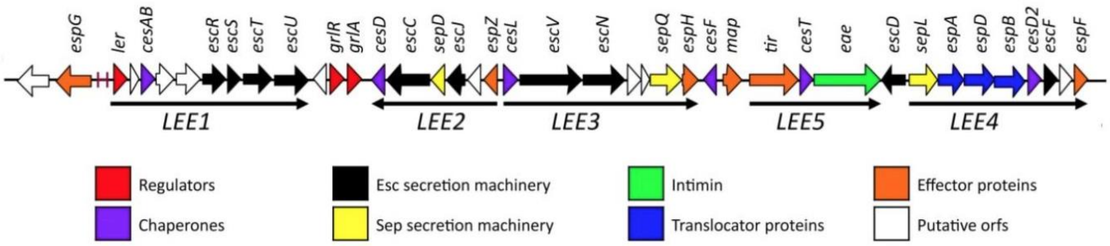


Figure 6 Schematic of the genetic organisation of the locus of enterocyte effacement.

The locus of enterocyte effacement (LEE) is organised into 5 polycistronic operons, indicated LEE1-LEE5, encoding up to 41 known genes. These genes include those encoding: regulator proteins (Red); a T3SS, composed of *E. coli* secretion (Esc; Black) and secretion of EPEC proteins (Sep; Yellow); translocator proteins (Blue); chaperones (Purple); the outer membrane protein Intimin (*eae*; Green); effector proteins, termed EPEC secreted/signalling proteins (Esp; orange; that include Tir & Map); and other putative open reading frames (Orf; White). Taken from Wong *et al.,* (Wong *et al.*, 2011).

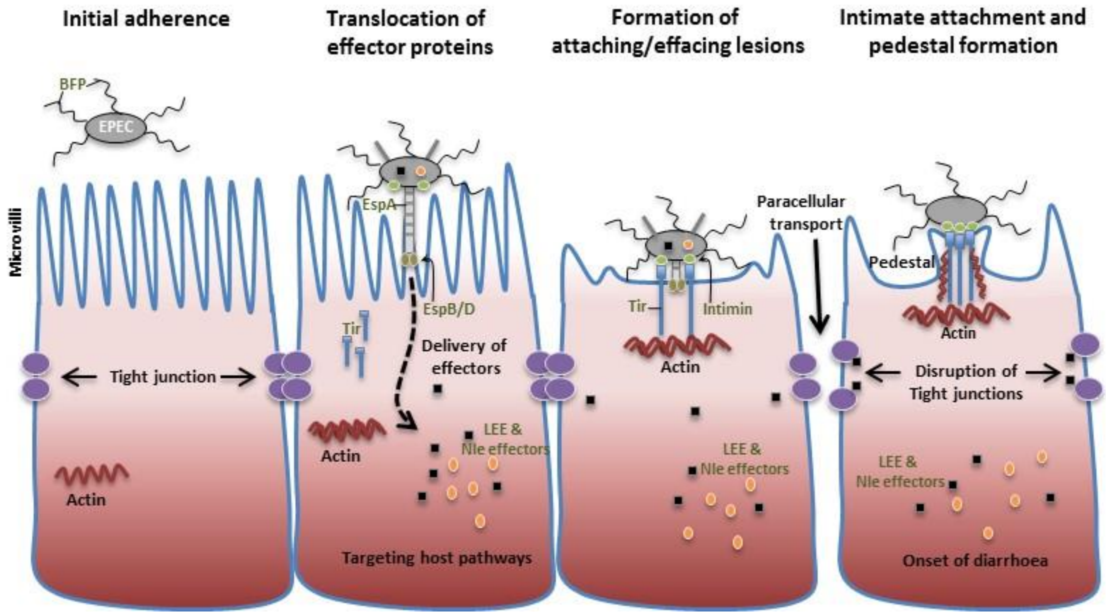


Figure 7 The four-stage model of EPEC infection.

(**1**) EPEC infection requires the initial attachment of EPEC to the apical surface of eneterocytes. Expression of the adhesin BFP aids this attachment process, forming discrete EPEC microcolonies and a characteristic localised adherence pattern. (**2**) EPEC, now in close proximity to the host cell membrane, delivers a plethora of effector proteins into the host cell via a type-3 secretion system (T3SS). The T3SS in addition to 6 effectors and 3 translocon proteins (critical for effector translocation, of which one [EspB] has effector function) are encoded on the pathogenicity island termed the locus of enterocyte effacement (LEE). Effectors translocated into the host that are not encoded on the LEE region are mostly termed non-LEE encoded (Nle) effectors, with some named Esp (e.g. EspG2, EspL & EspJ). (**3**) Through the cooperative action of LEE and Nle effector proteins, EPEC subverts many functions, in addition to disrupting the host cytoskeleton. This is evident by the extensive loss of absorptive microvilli on the apical surface of enterocytes known as effacement. (**4**) The final stage of EPEC infection is characterised by the formation of actin rich pedestals; the intimate attachment between bacterium and host cell membrane; and the onset of watery diarrhoea through increased paracellular diffusion, disruption of tight junctions, loss of absorptive microvilli and altered transporter function. Intimate attachment is mediated through binding of outer membrane protein Intimin and the T3SS delivered translocated intimin receptor (Tir) effector, which is inserted into the host cell membrane. Adapted from Clarke *et al.,* (Clarke *et al.*, 2003).

1.5.1 EPEC Pathogenesis

1.5.1.1 EPEC cell adherence and the bundle forming pilus

The initial adherence of EPEC to intestinal epithelial cells is known to occur in discrete clusters, forming what is described as a localised adherence pattern (Knutton *et al.*, 1987). This phenotype is rapidly promoted *in vitro* through pre-incubation of bacteria in tissue culture media (Vuopio-Varkila and Schoolnik, 1991). Formation of localised adherence is dependent on the expression of a number of EPEC genes encoded both on the chromosome and a plasmid subsequently named, EPEC adherence factor (EAF) (Baldini *et al.*, 1983). Encoded on the EAF plasmid is a cell surface adhesion structure known as the bundle forming pili (BFP) that was first described by Giron *et al* in 1991 as a bundle of rope like filaments (Giron *et al.*, 1991). Approximately 50-500 nm wide and extending from up to 14-20 μm long, the BFP filaments intertwine with those expressed on other localised EPEC forming a dense network that promotes microcolony formation (aggregation), stabilisation and non-intimate cell binding (Nataro and Kaper, 1998; Tobe and Sasakawa, 2001; Clarke *et al.*, 2003). Once bound to the cell, the BFP is thought to retract thereby bringing the bacterium closer to the cell surface for the effective translocation of effector proteins (Zahavi *et al.*, 2011). In addition to BFP, the EAF plasmid also encodes a cluster of regulatory genes known as plasmid encoded regulators A, B and C (*perA*, *perB* & *perC*) that regulate the expression of the BFP and other critical virulence factors including the T3SS via the LEE encoded regulator (LER) (Mellies *et al.*, 1999). However, not all EPEC strains carry the EAF plasmid; with those strains that do not unable to demonstrate a localised adherence pattern in cell culture. EPEC strains have therefore been classified into those that carry and those that do not carry the EAF plasmid, commonly referred to as typical and atypical EPEC respectively (Trabulsi *et al.*, 2002). Although atypical strains (EAF minus) are still capable of forming A/E lesions, these strains have been shown to be significantly delayed in lesion formation, inducing fewer cases of diarrhoea under human challenge (Levine *et al.*, 1985; Chen and Frankel, 2005; Bueris *et al.*, 2015). This suggests that expression of the BFP is not essential for full EPEC virulence, though may promote infection and EPEC colonisation. However, this has been challenged by studies in an intestinal *ex vivo* model, revealing the adhesion of EPEC strains lacking BFP (Hicks *et al.*, 1998).

1.5.1.2 Effector translocation and the type 3 secretion system

The T3SS is an important organelle common to many gram-negative bacteria, responsible for the delivery of effector proteins into host cells to subvert host cellular functions. Encoded on the LEE region, the T3SS of EPEC is a multi protein structure, composed of approximately 20 proteins arranged into a 'needle complex' (Figure 8) (Coburn *et al.*, 2007). These proteins named EPEC secretion (Esc) span both the inner and outer bacterial membrane. The inner membrane ring is composed of membrane spanning proteins EscR, EscS, EscT, EscU and EscV, whereas the outer membrane ring is formed from the subunit EscC. The two membrane rings are connected across the periplasmic space by the T3SS component EscJ. The external needle like structure is then formed from the secretion and polymerisation of the subunit EscF, which extends out from the outer membrane ring (EscC) a short distance (Garmendia *et al.*, 2005). At the base of the T3SS is the cytoplasmic ATPase EscN, which is critical for effector translocation (Donnenberg *et al.*, 1990). Hydrolysis of ATP to ADP by EscN is thought to provide the energy to release effector proteins from their chaperones, discussed below, prior to effector protein delivery (Akeda and Galan, 2005; Garmendia *et al.*, 2005).

The T3SS is connected to the host cell membrane by a 'translocon' structure (Figure 8). Together, the T3SS needle complex and the translocon provides a continuous conduit for the translocation of effector proteins across the bacteria and host cell membrane and directly into the host cell cytosol. The translocon is composed of EPEC secreted/signalling proteins (Esp) EspA, EspB and EspD (Frankel *et al.*, 1998). EspA is an important structural component of the secretion system, forming a hollow filamentous extension of the EscF needle structure (Knutton *et al.*, 1998; Daniell *et al.*, 2001). Transiently expressed at the bacterial cell surface, polymerised EspA can interact with the host cell membrane, prompting speculation it may contribute to initial bacterial-host cell adhesion. Pore forming components, EspB and EspD, are then secreted through the needle complex and EspA filament, before inserting themselves into the host cell membrane. The functionality of this membrane pore is indicated by its capacity to induce haemolysis of erythrocytes in an EspB/EspD dependent manner (Warawa *et al.*, 1999; Shaw *et al.*, 2001). Secretion of translocon proteins (EspA, EspB & EspD) and their collective interaction with the T3SS is therefore critical for successful translocation of effector proteins and EPEC virulence (Hartland *et al.*, 2000; Luo and Donnenberg, 2011). Once fully formed, the T3SS promptly switches from the secretion of translocator proteins to the secretion of EPEC effector proteins. This switch is controlled by cytoplasmic regulators SepD and SepL (Figure 8), though how this occurs is poorly understood. Indeed, mutations of SepD and SepL have been shown to cause the loss of effector secretion hierarchy and the hyper-secretion effector proteins through the T3SS (Deng *et al.*, 2004; Deng *et al.*, 2005).

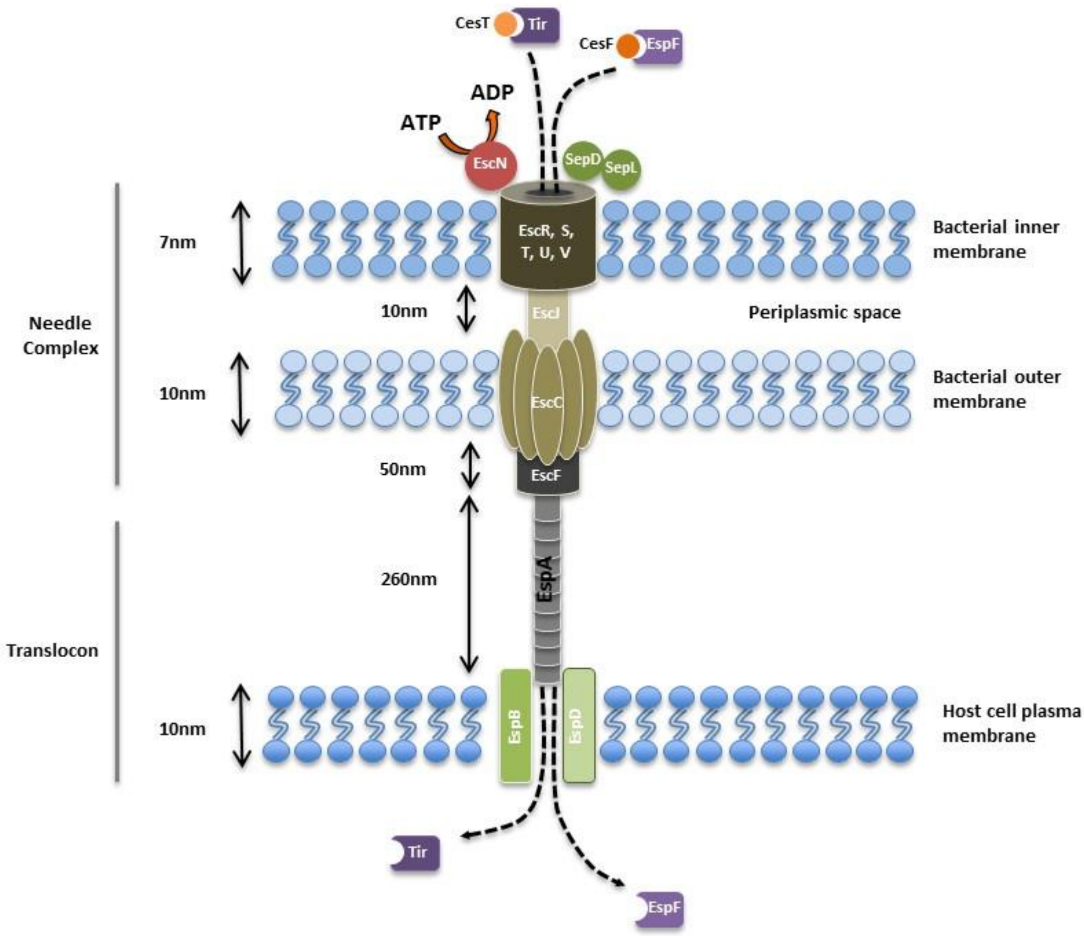


Figure 8 Simplified schematic of the EPEC T3SS.

The EPEC type 3 secretion system (T3SS) is a multiprotein complex comprised of *E. coli* secretion (Esc) and secretion of *E. coli* proteins (Sep). The T3SS needle complex is composed of proteins EscR, S, T, U and V, forming the inner membrane ring; EscJ, spanning the periplasmic space; EscC, spanning the outer membrane; and EscF, a short needle like structure extending away from the bacteria. This needle complex is connected to the host cell membrane via the translocaon. The translocon is formed of EspA, a long hollow filamentous structure, and membrane inserted proteins EspB and EspD, which are necessary for membrane pore formation. Together these proteins form continuous path for the delivery of effector proteins directly into the host cell cytosol, such as Tir and EspF. The bacterial cytosolic ATPase, EscN, provides the energy for this effector translocation through hydrolysis ATP to ADP. SepD, SepL and SepQ are proposed to regulate the switch from the secretion of translocator proteins to the secretion of EPEC effector proteins via the T3SS. Efficient translocation of most effector proteins is dependent on the chaperones CesF and CesT, though EspG, NleD and NleF are translocated independently of both chaperones. Adapted from Garmendia *et al.,* (Garmendia *et al.*, 2005).

1.5.1.3 EPEC effector proteins: LEE and Nle

Among the 41 genes contained within the LEE region include those encoding for three translocator proteins (EspA, EspB & EspD), six effector proteins (EspG, EspH, mitochondrial associated protein [Map], Tir, EspF & EspZ) and the outer membrane protein Intimin (*eae*). The functions of these translocator proteins have already been discussed. However, of these translocator proteins only EspB is reported to possess some effector activity, increasing the number of known LEE encoded effector proteins from six to seven (Iizumi *et al.*, 2007). Since Intimin is not delivered into host cells it is not considered to be a classical effector protein. However, the capacity of Intimin to trigger host cellular responses in both a Tir dependent and independent manner, through binding of host necleolin (Sinclair and O'Brien, 2002) and β-integin (Frankel *et al.*, 1996), highlights the importance of this virulent protein in EPEC pathogenesis (Liu *et al.*, 1999a; Phillips *et al.*, 2000; Dean and Kenny, 2004; Dean *et al.*, 2006).

In addition to the LEE region, other pathogenicity islands encoded on prophages (PP) and integrative elements (IE) have been identified throughout EPEC's genome; encoding up to 17 known effector proteins (Figure 9). These proteins encoded outside of the LEE region are therefore termed non-LEE encoded effectors (Nle), though a few retain the 'Esp' nomenclature. Together, these effector proteins (LEE & Nle) act to subvert a number of host cellular functions. Functional studies examining the impact of LEE and Nle effector proteins during EPEC infection have highlighted their complex and multifunctional roles. This is demonstrated through the number of overlapping, cooperative and antagonistic activities of the translocated effector proteins, used to manipulate several host cellular activities (Schmidt, 2010; Shames and Finlay, 2012). Examples of this will be discussed later on.

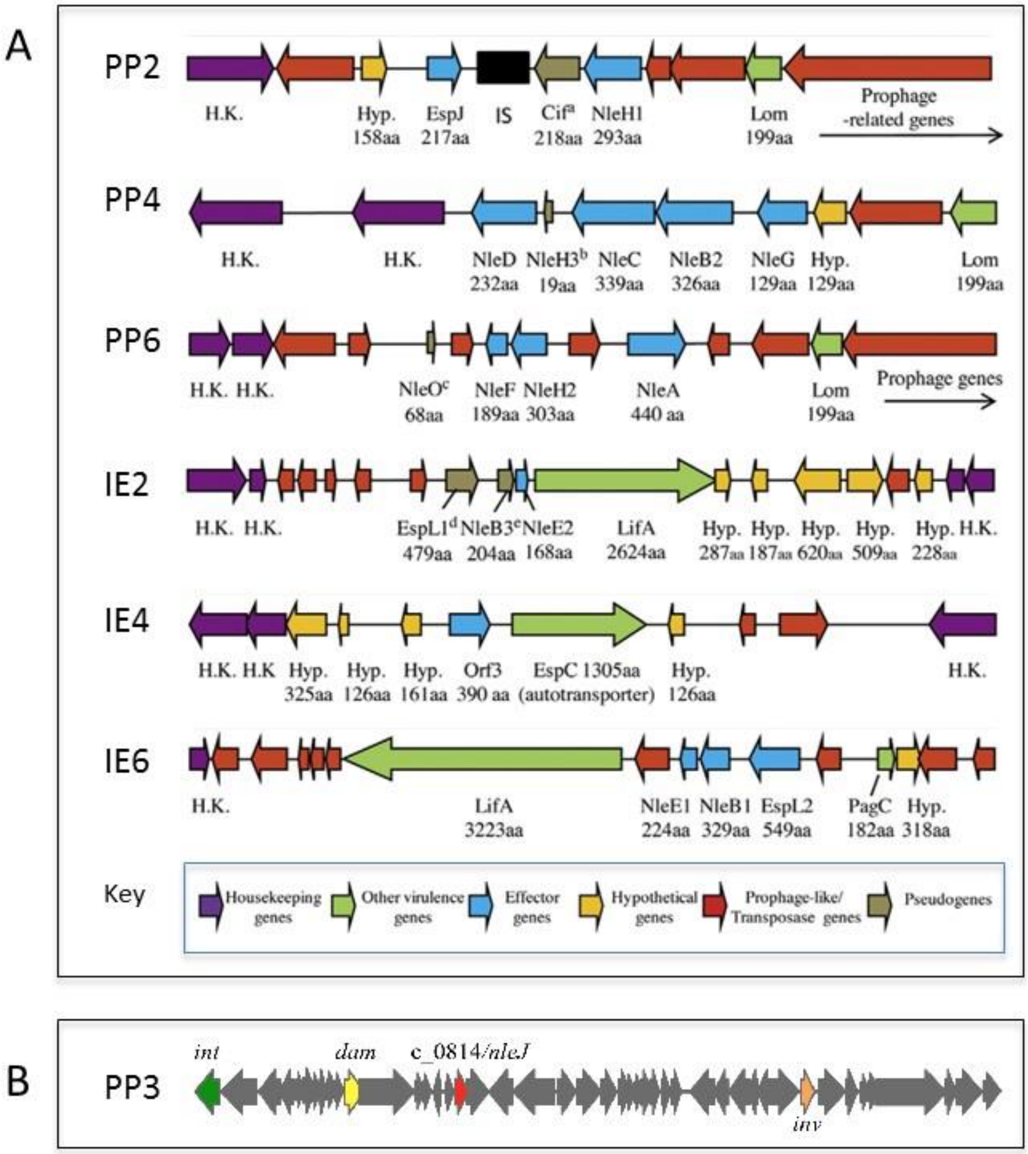


Figure 9 Non-LEE effector encoding pathogenicity islands.

Seventeen effector proteins are encoded outside of the LEE region. These effector proteins, termed non-LEE effector proteins (Nle), are encoded across three integrative elements (IE2, IE5 & IE6) and 4 prophages (PP2, PP3, PP4 & PP6) throughout EPEC's genome. Genes and strand direction are indicated using individual arrows. (**A**) Representation of the pathogenicity islands IE3, IE5, IE6, PP2, PP4 and PP6; encoding fourteen effectors (Blue); in addition effectors LifA (IE6; Green) and EspC (IE4; Green). Taken from Dean and Kenny *et al.,* (Dean and Kenny, 2009). (**B**) A simplified schematic of the prophage, PP3, encoding the most recently identified effector NleJ (Red). Taken from Deng *et al.,* (Deng *et al.*, 2012).

1.5.1.4 Chaperones and effector translocation

Effector translocation into target cells is a highly complex and regulated process, dependent on a functional T3SS and ancillary proteins known as chaperones encoded on the LEE region. Whilst these chaperones do not form part of the T3SS itself, it is becoming increasingly apparent they play a critical role in the efficient secretion of virulence proteins. Chaperones are implicated to stabilise virulent proteins within the bacterial cytosol, regulate gene expression, mediate trafficking/docking with the T3SS at the inner membrane and induce a hierarchical release of effector proteins (Cornelis *et al.*, 1998; Elliott *et al.*, 1999; Creasey *et al.*, 2003). EPEC encoded chaperones can be divided into three principal classes; those that bind effector proteins (Class I), those that bind translocon or pore forming proteins (Class II) and those that bind needle complex proteins (Class III). Class I type three secretion chaperones can be further divided into two categories, known as IA (single effector substrate binding) and IB (multiple effector substrate binding). EPEC encodes two Class I type three secretion chaperones on its LEE region (Figure 8); named chaperone of *E. coli* secreted Tir (CesT) and EspF (CesF) (Elliott *et al.*, 1999). CesF is a Class IA T3SC, which is thought to bind a single effector protein EspF. In contrast, CesT is a Class IB T3SC, which is recognised to bind and regulate the efficient translocation of at least 12 effector proteins. These effector proteins are either fully dependent (Tir, EspZ, EspH, Map, EspJ, NleG, NleH1 & NleH2) or only partially dependent (NleB1, NleB2, NleC & NleA) on the chaperone CesT for translocation into host cells (Mills *et al.*, 2013). The dependence of EspC, NleE1, NleE2 and EspL2 on both CesF and CesT chaperones has not been reported. Effector proteins NleD, NleF and EspG are translocated independently of CesF and CesT; however, whether the delivery of these effectors is indeed chaperone independent or dependent on a third as yet unknown Class I T3SC remains to be determined (Mills *et al.*, 2013).

The first EPEC effector protein delivered into host cells is Tir, followed by a temporal translocation order that demonstrates greater efficiency for LEE than most Nle encoded effector proteins (Mills *et al.*, 2013). The early delivery of Tir and other LEE effectors suggests the establishment of intimate attachment and A/E lesion formation is the most vital task during early EPEC infection. This is supported by the sufficiency of the LEE region to induce A/E lesions when expressed in a non-pathogenic *E. coli* K12 strains (McDaniel and Kaper, 1997). In contrast, Nle effectors are increasingly recognised to promote EPEC infection, targeting and subverting several host cell signalling pathways including those of cell survival and the inflammatory response (Krachler *et al.*, 2011; Alto and Orth, 2012; Clements *et al.*, 2012).

1.5.2 Subverting host cellular functions by EPEC effector proteins

1.5.2.1 Actin polymerisation and pedestal formation

The intimate attachment of EPEC to host cell surface is mediated through the binding of the EPEC outer membrane protein Intimin with the translocated intimin receptor (Tir). Originally named Hp90, the bacterial protein Tir represents the first documented example of a bacterium inserting its own receptor into host cell membranes for virulence (Kenny *et al.*, 1997). Subsequent binding of Intimin to Tir triggers the clustering of Tir beneath the bound bacterium, forming a strong bond between the bacterium and the host cell cytoskeleton, termed intimate cell attachment (Kenny *et al.*, 1997; Garmendia *et al.*, 2005). Tir is inserted into the host cell membrane in a hairpin like conformation; possessing two transmembrane domains, an extracellular domain for binding Intimin and intracellular amino and carboxy-termini. Once inserted into the host cell membrane, Tir is quickly phosphorylated by host kinases at several residues including Tyr474 within the carboxyl tail (Kenny, 1999; Campellone and Leong, 2005). This phosphorylation is reported to be mediated by Src family kinase c-fyn (SRK Fyn) (Phillips *et al.*, 2004), with additional studies implicating Abl (Swimm and Kalman, 2008) and Tec family kinases (Bommarius *et al.*, 2007). Phosphorylation of Tyr474 provides a docking site for the SH2 domain containing adaptor proteins Nck1 and Nck2 (Gruenheid *et al.*, 2001). Binding of Nck then facilitates the recruitment of neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin related protein (Arp)2/3 complex, resulting in the nucleation of actin formation of actin rich pedestals *in vitro* (Gruenheid *et al.*, 2001; Lommel *et al.*, 2001).

1.5.2.2 Mechanism of EPEC induced diarrhoea

It is well established that EPEC infection induces severe watery diarrhoea, though the precise mechanism by which this occurs remains poorly defined. The onset of diarrhoea is likely dependent on the collective action of multiple effector proteins, disrupting enterocyte cellular functions including: loss of absorptive microvilli (effacement), disruption of barrier function and alterations to ion/nutrient transporter activity (Figure 10).

The gastrointestinal epithelium is composed of a tightly packed monolayer of epithelial cells (enterocytes) that play a critical role in the absorption of nutrients from the gut. Often termed the brush border region, the apical membrane of enterocytes is made up of a number of finger like projections (microvilli) that increase their surface area, aiding absorption. These microvilli are constructed from bundles of actin filaments that are dependent on myosin for their formation, maintenance and stability. Loss of microvilli is a key characteristic in the formation of A/E lesions and a major contributor to the reduced absorptive capacity of enterocytes post infection. EPEC induced loss of microvilli is said to follow a two step process, whereby adherent bacteria first 'sink' into the brush border membrane prior to inducing extensive loss of cell surface microvilli (Dean *et al.*, 2006). The effacement of microvilli is linked to the cooperative actions of three LEE encoded effectors (Map, EspF & Tir) and the outer membrane protein Intimin (Figure 10) (Dean *et al.*, 2006). Although the mechanism and the contributing roles of each effector remain to be fully defined, depolymerisation of actin filaments is thought to play a principal role (Dean *et al.*, 2006). EspB also contributes to this effacement process through disruption of actin-myosin interactions leading to destabilisation of the actin bundles and loss of microvilli structure (Iizumi *et al.*, 2007).

The reduced capacity of enterocytes to absorb nutrients is also modulated by EPEC at the level of transporter function. Since water moves to areas of high salt concentration, it is unsurprising that ion gradients are manipulated by diarrheagenic bacteria. EPEC disrupts ion gradients in part by decreasing the activity of the sodium hydrogen exchanger (NHE), resulting in the reduced uptake of sodium ions (Na^+) from the gut lumen (Figure 10) (Hodges *et al.*, 2008). This T3SS dependent decrease of NHE activity is induced by the LEE effector EspF (Hodges *et al.*, 2008). Furthermore, decreased Na^+ uptake is exacerbated through reduced expression of sodium glucose co-transporter (SGLT1) at the apical membrane; triggered through the cooperative actions of Map, EspF, Tir and Intimin (Figure 10) (Dean *et al.*, 2006). In addition to sodium transport, EPEC can also reduce the uptake of chloride ions (CL^-) through decreased surface expression of the chloride bicarbonate exchanger (CL^-/$HCO3^-$) known as DRA (downregulated in adenoma). Dependent on effectors EspG and EspG2, this loss of DRA activity is mediated through disruption of microtubule networks necessary for protein trafficking to the membrane (Figure 10) (Gill *et al.*, 2007; Gujral *et al.*, 2015). EspG and EspF also contribute to the loss of water absorption through the redistribution of water channels (aquaporins) to intracellular compartments; away from the apical and lateral membrane (Figure 10) (Guttman *et al.*, 2007). Recent reports have also suggested that EPEC infection can decrease function and expression of the apical sodium dependent bile acid transporter (ABST), responsible for bile acid reabsorption within the gut, which may have implications for the development of diarrhoea (Annaba *et al.*, 2012). Together these effects likely contribute to the increase of water content within the gut lumen.

In addition to absorption, enterocytes play a critical role in barrier function; separating the contents of the gut lumen from that of the underlying tissues. Intercellular tight junctions are critical to this function, maintaining cell-cell adhesion and restricting the flow of molecules between cells known as paracellular diffusion. Tight junctions are complex structures composed of several transmembrane proteins including claudins, occludins, zonula occluden (ZO) and the junction adhesion molecule (JAM). It is increasingly recognised that EPEC induces barrier dysfunction through the redistribution of junctional proteins leading to the loss of tight junction integrity (Simonovic *et al.*, 2000; Guttman and Finlay, 2009; Zhang *et al.*, 2010). The mechanism by which this occurs is not understood, yet the activities of five LEE encoded proteins (Map, EspF, Tir, Intimin & EspG) and two Nle effectors (EspG2/Orf3 & NleA) have been implicated to play a significant role (Muza-Moons *et al.*, 2003; Dean and Kenny, 2004; Thanabalasuriar *et al.*, 2010; Glotfelty and Hecht, 2012). These effectors are not thought to act directly on junctional proteins themselves, though may modulate their function and distribution through altered actin assembly, disruption of microtubule networks and dysfunctional protein trafficking that are collectively required to maintain tight junction structures (Peralta-Ramirez *et al.*, 2008; Weflen *et al.*, 2009; Thanabalasuriar *et al.*, 2010; Glotfelty and Hecht, 2012).

The combined impact of decreased absorption and loss of barrier function are believed to be the principal factors contributing the development of severe watery diarrhoea during EPEC infection. Although the precise role and mechanism of each effector protein remain to be defined, it is clear the activities of LEE encoded effectors are central to the onset of diarrhoea.

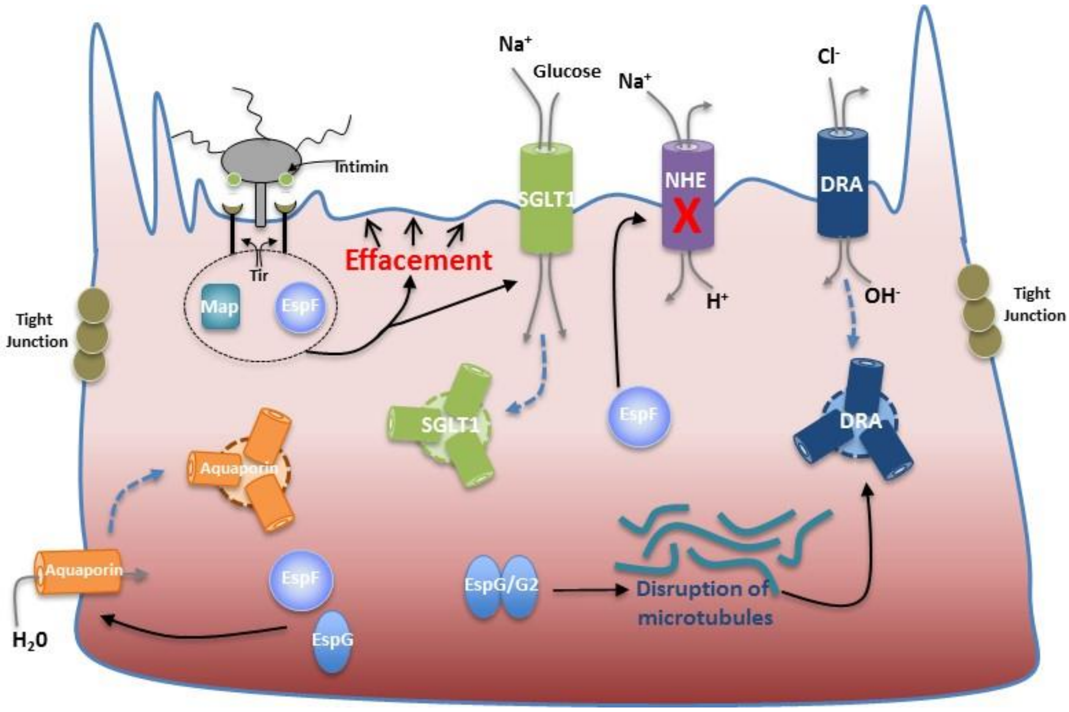


Figure 10 Disruption of cell absorption by EPEC effector proteins.

EPEC infection disrupts the absorption of ions, water and nutrients in a multifactorial process, dependent on the collective action of translocated effector proteins. EPEC effectors disrupt absorption through the loss of absorptive microvilii (effacement), induced by the cooperative activities of effectors Map, EspF, Tir and the outer membrane protein Intimin. These effectors also contribute to the reduced surface expression of sodium glucose transporter (SGLT1) by re-distribution to intracellular vesicles. Decreased water transport is mediated through the redistribution of water channels (Aquaporins) away from the apical and basolateral membrane, dependent on effectors EspF and EspG. The primary route of sodium uptake (Na^+) is the sodium hydrogen exchanger (NHE), which is disrupted by the effector EspF. Disruption of chloride (Cl^-) absorption through the chloride bicarbonate ($HCO3^-$) exchanger, known as downregulated in adenoma (DRA), is mediated by the effectors EspG and its Nle homolog EspG2. EspG and EspG2 disrupt the host cell microtubules, preventing protein trafficking, thus reducing DRA expression at the membrane. Adapted from Viswanathan *et al.,* (Viswanathan *et al.*, 2009).

1.5.2.3 EPEC inhibition of inflammation and the innate immune response

Intestinal epithelial cells play a critical role in immune surveillance within the gut, prompting initiation of the pro-inflammatory response following the detection of pathogenic bacteria. EPEC is detected through the recognition of bacterial surface proteins, including LPS and flagella by PRR TLR4 and TLR5 respectively (Zhou *et al.*, 2003; Edwards *et al.*, 2011). These receptors are predominantly located on the basolateral membrane of polarised epithelial cells and are thus largely inaccessible to EPEC during the early stages of infection (Ruchaud-Sparagano *et al.*, 2007; Ruchaud-Sparagano *et al.*, 2011). TLR agonist binding stimulates the activation of pro-inflammatory signalling pathways such as MAPK and NFκB, leading to the initial increase of cytokines such as IL8. EPEC inhibits these signalling pathways soon after infection through the cooperative action of several T3SS dependent effector proteins, prior to the disruption of barrier function, thereby reducing the pro-inflammatory response to infection (Figure 11).

EPEC effectors NleE and NleB are both reported to modulate the innate immune response by subverting the NFκB signalling pathway. These effector proteins act by stabilising the interaction between NFκB and its inhibitory subunit IκB, by preventing the phosphorylation of the latter by associated kinase IKK (Figure 11) (Nadler *et al.*, 2010; Newton *et al.*, 2010). This step is necessary for the dissociation/degradation of the IκB subunit, enabling the translocation of active NFκB to the nucleus for transcription of pro-inflammatory genes. The capacity of NleE to inhibit NFκB signalling is thought to depend on its S-adenosyl-L-methionine-dependent methyltransferase (SAM) activity (Zhang *et al.*, 2012). Upon TLR stimulation (Figure 11), the adaptor protein TNF receptor associated factor 6 (TRAF6) is recruited to the receptor complex, in addition to TGFβ-activated kinase 1 (TAK1) and TAK binding proteins 2 and 3 (TAB2/3). This interaction leads to the subsequent phosphorylation and activation of TAK1, which in turn recruits and activates the IKK complex (Takeda and Akira, 2005). NleE dependent methylation of TAB2/3 prevents the activation of IKK thereby inhibiting the phosphorylation and degradation of the IκB subunit (Figure 11) (Zhang *et al.*, 2012). In contrast to NleE, NleB inhibits NFκB signalling induced by TNFα, but not IL-1β, in a manner dependent on its N-acetyl-D-glucosamine (GlcNAc) transferase activity. NleB blocks TNF receptor associated factor 2 (TRAF2) but not TRAF6 dependent NFκB activation; mediated through inhibition by GlcNAcylation of the TNF receptor (TNFR) adaptor protein, known as TNFR1 associated death domain (TRADD) (Figure 11) (Li *et al.*, 2013; Pearson *et al.*, 2013).

Inhibition of the NFκB signalling pathway has also been attributed to the activity of effectors NleH1 and NleH2. Although less potent than NleE or NleB, both NleH effectors have been found to inhibit the nuclear translocation of NFκB for gene transcription. Royan *et al*, have demonstrated ectopic expression of NleH1 and NleH2 can supress IκB degradation and NFκB activation following stimulation with TNFα (Royan *et al.*, 2010). However, NleH1 and NleH2 may differentially regulate NFκB gene transcription through its interaction with cofactor, ribosomal protein s3 (RPS3). Necessary for the transcription of some NFκB dependent genes, NleH1 but not NleH2 can inhibit RPS3 nuclear translocation, thus preventing transcription of pro-inflammatory genes (Figure 11) (Gao *et al.*, 2009).

Disruption to NFκB signalling is further potentiated by the effector NleC, reported to inhibit translocation of NFκB to the nucleus through cleavage of proteins p50 and c-Rel, with specificity for the p65 subunit (Figure 11) (Yen *et al.*, 2010; Baruch *et al.*, 2011; Ruchaud-Sparagano *et al.*, 2011; Giogha *et al.*, 2015). This cleavage is dependent on the zinc-metalloprotease activity of NleC. NleD also possess zinc-metalloprotease activity, though in contrast to NleC cleaves the MAPK signalling components p38 and JNK, thereby preventing activation of the pro-inflammatory transcription factor AP1 (Figure 11) (Baruch *et al.*, 2011). Furthermore, the LEE effector Tir can also inhibit NFκB induced inflammation through supressing TRAF2 and TRAF6 mediated signalling, which is independent of Intimin binding (Ruchaud-Sparagano *et al.*, 2011). Interacting directly with TRAF2 and indirectly with TRAF6, via tyrosine phosphatases SHP1 and SHP2, Tir has been shown to inhibit their downstream activation of NFκB and inhibit IL8 expression (Yan *et al.*, 2012; Yan *et al.*, 2013).

The collective actions of these effector proteins effectively suppress the inflammatory response to EPEC infection. Interestingly, the EPEC effector protein NleF has been suggested to contribute to the activation of NFκB and induction IL8 secretion during infection (Pallett *et al.*, 2014). With the exception of Tir, it is interesting to note the prominent role of Nle effectors in manipulating MAPK/NFκB signalling pathways.

1.5.2.4 EPEC manipulates host cell survival

Apoptosis, also known as programmed cell death, is an important early host defensive strategy to infection; used to remove infected cells without emitting systemic alarm signals. Manipulating host cell survival mechanisms is an important feature of EPEC infection, necessary to promote colonisation and maintain an infective niche. EPEC infection is associated with early (altered membrane composition) but not late (cell shrinking, membrane blebbing & nuclear fragmentation) markers of apoptosis, suggesting a capacity to delay the onset of cell death (Santos and Finlay, 2015). This is achieved through the careful balance of pro- and anti-apoptotic effector proteins, delivered into host cells via the T3SS.

LEE encoded effectors, Map and EspF, trigger the induction of cell death by similar mechanisms. Targeted to the mitochondria via their N-terminal sequence, Map and EspF disrupt the mitochondrial membrane potential triggering release of cytochrome c (Figure 11) (Nougayrede and Donnenberg, 2004; Ma *et al.*, 2006; Papatheodorou *et al.*, 2006). This leads to the formation of the apoptosome and activation of initiator caspase-9, which in turn activates the executioner caspases, -3 and -7, that cleaves cellular proteins resulting in cell death (Zou *et al.*, 2003; Nougayrede and Donnenberg, 2004). In addition, EspF is also reported to target the ABC transporter AbcF2 for degradation that may contribute to the activation of caspases-9 and -3 (Nougayrede *et al.*, 2007).

EPEC effector proteins can also delay the onset of cell death to allow the establishment of infection, antagonising the activities of pro-apoptotic proteins. As described earlier, NleD can cleave JNK thus preventing the activation of transcription regulator AP1 and transcription of pro-apoptotic proteins (Figure 11) (Baruch *et al.*, 2011). NleB is also proposed to prevent apoptosis through the inhibition of death receptor signalling, dependent on by its GlcNAc transferase activity. Ligand binding of death receptors, such as TNFα-TNF receptor or FAS ligand-FAS receptor, triggers recruitment of death domain adaptor proteins including TRADD and Fas associated death domain (FADD) respectively (Figure 11). Both receptor pathways induce apoptosis through the activation of protein caspase cascade. NleB GlcNAcylates the death domains of TRADD and FADD, thereby preventing their recruitment and activation of pro-apoptotic signals (Li *et al.*, 2013; Pearson *et al.*, 2013). NleH1 and NleH2 are reported to inhibit apoptosis by reducing the levels of active caspase-3 (Figure 11). Furthermore, NleH1 has been shown to interact with the Bax inhibitor 1 (BI-1) thus inhibiting Bax mediated permeablisation of the mitochondrial membrane, release of cytochrome c and activation of caspase-3 (Figure 11) (Hemrajani *et al.*, 2010). NleF is also reported to play a role in preventing cell death through binding and inhibiting the catalytic activity of initiator caspase-9, thereby decreasing caspase-3 activation (Figure 11) (Blasche *et al.*, 2013).

Interestingly, the LEE effector EspZ is proposed to promote the survival of host cells by multiple mechanisms. EspZ has been shown to interact with the transmembrane protein CD98, promoting β1-integrin/FAK mediated host survival signals in addition to AKT phosphorylation (Figure 11) (Shames *et al.*, 2010). However, recent reports have suggested this is likely an artefact of the cell line (HeLa) used, with the precise influence of EspZ on AKT phosphorylation remaining inconclusive (Roxas *et al.*, 2012). Additionally, EspZ may also counteract the pro-apoptotic activities of effectors Map and EspF through its interaction with the mitochondrial membrane translocase Tim17b. Shames *et al*, have suggested this interaction with Tim17b stabilises the mitochondrial membrane potential, preventing the release of cytochrome c (Shames *et al.*, 2011; Roxas *et al.*, 2012). Moreover, EspZ is also proposed to regulate effector protein translocation into host cells via the T3SS through its interaction with EspD in the membrane pore (Berger *et al.*, 2012). This regulation of effector protein delivery is thought to control the balance of pro- and anti-apoptotic effectors, thus delaying the onset of cell death.

During the initial stages of EPEC infection, pro-survival signalling is also mediated through the cell surface EGFR receptor; stimulating the activation of the PI3K/AKT signalling pathway and expression of anti-apoptotic genes (Roxas *et al.*, 2007). EGFR signalling is subsequently terminated by cleavage of the receptor after prolonged infection in a caspase dependent manner. This T3SS dependent degradation of the EGFR is dependent on the effector protein EspF; however, this activity is prevented during the early stages of infection by the effector EspZ (Roxas *et al.*, 2014). The conflicting activities of these effector proteins manipulating host survival signalling highlights the complexities of the EPEC infection strategy. Whilst these antagonistic and overlapping functions are hard to reconcile, they may represent the hierarchy of effector translocation and timing of subversive activities.

1.5.2.5 EPEC Inhibition of phagocytosis

Phagocytosis is a key innate immune mechanism responsible for the internalisation and degradation of invading pathogens. Phagocytosis is triggered either directly from recognition of bacterial structures known as cis-phagocytosis (TLR and Savenger receptor mediated detection of LPS) or indirectly through detection of attached immune opsonins known as trans-phagocytosis (Fcγ and complement receptor 3 [CR3] mediated detection of IgG and C3bi respectively). EPEC infection maintains a predominantly extracellular lifestyle whilst evading immune detection by preventing their uptake through intestinal microfold cells (M-cells) and professional phagocytes such as macrophages. This inhibition is dependent on the collective action of effector proteins, targeting both the cis and trans phagocytosis signalling pathways. EspH has recently been identified as an inhibitor of both cis and trans mediated phagocytosis, likely mediated through its capacity to bind and inhibit the DH-PH domain of RhoGEFs (Dong *et al.*, 2010). This prevents the activation of RhoGTPase, thereby preventing actin remodelling and phagosome formation. Interestingly, the Nle effector protein EspJ is recognised to inhibit trans-phagocytosis of both IgG and iC3b opsonised bacteria. This inhibition is proposed to occur through the inhibition of Src family kinases (SFK) by EspJ, preventing actin driven opsono-phagocytosis (Marches *et al.*, 2008; Young *et al.*, 2014).

In addition to EspB's critical role in T3SS effector translocation, this effector protein is also implicated in the inhibition EPEC phagocytosis. As discussed earlier, EspB can bind myosin motor domains of myosin family proteins (myosin-1c, -2, -5, -6 & -10), thus preventing their interaction with actin filaments (Iizumi *et al.*, 2007). This disruption to actin dynamics therefore prevents pseudopod extension of the membrane, around the invading bacterium, and loss of myosin dependent closure of the phagocytic cup. Additionally, the effector protein EspF can also inhibit cis-phagocytosis by targeting the PI3K signalling pathway. EspF inhibits the activation but not the recruitment of PI3K to the membrane, which is thought to prevent actin cytoskeletal rearrangements necessary for phagosome formation and internalisation of the bacterium (Celli *et al.*, 2001). This inhibition of PI3K activity was also correlated with the loss of AKT phosphorylation at Ser473. However, studies by Quitard *et al.*, have since uncoupled the two events, indicating the loss of AKT phosphorylation occurs independently of EPEC manipulation of PI3K activity (Quitard *et al.*, 2006). Whilst the mechanism by which EPEC inhibits PI3K activity is unknown, it has been shown this activity of EspF is dependent on the N-terminal region (101 residues) (Quitard *et al.*, 2006).

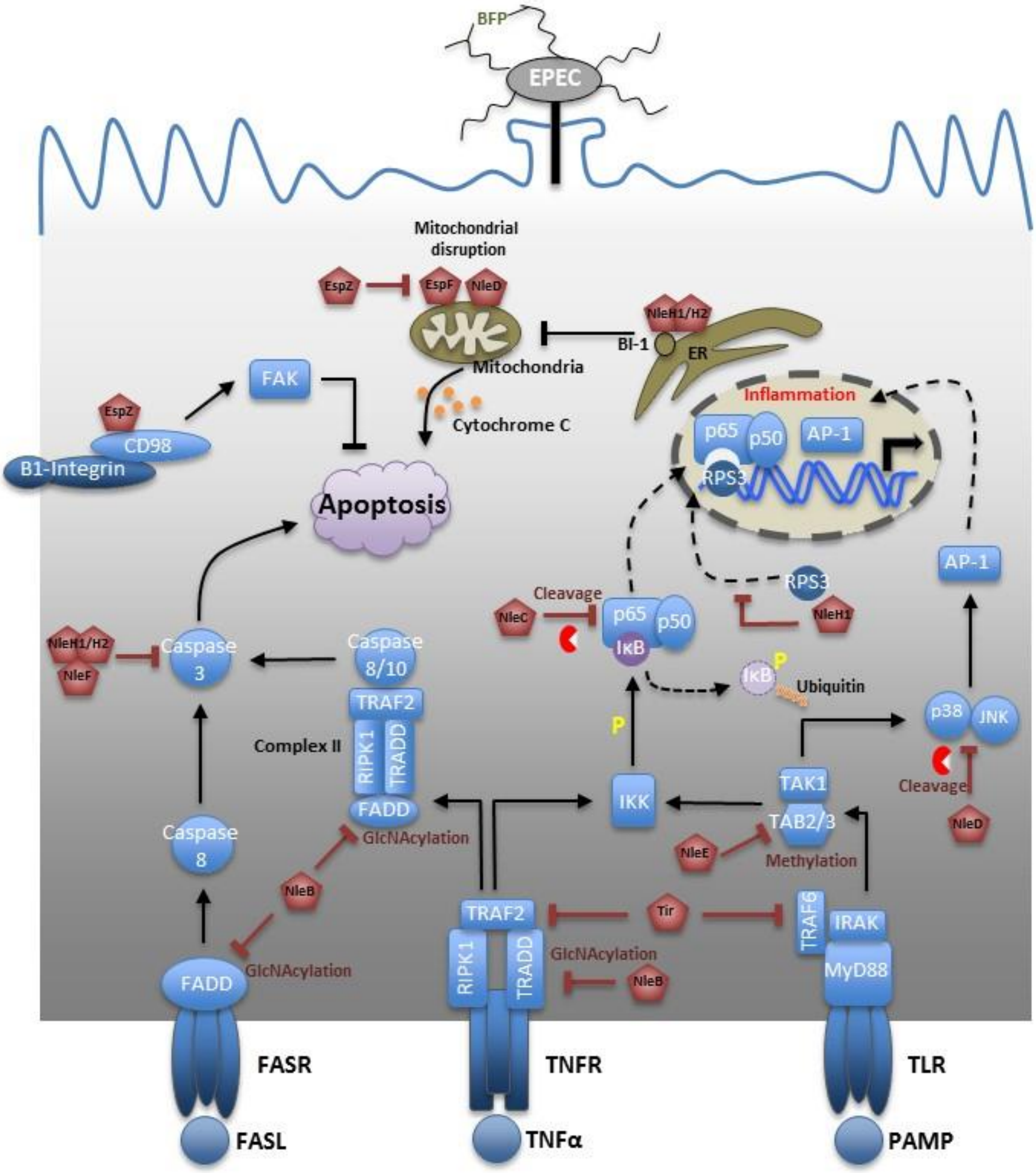


Figure 11 Modulation of cell death and inflammatory signalling pathways by EPEC.

The disruption of mitochondria or activation of TNFα receptor (TNFR), FAS receptor (FASR) and toll like receptors (TLR) signalling triggers the onset of cell death and expression of pro-inflammatory genes. EPEC effector proteins disrupt these inflammatory signalling pathways and delay host cell apoptosis by targeting multiple components of the MAPK (Tir & NleD), NFκB (Tir, NleB, NleC, NleE & NleH1) and caspase (Tir, NleB, NleF & NleH1/2) signalling cascade. EPEC inhibits these signalling pathways through the cleavage of host proteins or inhibiting their activation by effector mediated post-translational modification (methylation or GlcNAcylation) as indicated. EPEC induces host cell death by apoptosis through disruption of mitochondria membrane potential (EspF & NleD). However, EPEC can antagonise this effector induced cell death through inhibition of mitochondria membrane disruption (EspZ & NleH1/2) or by promoting host cell survival signals (EspZ) triggered downstream of β1-integrin and FAK mediated signalling. See text for further explanation. ER is endoplasmic reticulum and BI-1 is Bax inhibitor 1. Adapted from Wong *et al.,* (Wong *et al.*, 2011).

1.5.2.6 EPEC subverts phosphoinositide metabolism

EPEC infection is increasingly recognised to involve the modulation of phosphoinositide metabolism to promote colonisation. As discussed earlier, phosphoinositides act as key signalling molecules (second messengers) by maintaining diverse lipid-protein and protein-protein interactions. EPEC infection has been shown to induce the transient accumulation of PI[4,5]P2 and PI[3,4,5]P3 directly beneath its attachment site in a T3SS dependent manner (Sason *et al.*, 2009). This accumulation of phosphoinositides is associated with the formation of cholesterol enriched lipid rafts, thought to promote effector translocation and pedestal formation. Indeed, PI[4,5]P2 has been associated with the recruitment of proteins key to pedestal formation, including Nck, WASP, Arp2/3, actinin, clathrin and dynamin (Sason *et al.*, 2009). The transient increase of PI[4,5]P2 suggests that EPEC induces an elimination phase, proposed to occur by two possible mechanisms: Tir-Intimin induced phosphorylation and activation of phospholipase C (PLC), converting PI[4,5]P2 to IP3; and/or Tir dependent activation of PI3K, triggering phosphorylation of PI[4,5]P2 to PI[3,4,5]P3 (Sason *et al.*, 2009). Interestingly, Sason *et al* have shown that EPEC can recruit and activate of PI3K in a manner dependent on the phosphorylation of Tir residue Tyr454; likely mediated through binding of SH2 domains of PI3K regulatory subunit p85 (Sason *et al.*, 2009). These results contradict previous reports supporting the ability of EPEC to inhibit PI3K activation in a manner dependent on the effector protein EspF (Celli *et al.*, 2001; Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007). However, activation and recruitment of PI3K was correlated with the transient increase of PI[3,4,5]P3, thought to promote actin polymerisation and protrusion from the membrane (pedestal formation) (Sason *et al.*, 2009). This elongation and polymerisation is then suggested to be terminated by the recruitment of SH2 domain containing inositol 5 phosphatase 2 (SHIP2) to Tir, inducing the dephosphorylation of PI[3,4,5]P3 to PI[3,4]P2 (Campellone, 2010). This recruitment is thought to occur through Tir associated immune receptor tyrosine based inhibition motifs (ITIMs), known to recruit SH2 domain containing phosphatases (Smith *et al.*, 2010).

1.6 Summary and project aims

Pathogenic bacteria such as EPEC employ a number of strategies to promote their colonisation and survival. The ability of EPEC to subvert host cellular functions is dependent on its capacity to translocate up to 24 known effector proteins via a T3SS into target cells. These effector proteins subvert numerous cell signalling pathways, including those regulating the innate immune response and host cell survival. The mechanisms by which EPEC disrupts these signalling cascades is slowly being unravelled, providing valuable insight into the many components and complexity of these signalling pathways controlling the cell response. An interesting strategy identified during infection was the ability of EPEC to manipulate PI3K activity, phosphoinositide metabolism and AKT phosphorylation. Although effector proteins and mechanisms have been proposed through which EPEC targets and subverts PI3K activity, the precise mechanism for terminating AKT phosphorylation remains to be identified. AKT signalling is implicated in many cellular functions, including cell proliferation, apoptosis, transcription and metabolism. Understanding how EPEC disrupts AKT activity would provide greater understanding of the EPEC infection strategy and provide critical insight into the control of AKT signalling. This could prove useful in the development of agonists and/or antagonists where AKT signalling is overactive such as cancer.

Due to the critical role of both LEE and Nle effector protiens to subvert host cell signalling pathways, it is hypothesised that one or more of these effectors may be required to disrupt AKT phosphorylation, and thus its activity. Therefore, the project aims are to examine the role of both LEE and Nle effector proteins, and to investigate the mechanism by which EPEC triggers the rapid decline of phosphorylated AKT.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1 Bacterial strains and culture

Bacterial strains used in this study are listed in Table 1. Bacteria cultures were streaked from -80˚C frozen culture stocks onto Luria-Bertani (LB) agar plates supplemented with antibiotic(s) (Nalidixic acid [50µg/ml], Carbenicillin [100µg/ml], Kanamycin [25µg/ml], Chloramphenicol [25µg/ml] and Tetracycline [12µg/ml] at final concentration) as appropriate for selection. For infection studies, single colonies were picked from LB agar plates and cultured in LB broth supplemented with selective antibiotic(s) and incubated overnight (16-18 h) at 37°C without shaking. For long-term storage, overnight LB broth cultured bacteria with antibiotic selection were diluted with 50% glycerol to a final concentration of 10% before storing at -80°C.

On the day of experimentation, overnight bacterial cultures (37°C standing) were pre-activated by dilution (1/10) in serum free Dulbecco's minimal Eagles medium (DMEM; Sigma) supplemented, when needed, with antibiotic(s) for selection and incubated at 37°C with 5% CO_2 for 3 h, unless otherwise indicated. The pre-activation of EPEC strains induces the synthesis of virulence proteins (including those of the T3SS), promoting rapid host cell binding and formation of characteristic disease phenotypes including A/E lesion formation, as described elsewhere (Rosenshine *et al.*, 1996a).

2.1.2 Mammalian cell culture

Cell culture was performed under aseptic conditions in a Class 2 laminar flow hood (BioMat 2). Cells were passaged in tissue culture flasks with a surface area of 25 cm^2, 75 cm^2 or 175 cm^2 (Corning) and maintained under sterile conditions in a humidified incubator at 37°C and 5% CO_2. J774.A1 macrophages (ATCC TIB-67) and HeLa cells (ATCC CCL-2) were cultured in DMEM, supplemented with 10% fetal calf serum (FCS; GIBCO). Cells were routinely cultured to ~80% confluence before passaging into a new tissue culture flask, at a 1:6 dilution. J774.A1 macrophages were isolated from tissue culture flasks using a rubber cell scraper following the replacement of cell culture media with fresh DMEM (5-15 ml). In contrast, HeLa cells were recovered from tissue culture flasks by trypsinisation (1 x Trypsin-EDTA [ethylenediamineacetic acid]; Sigma) for 10-15 min at 37°C and 5% CO_2, following the removal of media by washing cells with sterile 1x phosphate buffered saline (PBS; 137mM sodium chloride [NaCl], 2.7 mM potassium chloride [KCl], 10 mM disodium phosphate and 1.8 mM monopotassium phosphate; pH 7.4). The resulting cell suspension of both cell lines were collected and the cell density determined by a 1:1 dilution in Trypan Blue (Sigma), with non-permeable and viable cells calculated by haemocytometer under a light microscope.

Cell passage number was recorded, with cells passaged up to 30 times before cells were discarded. J774A.1 macrophage and HeLa cell stocks were prepared for long term storage in 10% DMSO and 90% FCS at a concentration of $1x10^6$/ml, before storing in liquid nitrogen.

2.2 Molecular biology

2.2.1 Plasmid extraction

Bacteria carrying the plasmid for isolation were cultured overnight in 5 ml LB broth with appropriate antibiotic(s) for selection at 37°C shaking (~250 rpm). Bacteria were then harvested by centrifugation at 12,000 xg for 5 min at room temperature (RT) before isolating plasmid using the AccuPrep plasmid miniprep kit (Bioneer) according to manufacturer's instructions. Concentration and purity of isolated plasmids was determined by using NanoDrop 100 spectrophotometer (Thermo Scientific). Concentration of the sample was measured using a reading at A_{260}. Purity of the isolated plasmid was estimated by a ratio of A_{260}:A_{230}, with a value of ≥1.6 considered satisfactory.

2.2.2 Preparation of electrocompetent bacteria

Bacterial strains were cultured overnight at 37°C standing before diluting (1:100) in 100 ml LB broth with appropriate antibiotic(s) for selection at 37°C shaking (~250 rpm) for 2-4h, until an optical density (OD_{600}) of 0.4-0.6. The resulting bacterial culture was centrifuged at 12,000 xg for 15 min at 4°C before discarding the supernatant and re-suspending the bacterial pellet in ice cold 50 ml distilled H_2O (d H_2O). This bacterial cell suspension was again centrifuged at 12,000 xg 4°C for a further 15 min before repeating this wash step once more. Once complete, the resulting supernatant was discarded and remaining pellet re-suspended in 20 ml sterile 10% glycerol and centrifuged at 12,000 xg and 4°C. Again, the supernatant was discarded and pellet re-suspended in 100 µl of 10% glycerol before aliquots of 40 µl were 'snap frozen' in liquid nitrogen and stored at -80°C. All solutions were chilled to 4°C and kept on ice between each step.

2.2.3 Electroporation of bacteria

Electrocompetent bacteria (40µl aliquots) were thawed on ice and incubated with 25-50 ng/µl of plasmid DNA for 5-10 min, on ice. Bacteria-DNA suspension was then transferred to a 2 mm gap electroporation cuvette and electroporated at 2.5 kV, 200 Ω, 25 mF for 4.5 µs (GenePulser II; BioRad). Immediately after electroporation, transformed bacteria were re-suspended in 1 ml super optimal broth with catabolite repression (SOC; 0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM MgSO4 & 20 mM Glucose) media pre-warmed to 37°C and incubated for a minimum of 1 h at 37°C. Post incubation, 50-1000 µl of transformed bacterial suspension was plated onto LB agar plates with appropriate antibiotic(s) for the selection of plasmid(s) and incubated at 37°C overnight (16-18 h). Single colonies were picked and screened for expression of appropriate genes either by PCR and/or western blot analysis. Plasmids used in this study are listed in Table 2.

2.2.4 PCR amplification of EPEC genes

Oligonucleotide primers were designed using Clone Manager Professional Suite 8.0 (Scientific and Educational Software) and are presented in Table 3. Colony PCR was performed using standard 25 µl PCR reactions prepared as follows: Template DNA (<1000 ng), 1x Taq reaction buffer (New England Biolabs), dNTPs (200 µM; New England Biolabs), Taq DNA Polymerase (0.625 units; New England Biolabs) forward and reverse primers (0.2 µM). Template bacterial DNA was obtained by boiling a single bacterial colony in 10 µl dH_2O for 5 min at 100°C. Thermocycling was performed on a Veriti 96 well thermocycler (Applied Biosystems) using the cycling conditions listed in Table 4.

2.2.5 Agarose gel electrophoresis

PCR reaction products were analysed by agarose gel electrophoresis, prepared with 0.7-1% w/v agarose in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer, supplemented with fluorescent nucleic acid stain GelRed^{TM} (1:30,000; Biotium). DNA was mixed with 10x ficoll loading dye and loaded onto the gel alongside a 1 Kb DNA ladder (New England Biolabs). Samples were separated at 100 volts and products visualised using a UV transilluminator (Biorad) for image capture.

2.3 Infection of mammalian cells

2.3.1 Infection of J774.A1 macrophages

2.3.1.1 Standard AKT phosphorylation assay with J774.A1 macrophages for protein extraction

J774.A1 macrophages were seeded onto 6 well tissue culture plates at a density of $5x10^5$ cells per well 2 days prior to infection. Approximately 18 h before infection, cells were serum starved by replacing media with DMEM supplemented with 0.5% FCS, before starving cells in serum free DMEM for 1 h before infection. Where appropriate, cells were pre-treated with drugs at various concentrations (Table 5) in serum free DMEM for 1 h prior to infection and maintained in culture throughout the infection protocol. The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacterial strains (Table 1) at a multiplicity of infection (MOI) of 200:1 in a total volume of 2 ml and placed in an incubator with 5% CO_2 at 37°C. Infections were stopped at various time points over a 2 h infection period by removing media and washing cells three times with chilled 1x PBS (4°C), to remove unbound bacteria, ready for isolation of protein extracts.

2.3.1.2 Two-wave infection AKT phosphorylation assay with J774.A1 macrophages for protein extraction

J774.A1 macrophages were seeded onto a 60 mm tissue culture dish at a density of $1.5x10^6$ cells per well 2 days prior to infection. Approximately 18 h before infection, cells were serum starved in DMEM supplemented with 0.5% FCS, before starving cells in serum free DMEM for

1 h before infection. The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacterial strains (Table 1) at an MOI of 200:1 in a total volume of 3 ml and left to incubate in 5% CO_2 at 37°C. Infections were stopped after 1 h by removing DMEM, washing cells with 1x PBS and incubating in 5% CO_2 at 37°C with serum free DMEM supplemented with gentamicin (100 µg/ml; bactericidal levels) to kill extracellular bacteria, with or without inhibitors (when needed). After incubation, cells were again washed with 1x PBS and allowed to recover in fresh serum free DMEM for 1 h before infecting cells with the pre-activated EPEC strain, *cfm-14*, at an MOI of 100:1 in 5% CO_2 at 37°C (Figure 12). Infections were stopped at various time points over a 1 h infection (15, 30 & 60 min) by removing media and washing cells three times with chilled 1x PBS (4°C) on ice, to remove unbound bacteria, ready for isolation of protein extracts.

2.3.1.3 Phagocytosis Assay

2.3.1.3.1 Single infection phagocytosis assay

J774.A1 macrophages were seeded onto glass coverslips in a 24 well plate at a density of $1.1x10^5$ cells per well 2 days prior to infection. Approximately 1 h before infection, cells were incubated in fresh serum free DMEM. The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacterial strains (Table 1) at an MOI of 100:1 in a total volume of 1 ml and left to incubate in 5% CO_2 at 37°C for 1 h. Infections were stopped by washing cells three times in 1x PBS and media replaced with fresh serum free DMEM supplemented with chloramphenicol (25 µg/ml; bacteriostatic levels) for 1 h to prevent further synthesis of EPEC effector proteins for T3SS dependent delivery into host cells. Cells were then washed three times in 1x PBS and fixed in 2.5% paraformaldehyde (PFA) for 20 min at RT and stored at 4°C prior to immunofluorescence staining.

2.3.1.3.2 Two-wave infection phagocytosis assay

J774.A1 macrophages were seeded onto glass coverslips in a 24 well plate at a density of $1.1x10^5$ cells per well 2 days prior to infection. Approximately 1 h before infection, cells were incubated in fresh serum free DMEM. The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacteria strains (Table 1) at an MOI of 100:1

in a total volume of 1 ml and left to incubate in 5% CO_2 at 37°C for 1 h. Infections were stopped by replacing media with fresh serum free DMEM supplemented with gentamicin (100 µg/ml; bactericidal levels) for 1h to kill extracellular bacteria. Cells were then washed with 1x PBS and left to recover in fresh DMEM supplemented with 10% FCS for 50 min, before replacing with serum free DMEM for a final 10 min incubation. After incubation, cells were infected with the pre-activated T3SS defective mutant carrying a plasmid encoding the green fluorescent protein (*cfm-14/pGFP*) in serum free DMEM at an MOI of 100:1 for 1 h, in a total volume of 1 ml, incubated in 5% CO_2 at 37°C. Infections were stopped by washing cells three times in 1x PBS and media replaced with fresh serum free DMEM supplemented with chloramphenicol (25 µg/ml; bacteriostatic levels) for 1h to prevent further EPEC protein synthesis for T3SS dependent delivery of effector proteins into host cells. Cells were then washed three times in 1x PBS and fixed in 2.5% paraformaldehyde (PFA) for 20 min at RT and stored at 4°C prior to immunofluorescence staining (see 2.4).

2.3.2 Cell viability

2.3.2.1 Trypan blue assay

J774A.1 macrophages were seeded onto 6 well plates and prepared for infection as described earlier (see 2.3.1.1). The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacterial strains (Table 1) at an MOI of 200:1 for 2 h in 5% CO_2 at 37°C. Infections were stopped by washing cells three times in 1x PBS before treating cells with a 1:1 mixture of trypan blue solution (Sigma) in 1x PBS and incubating in 5% CO_2 at 37°C for 5 min. Post incubation, cells were washed three times in 1x PBS before viewing by light microscope. Evaluation of cell viability and membrane integrity was assessed by visualising the permeability of cells to trypan blue dye.

2.3.2.2 Lactate dehydrogenase (LDH) cytotoxicity assay

The release of lactate dehydrogenase (LDH) from host cells was determined using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific #88953); measuring the activity of LDH by formation of red Formazan product. Briefly, J774A.1 macrophages were seeded onto 24 well plates and prepared for infection as described earlier (See section 2.3.1.3.1). The OD_{600}

of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells with bacterial strains (Table 1) at an MOI of 200:1. Cells were left to incubate in 5% CO_2 at 37°C before infections were stopped after 15, 30, 60, 90 and 120 min and supernatants collected. As per manufacturer's instructions, uninfected cells were treated with a final concentration of 1x lysis buffer for 45 min prior to collecting supernatant for maximal LDH release. Sample supernatant was centrifuged at 250 xg for 3 min prior to transferring 50 µl to a flat bottomed 96 well plate. Each sample was then mixed with 50 µl of reaction mixture (see manufacturer's instructions) and left to incubate for 30 min protected from light. The reaction was stopped by the addition of 50 µl of stop solution before measuring the absorbance of the red reaction product Formazan at 490nm and 680nm using a FLUOstar opitma microplate reader (BMG Labtech). Percentage cell cytotoxicity induced by EPEC was calculated as follows: spontaneous release of LDH in uninfected cells is subtracted from that released in EPEC treated samples; this is then divided by maximum LDH activity (minus spontaneous LDH release) before multiplying by 100. Importantly, bacterial cell culture media did not alter the absorbance readings compared to DMEM alone.

2.3.3 Infection of HeLa cells

2.3.3.1 AKT phosphorylation assay with HeLa cells for protein extraction

HeLa cells were seeded onto 24 well plates at a density of $2.5x10^5$ cells per well 2 days before infection. Approximately 1 h prior to infection and/or treatment, cells were incubated in fresh serum free DMEM. Cells were either treated with epidermal growth factor (EGF; 100 ng/ml final concnetration), Tumour necrosis factor α (TNFα; 10, 15 or 50 ng/ml final concentration) or infected with pre-activated EPEC strains in a total volume of 1 ml (Table 1). The OD_{600} of pre-activated bacterial cultures (see 2.1.1) were measured before infecting cells at a multiplicity of infection (MOI) of 100:1. Cells were left to incubate in 5% CO_2 at 37°C for the indicated times before cells were washed three times in 1x PBS (4°C) on ice, ready for isolation of protein extracts.

Alternatively, cells were left uninfected or infected with pre-activated EPEC strains at an MOI of 100:1 and left to incubate in 5% CO_2 at 37°C for indicated times (1-5 h). Post incubation, cells were treated with agonists EGF (100 ng/ml) for 1 and/or 2 min before cells were washed three times in 1x PBS ready for isolation of protein extracts.

2.4 Immunofluorescence staining

Following fixation of cells (2.5% paraformaldehyde) for 30 min, cells were washed three times in 1x PBS and probed for extracellular bacteria using appropriate primary antibodies against EPEC or *E. coli* (Table 6) for 1 h. Post incubation, cells were washed three times in 1x PBS and incubated with Alexa555 (Red) conjugated α-rabbit secondary antibody for 1 h. Again, cells were washed three times in 1x PBS and permeabilised in 1% Triton X-100 for 30 min at RT, before incubating in appropriate primary antibody against EPEC or *E. coli* (Table 6). Cells were then washed three times in 1x PBS before probing for total cell associated bacteria using Alexa488 (Green) α-rabbit secondary antibody. Host cellular and bacterial DNA was visualised by treating with the nuclear stain 4’6-diamidino-2-phenylinodole (DAPI; Blue). For two-wave infection studies, the total number of second-wave infected T3SS mutant bacteria (*cfm-14*) was determined by their expression of a plasmid encoded green fluorescent protein (*pGFP*; Green). Extracellular bacteria were labelled with anti-EPEC antibodies followed by Alexa 555 conjugated (Red) anti-rabbit antibodies. Coverslips were mounted onto glass slides using FluorSave reagent and imaged using Zeiss Axioskop epifluorescent microscope. The percentage of internalised bacteria was calculated by subtracting the number extracellular bacteria (labelled Red) from the total number of cell associated bacteria (labelled Green), divided by the total and then multiplied by 100. A total of 50 randomly selected cells per coverslip, each with a minimum of 20-30 cell-associated bacteria, were quantified in a semi-blind manner (identity of sample revealed post quantification).

2.5 Protein analysis

2.5.1 Isolation of cellular protein fractions

Host cell proteins were isolated after appropriate infection protocols as described below. Using a cell scraper, total cellular protein extract was obtained by lysis of cells in 1X Laemmli sample buffer (60 mM Tris-HCl pH 6.8, 1% w/v SDS, 5% v/v glycerol, 5% v/v β-mercaptoethanol & 0.01 % w/v bromophenol blue) for a concentration of ~10,000 cells/µl.

The isolation of host cellular protein fractions (cytoplasmic, membrane and insoluble proteins) was as previously described (Kenny *et al.*, 1997; Kenny, 1999; Warawa and Kenny, 2001). Briefly, cells were scraped into saponin lysis buffer (0.2% saponin, 1 mM PMSF, 1 mM NaF & 1 mM NaVO4 in ice cold 1x PBS) and centrifuged at 12,000 xg for 3 min at 4°C. The resulting cell supernatant, labelled host cytoplasmic proteins, was collected and stored on ice. The remaining cell pellet was washed in ice cold 1x PBS before re-suspending in Triton lysis buffer (1% Triton, 1 mM phenylmethylsulphonyl fluoride [PMSF], 1 mM sodium fluoride [NaF] & 1 mM sodium orthvanadate [NaVO4] in ice cold 1x PBS) and centrifuged at 12,000 xg for 3 min at 4°C. Again the resulting supernatant, labelled host membrane proteins, was isolated and stored on ice. The remaining cell pellet containing cell adherent bacteria, host nuclei and cytoskeletal proteins (insoluble proteins) was washed in 1x PBS 4°C before re-suspending in 1X Laemmli sample buffer. Host cytoplasmic and membrane protein fractions were diluted in 5x Laemmli sample buffer for a final 1x concentration. Protein samples (total and fractionated proteins) were then boiled for 10 min at 100°C, before samples were vortexed and centrifuged at 13,000 xg at RT. All protein samples were stored at -20°C before subjecting to SDS-PAGE and western blot analysis.

2.5.2 SDS-PAGE

Isolated protein samples were denatured at 100°C for 10 min in Laemmli sample buffer and separated by polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples of equal loading (derived from ~50,000 mammalian cells/well) were separated using a 10% polyacrylamide resolving gel (0.375 M Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate & 0.1% v/v TEMED) and a 5% polyacrylamide stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate & 0.1% v/v TEMED); all gels were prepared in dH_2O. Polyacrylamide gels were loaded in to a vertical electrophoresis cell (Bio-Rad) system, with electrophoresis performed using 1X SDS running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1 % w/v SDS; pH 8.3) for 1 h at 200 v. Once complete, gels were prepared for transfer to polyvinylidene fluoride (PVDF) membrane (GE Healthcare) for western blotting by tank (Wet) electrotransfer.

2.5.3 Western blot and quantification

Transfer of proteins (separated by SDS-PAGE) onto PVDF membrane was performed in 1X transfer buffer (25 mM Tris & 192 mM glycine; pH 8.3) using the Mini Trans-Blot cell (Bio-Rad) system for 60 min at 110 volts. Transfer success and gel loading levels was evaluated using Ponceau S, a staining solution designed for detecting proteins on nitrocellulose membranes (Sigma). Following transfer of proteins, PVDF membranes were removed of Ponceau S stain (1x PBS wash) and blocked in 5% (w/v) skimmed milk powder prepared in Tris buffered saline with Tween-20 (TBST; 150 mM NaCl, 10 mM Tris-HCl & 0.05% v/v Tween-20; pH 7.5) for 1h at RT. PVDF membranes were removed from blocking buffer and washed 3x 5 min washes in TBST, before incubating with primary antibody prepared in TBST with 5% (w/v) bovine serum albumin (BSA) overnight at 4°C. Primary antibodies and relevant dilutions used in this study are detailed in Table 6. Post incubation, membranes were removed and washed 3x 5min in TBST before incubating with appropriate HRP conjugated secondary antibody (Table 6) in TBST with 5% (w/v) skimmed milk powder for 1 h at RT, followed by a final wash step with 3x 5min in TBST. PVDF membranes were then exposed to ECL chemiluminesence substrate (Thermo Fisher Scientific) as per manufacturer's instructions and imaged using a ChemiDoc imaging system (Bio-Rad). Quantification of protein signal, identified by western blot, was performed by densitometry analysis. Protein(s) identified by chemiluminescent signal of bound HRP-conjugated antibodies was quantified using Image Lab software (BioRad; V5.2.1), measuring the signal intensity of each protein band, adjusted for background signal and labelled as arbitrary units. The signal intensity of query/target protein was then normalised against a protein loading control (actin) before calculating as a fold change against uninfected control cells (unless otherwise stated).

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism V6.0 (GraphPad software) to analyse data sets using a one-way or two-way analysis of variance (ANOVA) with additional Dunnett's (*) or Sidaks (#) post-tests, as indicated respectively. P-values of $p<0.05$ were considered significant.

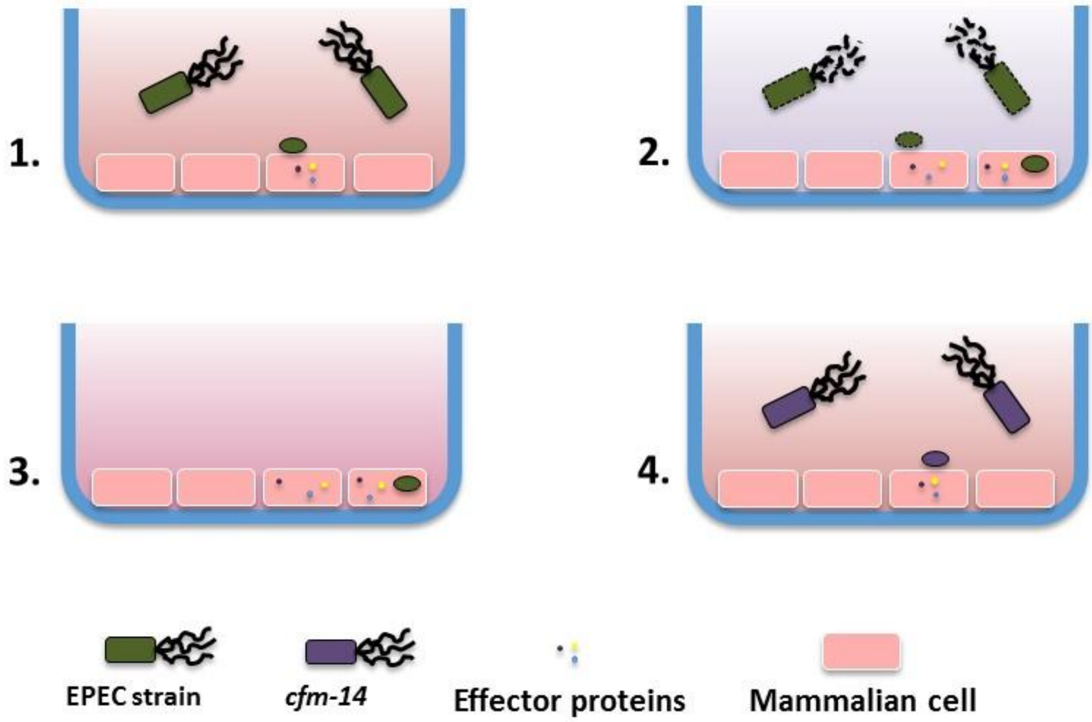


Figure 12 EPEC two-wave infection model.

An illustration for the two-wave infection of the J774A.1 macrophage cell model to examine the impact of cells pre-infected with EPEC to trigger AKT phosphorylation or the phagocytosis of the T3SS mutant (*cfm-14*) following a second infection. (**1**) J774A.1 macrophages are infected with selected EPEC strains. (**2**) Infections are stopped and extracellular bacteria killed by gentamicin treatment. (**3**) Cells are allowed to recover in cell culture media. (**4**) Cells pre-infected with EPEC strains are challenged with the T3SS defective mutant (*cfm-14 ± pGFP*) before analysing for AKT phosphorylation or percentage of bacteria uptake.

Bacterial Strain	Description	Antibiotic Selection	Reference
Wild-type (WT) EPEC E2348/69 (0127:H6)	Prototypical EPEC strain.	Nal^R	(Levine *et al.*, 1985)
Δcfm-14	Transposon-disrupted *escN* gene. Lacks functional Type 3 secretion system.	Nal^S/Km^R	(Donnenberg and Kaper, 1991)
***ΔsepB* (*CVD452*)** **E2348/69**	Lacks functional *sepB* gene. Type-3-secretion system defective strain.	Nal^R	(Jarvis *et al.*, 1995)
Class5 30-5-1(3) **E2348/69**	Transposon-disruption which inactivates the Type 3 secretion system.	Km^R	(Donnenberg *et al.*, 1990)
***ΔespA* (*UMD872*)**	Lacks EspA translocator protein (can't deliver effector proteins).	Nal^R	(Kenny *et al.*, 1996)
***ΔespB* (*UMD864*)**	Lacks EspB translocator protein (can't deliver effector proteins).	Nal^R	(Donnenberg *et al.*, 1993)
***ΔespD* (*UMD870*)**	Lacks EspD translocator protein (can't deliver effector proteins).	Nal^R	(Lai *et al.*, 1997)
ΔcesF	Lacks EspF chaperone, CesF (*cesF* gene disrupted by introduced kanamycin cassette).	Nal^R/Km^R	Kenny Lab, provided by B. Kenny (unpublished)
ΔcesT::km	Lacks chaperone, CesT (*cesT* gene disrupted by introduced kanamycin cassette) that promotes the delivery of many effectors.	Nal^R/Km^R	Kenny Lab, provided by B. Kenny (unpublished)
Δquad **E2348/69**	Lacks Map, EspF, Tir effectors and Intimin (*eae* gene) surface protein	Nal^R	(Quitard *et al.*, 2006)
Δmfzgorf3eh::km	Lacks EspG, Orf3/EspG2, Map, EspF, EspZ, EspH effectors plus Intimin (*eae* gene) surface protein. *espH* gene disrupted by introduced gene encoding Kanamycin resistance.	Nal^R/Km^R	Kenny Lab, provided by Azzeldin Madkour (unpublished)
Δcore::km	Lacking LEE region (replaced with kanamycin cassette) encoding 3 effectors (EspH, Map, Tir) 2 chaperones (CesT, CesF) and Intimin (*eae* gene) surface protein.	Nal^R/Km^R	(Dean *et al.*, 2010)
gorf3Δcore::km	*Δcore::km* mutant also lacking another two effectors (EspG and	Nal^R/Km^R	(Ruchaud-Sparagano *et al.*,

Bacterial Strain	**Description**	**Antibiotic Selection**	**Reference**
	its homologue Orf3/EspG2).		2007)
ΔespZ	Lacks the EspZ effector.	Nal^R	Kenny Lab, provided by B. Kenny (unpublished)
WT EPEC Japan E2348/69 (0127:H6)	Prototypical EPEC strain provided by Tobe *et al*.	Nal^S	(Yen *et al.*, 2010)
TOE A1	Lack PP2 effector gene cluster.	Nal^S	(Yen *et al.*, 2010)
TOE A2	Lack PP2 & IE2 effector gene clusters.	Nal^S	(Yen *et al.*, 2010)
TOE A3	Lack PP2, IE2 & PP4 effector gene clusters.	Nal^S	(Yen *et al.*, 2010)
TOE A4	Lack PP2, IE2, PP4 & IE6 gene clusters.	Nal^S	(Yen *et al.*, 2010)
TOE A5	Lack PP2, IE2, PP4, IE6 & PP6 gene clusters.	Nal^S	(Yen *et al.*, 2010)
TOE A6	Lack PP2, IE2, PP4, IE6, PP6 & IE5 gene clusters.	Nal^S	(Yen *et al.*, 2010)
TOE A7	Lack PP2, IE2, PP4, IE6, PP6, IE5 gene clusters and LEE *espG* gene.	Nal^S	(Yen *et al.*, 2010)
TOE A7_*Δcore::km*	Lacking genes *espH*, *cesF*, *map*, *tir*, *cesT* & *eae* [Intimin] from a TOEA7 strain.	Nal^S/Km^R	Kenny Lab, provided by B. Kenny (unpublished)
TOB01 (*E. coli K12*)	*E. coli* K12 strain carrying the pTOK-01 plasmid, encoding the *bfp* operon and *perABC* regulatory genes. All EPEC genes encoded on the plasmid originate from the EPEC B171-8 strain.	Km^R/Cmp^R	(Yen *et al.*, 2010)
TOBO2 (*E. coli K12*)	*E. coli* K12 carrying plasmids pTOK-01, encoding the *bfp* operon and *perABC* regulatory gene; and pTOK-02 encoding the LEE region. All EPEC genes encoded on plasmids originate from the EPEC B171-8 strain.	Km^R/Cmp^R	(Yen *et al.*, 2010)
DH10B (*E. coli K12*)	Innocuous *E. coli* K12 strain.	NA	Thermo Fisher Scientific

Table 1 Bacterial strains used in this study.

Bacteria strains and their selective antibiotics: Nalidixic acid (Nal^R), Kanamycin (Km^R), Carbenicillin ($Carb^R$) and Chloramphenicol (Cmp^R).

Plasmid	Description	Antibiotic Selection	Reference
pLG-HcFM (*pHcFM*)	Carrying LEE genes *espH, cesF* & *map*.	$Carb^R$	Kenny Lab, provided by B. Kenny (unpublished)
pACYC-TcT (*pTcT*)	Carrying LEE genes *tir* & *cesT*.	Cmp^R	Kenny Lab, provided by B. Kenny (unpublished)
pGFP	Carrying *gfp* gene.	$Carb^R$	Kenny Lab, provided by B. Kenny (unpublished)

Table 2 Plasmids used in this study.

Plasmids used in this study (unpublished) were provided by B. Kenny. Antibiotic selection is indicated; Carbenicillin ($Carb^R$) and Chloramphenicol (Cmp^R).

Name	Oligonucleotide sequence 5’-3’
***espH* Forward Primer**	CCCTTTGGCAACCGTAAAGC
***espH* Reverse Primer**	AAATATCGTCCCCAGAACAG
***espF* Forward Primer**	ATGGAATTAGTAACGCTGCTTCTACAC
***espF* Reverse Primer**	TTGGTTACCCTTTCTTCGATTGCTCATAG
***espG* Forward Primer**	ACAAAAACTATGGCTGACGCATCAC
***espG* Reverse Primer**	TTCAGCGCATGACATCTCATCCCAG
***espG2/orf3* Forward Primer**	TAGGTATAACCCTATGCCTGTGTTC
***espG2/orf3* Reverse Primer**	AACAAATTCAGGCTGACACAGTACC
***nleE1* Forward Primer**	CCAGTATGTATACCAGCAGTTCATGGTAAG
***nleE1* Reverse Primer**	ATTGGGCGTTTTCCGGATATAACTG
***nleD* Forward Primer**	GCTTTATTATCGGGTTTGGTGACCGCCTTG
***nleD* Reverse Primer**	AATAAGAGCTGAGTCGTGCGGGTAG
***nleE2* Forward Primer**	TTATTTCCACAGGCATGTAG
***nleE2* Reverse Primer**	TCCTGTTACTAATACTCAGGG
***espJ* Forward Primer**	AAATAACCACCACTCCCACACCAGCGAAAC
***espJ* Reverse Primer**	ATACCAAATGCGTTTTTTTGTGGGTTATAC
***nleF* Forward Primer**	AAGGGGGTTTTGATATGTTACCAACAAGTG
***nleF* Reverse Primer**	CCACGAGGCATTTCATTGCTCGTAG

Table 3 Oligonucleotides used in this study.

List of oligonucleotides designed in this study for confirmation of functional genes by PCR analysis.

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

Table 4 PCR Thermocycling conditions.

Routine thermocycling conditions used for colony PCR analysis of genes.

Inhibitor	Description	Working concentration	Source
Calyculin A	Selective inhibitor of serine/threonine protein phosphatases, PP1 and PP2A.	5-100 nM	Calbiochem; #208851
Okadaic Acid	Selective inhibitor of serine/threonine protein phosphatases, PP2A.	100-400 nM	Sigma; # O-7885

Table 5 Serine/Threonine phosphatase inhibitors used in this study.

List of inhibitors and used in this study with respective working concentrations.

Antibody Target	Origin	Dilution (IF)	Dilution (WB)	Company & Catalogue #
Primary Antibodies				
EPEC 0127	Rabbit	1/100	NA	Statens Serum Institute '*E. coli* OK127 monospecific OK rabbit antiserum; #44305
E. coli (polyclonal)	Rabbit	1/100	NA	AMS Biotech; #

Antibody Target	Origin	Dilution (IF)	Dilution (WB)	Company & Catalogue #
				B65001R
Tir	Rabbit	NA	1/5000	Kenny Lab
EspF	Rabbit	NA	1/2000	Kenny Lab
β-Actin	Rabbit	NA	1/2000	Sigma; A2066
p-AKT$_{ser473}$	Rabbit	NA	1/500	Cell Signalling; #4060
p-AKT$_{Thr308}$	Mouse	NA	1/500	Cell Signalling; #13038
AKT (Pan isoforms 1, 2 & 3)	Rabbit	NA	1/1000	Cell Signalling; #4691
AKT1	Rabbit	NA	1/1000	Cell Signalling; #2938
AKT2	Rabbit	NA	1/1000	Cell Signalling; #3063
AKT3	Rabbit	NA	1/1000	Cell Signalling; #8018
p-PDK1$_{Ser241}$	Rabbit	NA	1/1000	Cell Signalling; #3438
p-PTEN$_{Ser380}$	Rabbit	NA	1/1000	Cell Signalling; #9551
p-C-Raf$_{Ser259}$	Rabbit	NA	1/1000	Cell Signalling; #9421
p-GSK3β$_{Ser9}$	Rabbit	NA	1/1000	Cell Signalling #5558
EGFR	Rabbit	NA	1/200	Santa Cruz Biotechnology; #sc-03
LDH	Mouse	NA	1/200	Abcam; #ab54288
Secondary Antibodies				
Anti Rabbit IgG-HRP	Goat	NA	1/5000	Jackson Immuno Research; #111-035-003
Anti Mouse IgG-HRP	Goat	NA	1/5000	Jackson Immuno Research; #111-035-003
Anti Rabbit Alexa Fluor 555	Goat	1/200	NA	Molecular Probes; #A11008
Anti Rabbit Alexa Fluor 488	Goat	1/200	NA	Molecular Probes; #A21428
Nuclear Stain				
DAPI	NA	1/1000	NA	Invitrogen; #D1306

Table 6 Antibodies for western blot or immunofluorescence analyses.

List of antibodies selected in this study and their respective dilutions.

Chapter 3 Screening for the EPEC effectors required to trigger the rapid decline of phosphorylated AKT

3.1 Introduction

3.1.1 The EPEC effector repertoire

The importance of EPEC effector proteins to promote colonisation and subvert host cell function has become increasingly recognised over the past 2 decades (Dean *et al.*, 2005). The first effector proteins identified were those encoded on the pathogenicity island known as LEE, which is highly conserved amongst A/E pathogens. In addition, effector proteins have been identified that are encoded outside the LEE region, known as non-LEE encoded (Nle) effectors, though some have retained the nomenclature ‘Esp’ (Elliott *et al.*, 2001; Gruenheid *et al.*, 2004; Dean and Kenny, 2009). The complete genome sequence of the prototypic EPEC strain (E2348/69) in 2009 led to the recognition of 21 effector proteins (7 LEE & 14 Nle) (Iguchi *et al.*, 2009). Since then the EPEC effector repertoire has expanded further with the identification of additional effectors EspC (Vidal and Navarro-Garcia, 2008), NleJ and LifA (Deng *et al.*, 2012). Furthermore, studies within the Kenny lab have identified 6 additional experimentally supported effectors (Dean *et al*., unpublished), suggesting the full EPEC effector repertoire is yet to be identified.

Although the functions of many LEE and Nle effector proteins have been reported, full characterisation of effector activity and their role in EPEC pathogenesis remains to be defined (Dean and Kenny, 2009; Santos and Finlay, 2015). EPEC effector proteins function in a carefully orchestrated manner, exhibiting many examples of antagonistic actions, overlapping function (functional redundancy) and cooperative activity to subvert host cellular functions (Dean and Kenny, 2009; Schmidt, 2010; Santos and Finlay, 2015). This is exemplified by the cooperative and redundant activities of 3 effectors (Tir, Map & EspF) and the outer membrane protein Intimin to induce the effacement of absorptive microvilli of enterocytes (Dean *et al.*, 2006). This not only highlights the complexity of host pathogen interactions but also demonstrates the difficulty in defining the contribution of specific effectors to subvert host cellular pathways.

3.1.2 EPEC anti-phagocytosis

EPEC inhibition of phagocytosis was initially identified using the rabbit form of EPEC (RDEC-1); revealing the capacity of these bacteria to inhibit their uptake by intestinal microfold cells

(M-cells) (Inman and Cantey, 1983; Siebers and Finlay, 1996). Studies examining this mechanism of inhibition have predominantly utilised macrophages (J774A.1 & RAW264.7) as a more convenient and simplistic model to examine the interaction of EPEC with phagocytic cells (Law *et al.*, 2013). These studies demonstrated that EPEC has the ability to inhibit its phagocytosis by the PI3K mediated pathway, dependent on a functional T3SS (Goosney *et al.*, 1999). This inhibition of PI3K mediated phagocytosis was correlated with the rapid decrease of phosphorylated AKT (Ser473), routinely used as a marker of PI3K activity (Celli *et al.*, 2001). However, the identification of EspF as the effector protein responsible for inhibiting PI3K dependent phagocytosis has uncoupled these two signalling events (Quitard *et al.*, 2006). Quitard *et al.*, were the first to identify the critical role of EspF, revealing EPEC strains lacking the EspF effector are unable to inhibit their uptake by the PI3K mediated pathway. These results were later supported by two independent studies in a macrophage (Tahoun *et al.*, 2011) and M-cell model (Martinez-Argudo *et al.*, 2007). Crucially, strains lacking EspF retained the capacity to induce the rapid loss of phosphorylated AKT. This latter finding suggests that EPEC induced inhibition of PI3K mediated phagocytosis and the rapid decrease of phosphorylated AKT (Ser473) are triggered by independent mechanisms.

It is hypothesised that the rapid decline of infection-induced phosphorylated AKT is dependent on one or more of the 24 known EPEC effectors translocated into host cells. Therefore, to identify the critical virulence factors necessary to subvert AKT phosphorylation, bacterial strains that express or lack specific EPEC virulence factors were examined for their ability to decrease infection-induced phosphorylated AKT.

3.2 Results

3.2.1 EPEC triggers a rapid T3SS dependent loss of phosphorylated AKT

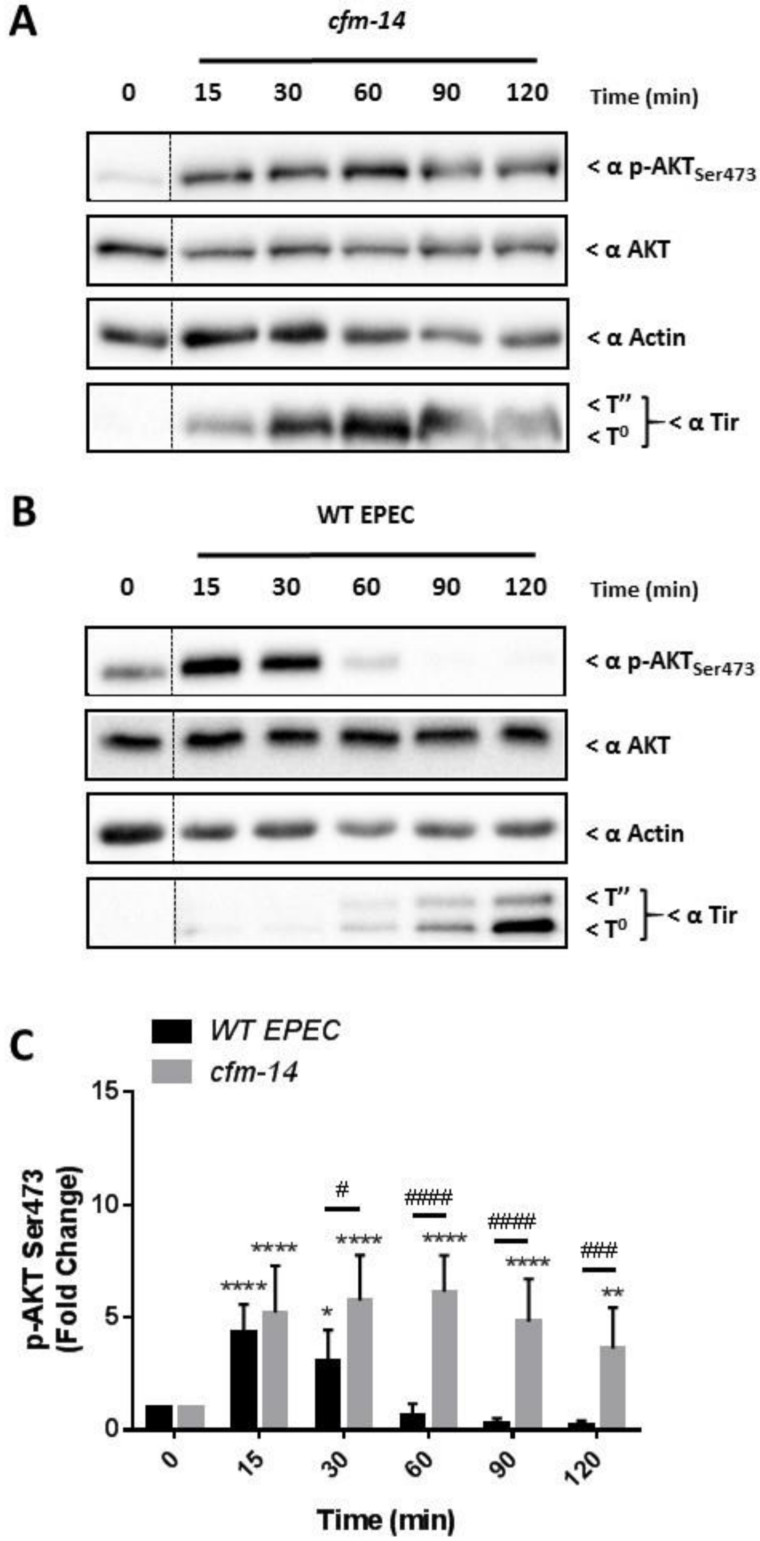
EPEC induced loss of AKT phosphorylation at Ser473 (p-AKT_{ser473}) is reported to be dependent on a functional T3SS (Celli *et al.*, 2001; Quitard *et al.*, 2006). Prior to screening for EPEC virulence factors responsible for this rapid decrease, it was first necessary to re-establish this infection model. Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated T3SS defective mutant, *cfm-14* (unable to translocate effectors) or WT EPEC strain at an MOI of 200:1, before isolating total cell extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

Infection with the *cfm-14* mutant induced a strong increase in AKT phosphorylation (Figure 13A). Increased levels of p-AKT_{ser473} were detected at 15 min post infection and maintained above the level of uninfected control over the 120 min infection period (Figure 13A). In contrast, infection with WT EPEC strain induced only a transient increase in phosphorylated AKT, triggering the rapid decrease of p-AKT_{ser473} signal at 60 min post infection (Figure 13B). These results are consistent with previous studies (Celli *et al.*, 2001; Quitard *et al.*, 2006). Total AKT and Actin (loading control) remained constant throughout the 120 min infection period with the *cfm-14* mutant and WT EPEC strain (Figure 13A & Figure 13B). This sustained increase of p-AKT_{ser473} levels over the 120 min infection period was also evident using alternative T3SS defective mutant's *ΔsepB* (*CVD452*) and *class5* mutant (Appendix Figure 1).

The LEE effector Tir was used as a marker of T3SS effector protein translocation, representing the first and most abundantly delivered EPEC effector into target cells (Mills *et al.*, 2008). Tir delivered into host cells undergoes modification by host cell kinases resulting in a shift in Mw from its unmodified (T^0; 78 kDa) to modified form (T''; 90 kDa) (Kenny *et al.*, 1997). As expected (Kenny *et al.*, 1997), both forms of Tir (T^0 & T'') were detected in cells infected with the WT EPEC strain, with only the unmodified form evident post infection with the T3SS mutants: *cfm-14*, *ΔsepB* (*CVD452*) and *class 5* (Figure 13 & Appendix Figure 1). The host modified form of Tir (T'') was detected after 60 min of infection with the WT EPEC strain and correlated with the rapid loss of p-AKT_{ser473} signal (Figure 13B). Comparison of these strains reveals the detection of the unmodified form of Tir (T^0) by 15-30 min post infection with nalidixic acid sensitive (Nal^S) EPEC strains (*cfm-14* & *class5*), in contrast to 60-90 min of infection with nalidixic acid resistant (Nal^R) strains (WT EPEC & *ΔsepB*). The delay in detection of the unmodified form of Tir (T^0) with the WT EPEC and *ΔsepB* mutant was associated with a comparatively slower rate of growth (data not shown); linked to the acquired resistance to the antibiotic nalidixic acid (Nisa *et al.*, 2013). This faster growth of Nal^S EPEC strains presumably accelerates the formation of stable EPEC-macrophage interactions, enabling the early detection of Tir (T^0). For the remainder of this study all strains examined were of Nal^R genetic background, unless stated otherwise.

To support the development of a reproducible and quantifiable method of analysing AKT phosphorylation (Ser473), western blots from multiple independent experiments were subjected to densitometry analysis; quantifying changes in p-AKT_{ser473} signal, normalised to loading control (Actin) and calculated as a fold change of uninfected cells (see Materials and Methods). Actin, and not total AKT, was selected as a loading control to ensure normalisation of p-AKT_{ser473} signal within the same blot; avoiding complications of comparing separate blots, or the stripping and re-probing of the same blot which can lead to loss or variable protein signal. Densitometry analysis supports the T3SS dependent decrease of phosphorylated AKT, revealing a significant increase of p-AKT_{ser473} above the level uninfected control throughout the 120 min infection period with the *cfm-14* mutant (Figure 13C). In contrast, this significant increase of p-AKT_{ser473} signal is lost at 60 min post infection with the WT EPEC strain (Figure 13C). Furthermore, comparison of results reveals significantly reduced levels of p-AKT_{ser473} at 30, 60, 90 and 120 min post infection with the WT EPEC strain than induced by the *cfm-14* mutant (Figure 13C). Although not obvious from western blots, quantification data indicates a subtle decrease of p-AKT_{ser473} levels towards latter stages of infection with the *cfm-14* mutant (Figure 13C). This gradual decrease may represent the natural host mediated down regulation of phosphorylated AKT, possibly due to internalisation of the activating receptor (e.g. TLR, RTK, etc) (Husebye *et al.*, 2006; Sorkin and von Zastrow, 2009) and/or dephosphorylation of AKT by protein phosphatases (Hers *et al.*, 2011). This decrease of p-AKT_{ser473} signal has previously been demonstrated over a similar time course following LPS stimulated AKT phosphorylation in macrophages (McGuire *et al.*, 2013).

Taken together, these results support the re-establishment of a robust and re-producible model to study the EPEC induced T3SS dependent loss of phosphorylated AKT.


A
cfm-14
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α AKT
< α Actin
< T''
< T0
< α Tir
B
WT EPEC
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α AKT
< α Actin
< T''
< T0
< α Tir
C
WT EPEC
cfm-14
p-AKT Ser473 (Fold Change)
15
10
5
0
0 15 30 60 90 120
Time (min)
#
####
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*
**

Figure 13 EPEC induced loss of phosphorylated AKT requires a functional T3SS.

(**A & B**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated WT EPEC or T3SS mutant (*cfm-14*) strains. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT_{ser473}, anti-pan-AKT (detect all 3 isoforms), anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**C**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.2.2 The T3SS dependent loss of phosphorylated AKT requires functional translocator proteins

In addition to the T3SS, effector protein translocation into host cells is dependent on 3 translocator proteins, EspA, EspD and EspB; of which only EspB has known effector functions (Iizumi *et al.*, 2007). To confirm the assumption that translocator proteins are critical for EPEC induced loss of phosphorylated AKT, J774A.1 macrophages were left uninfected or infected with the pre-activated *ΔespB* (lacking EspB) mutant at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

Infection with the *ΔespB* mutant induced a robust increase in p-AKT$_{ser473}$ signal over the 120 min infection period (Figure 14A), similar to that induced by the *cfm-14* strain (Figure 13A). Lack of effector protein delivery with the *ΔespB* mutant was supported by detection of only the unmodified form of Tir (T^0) in infected cell extracts (Figure 14A). Similar results were obtained with the additional translocator mutants, *ΔespA* and *ΔespD* (lacking EspA & EspD respectively), revealing a sustained increase in p-AKT$_{ser473}$ signal above the level of uninfected control over the 120 min infection period (Appendix Figure 2). Both strains were positive for the detection of the unmodified form of Tir only (T^0) (Appendix Figure 2).

Quantification analysis revealed the significant increase of p-AKT$_{ser473}$ levels at 60, 90 and 120 min post infection with the *ΔespB* strain (Figure 14B). Furthermore, this increase of p-AKT$_{ser473}$ levels were significantly greater than that induced by the WT EPEC strain after 60, 90 and 120 min of infection. This p-AKT$_{ser473}$ profile was similar to that induced by the *cfm-14* mutant (Figure 13C), though notably more variable (note error bars); possibly due to the variability of cell binding with the *ΔespB* strain.

This work reveals a critical role for translocator proteins in EPEC induced rapid loss of p-AKT$_{ser473}$ signal, supporting the requirement for a functional effector delivery system for effector protein translocation into host cells. Collectively these findings suggest that one or more T3SS/translocator dependent effector protein(s) may affect AKT phosphorylation.

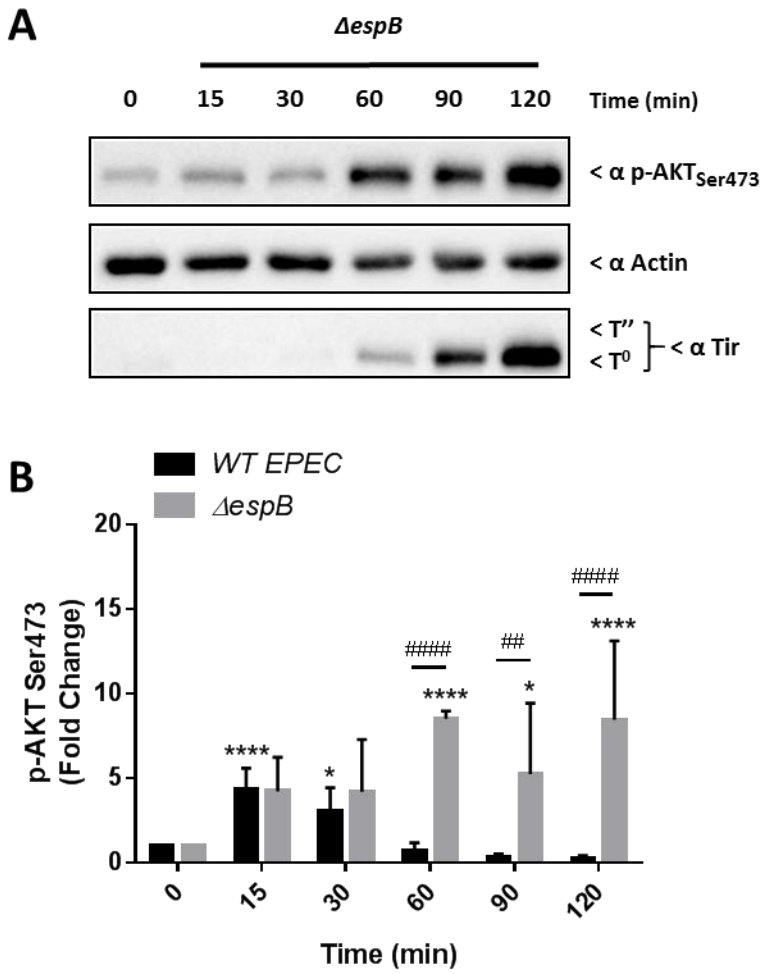


Figure 14 EPEC requires a functional effector delivery system to decrease phosphorylated AKT.

(**A**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated *ΔespB* (lacking EspB translocator protein) strain. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.2.3 EPEC induced loss of phosphorylated AKT is independent of 6 LEE effectors, the outer membrane protein Intimin and non-LEE effector EspG2/Orf3

Studies by Quitard *et al.*, have previously demonstrated the rapid loss of p-AKT$_{ser473}$ using the EPEC *Δquad* mutant (lacks 3 LEE effectors [Map, EspF & Tir] & the outer membrane protein Intimin), similar to that of the WT EPEC strain (Quitard *et al.*, 2006). To examine the role of LEE effector proteins, it was important to re-establish these results using the *Δquad* mutant prior to screening the recently generated more complex LEE effector deficient strain, *Δmfzgorf3eh::km* (lacks the Nle effector EspG2/Orf3 & all known LEE effectors except Tir and EspB; Figure 15). Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated *Δquad* or *Δmfzgorf3eh::km* mutant at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

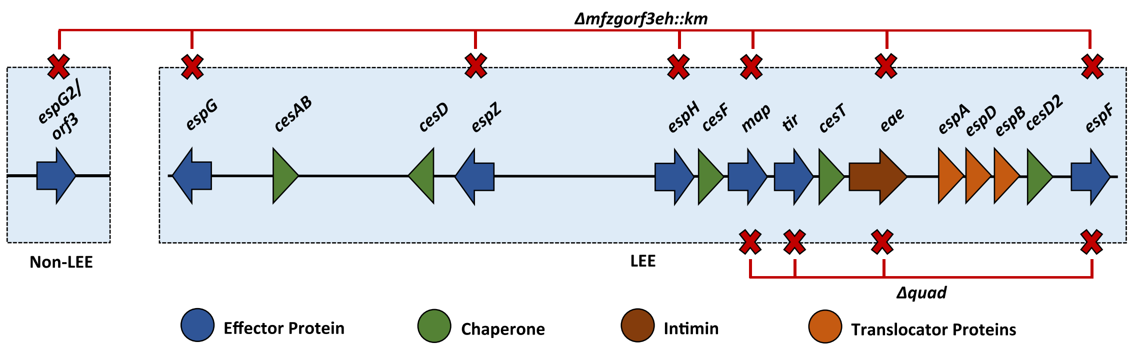


Figure 15 Schematic of the complex LEE effector *Δquad* and *Δmfzgorf3eh::km* mutants.

An illustration of the LEE region encoding T3SS dependent effector proteins (Blue), T3SS effector chaperones (Green), translocator proteins (Orange) and the outer membrane protein Intimin (Brown). Also presented is the non-LEE encoded (Nle) effector EspG2/Orf3 encoded on the pathogenicity island IE5. EPEC strains *Δquad* (Quitard *et al.*, 2006) and *Δmfzgorf3eh::km* (A. Madkour *et al.*, unpublished) were created by the disruption of LEE effector genes in addition to *espG2/orf3*; these strains and corresponding disrupted genes are indicated with a 'x'.

As reported (Quitard *et al.*, 2006), infection with *Δquad* mutant induced a transient increase in AKT phosphorylation (Figure 16A), similar to that of the WT EPEC strain (Figure 16B). Increased p-AKT$_{ser473}$ levels were detected at 15 and 30 min post infection, followed by a rapid decrease below uninfected control after 60 min of infection (Figure 16A).

Quantification analysis supports the significant increase of p-AKT$_{ser473}$ levels at 15 min post infection only (Figure 16B). Unexpectedly, this increase was significantly greater than that induced by the WT EPEC strain at 15 min post infection (Figure 16B). Importantly, infection with the *Δmfzgorf3eh::km* mutant displayed a similar capacity to rapidly decrease the level of p-AKT$_{ser473}$ below uninfected control by 60 min post infection (Figure 16C). As a result, it was decided further repeat experiments were not required for quantification analysis to support this WT EPEC like p-AKT$_{ser473}$ profile. Detection of both the unmodified (T^0) and modified form of Tir (T'') was evident in cells infected with the *Δmfzgorf3eh::km*, but not *Δquad* mutant, supporting the loss of a functional *tir* gene in the latter strain only (Figure 16A & Figure 16C). The *Δmfzgorf3eh::km* strain genotype was supported by PCR analysis for the disruption of the appropriate genes (Madkour *et al*., data not shown). For example, Figure 16D reveals the increase in size of the *espH* gene product by PCR analysis for the *Δmfzgorf3eh::km*, but not the *Δquad*, strain. This supports the disruption of the *espH* gene by insertion of a kanamycin antibiotic cassette (Figure 16D).

This work supports the non-essential roles (cooperative or individual) for 5 LEE effectors (EspZ, EspH, Map, EspF & EspG), the outer membrane protein Intimin and the Nle effector EspG2/Orf3, for EPEC to induce the rapid loss of phosphorylated AKT. Crucially, these strains do not discount a role for the LEE effector EspB, individually or in cooperation with Tir; maintained in the strain as a marker of T3SS functionality.

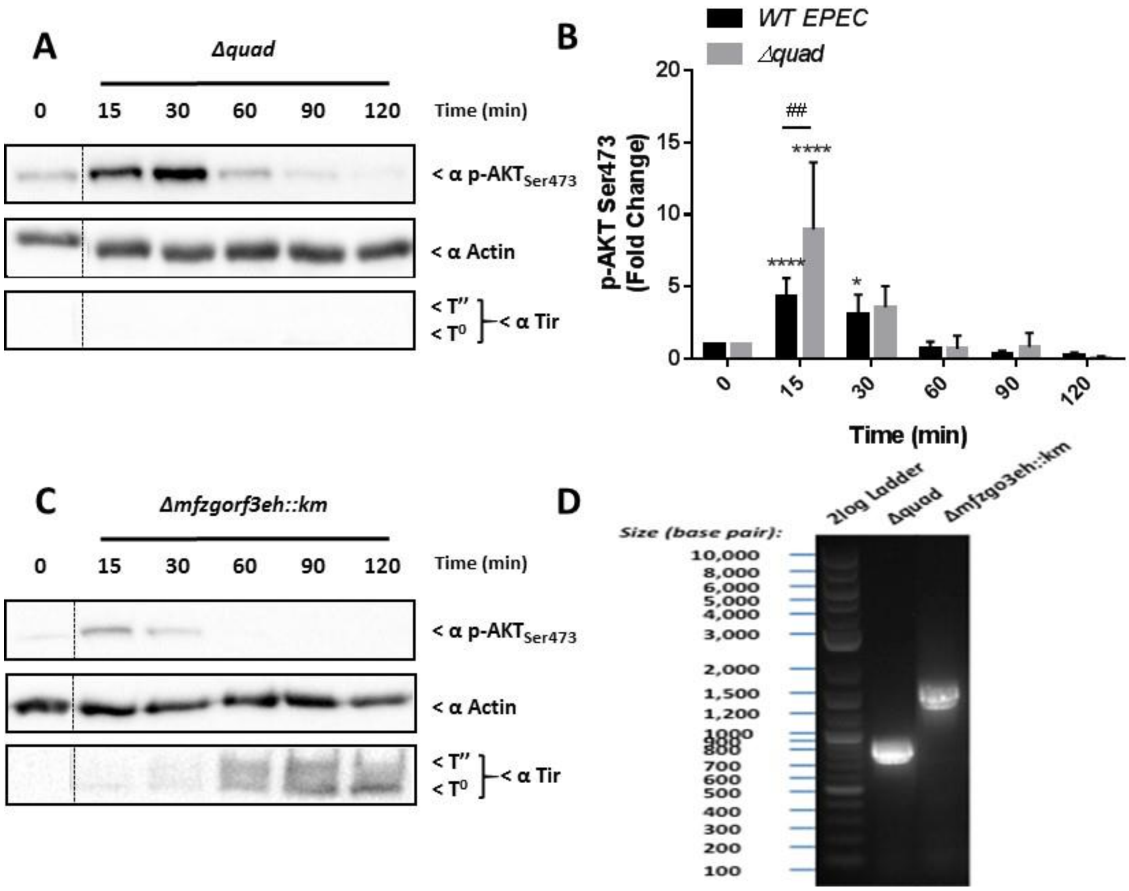

A
Δquad
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T0
< α Tir
B
WT EPEC
Δquad
p-AKT Ser473 (Fold Change)
20
15
10
5
0
##
****
****
*
0 15 30 60 90 120
Time (min)
C
Δmfzgorf3eh::km
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T0
< α Tir
D
Size (base pair):
2log Ladder
Δquad
Δmfzgo3eh::km
10,000
8,000
6,000
5,000
4,000
3,000
2,000
1,500
1,200
1000
900
800
700
600
500
400
300
200
100

Figure 16 EPEC strains lacking a subset of LEE encoded effector proteins do not prevent the rapid decrease of AKT phosphorylation.

(**A & C**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated EPEC *Δquad* (lacking LEE genes *espF*, *map*, *tir* & *eae* [intimin]) or *Δmfzgorf3eh::km* (lacking LEE genes *eae* [Intimin], *map*, *espF*, *espZ*, *espH*, *espG* & the EspG homologue *espG2/orf3*) mutant strains. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots presented are representative of three (**A**; *Δquad*) and one (**C**; *Δmfzgorf3eh::km*) independent experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Values shown are the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (#p<0.05). (**D**) PCR analysis of the EPEC *Δquad* and *Δmfzgorf3eh::km* mutants, probing for the disruption (by kanamycin gene; km) of the *espH* gene, indicated by a larger PCR gene product. PCR products were separated by agarose gel (1%) electrophoresis, stained with Gel Red and imaged by UV illumination.

3.2.4 *E.coli* K12 bacteria carrying the LEE region can inhibit PI3K mediated phagocytosis

The effector activity of EspB cannot be distinguished from its critical role in effector translocation by a gene deletion strategy. To examine the effector activity of EspB and possible redundant activities of all LEE effectors, the ability of the LEE region to trigger the rapid loss of p-AKT$_{ser473}$ signal was examined using the *E. coli* K12 strain carrying the LEE region on a plasmid (Yen *et al.*, 2010). This strategy has previously confirmed the sufficiency of the LEE region to induce A/E lesion formation (McDaniel and Kaper, 1997) and barrier disruption (Simonovic *et al.*, 2000). The ability of the LEE region to rapidly reduce p-AKT$_{ser473}$ levels was interrogated using the available strains TOB02, TOB01 and DH10B. As described (Yen *et al.*, 2010), the TOB02 strain carries two plasmids, one encoding the LEE region (*pLEE*) and the other (*pBFP_Per*) encoding the *bfp* operon (coding for the bundle forming pilus) and *per*ABC regulatory genes (encoding regulatory genes for BFP and LEE transcription) (Mellies *et al.*, 1999; Mellies *et al.*, 2007). In contrast, the TOB01 strain carries the plasmid *pBFP_Per* only (Yen *et al.*, 2010), while the *E. coli* K12 strain (DH10B) is lacking of both these plasmids.

Before interrogating the impact of LEE carrying TOB02 on AKT phosphorylation, it was first necessary to confirm whether this strain could induce a rapid LEE effector dependent subversive event. This was achieved by examining the ability of the TOB02 (K12/*pLEE* + *pBFP_Per*) strain to inhibit its uptake (phagocytosis) by J774A.1 macrophages. This inhibition of phagocytosis by the PI3K mediated pathway is linked to the activities of 3 LEE effector proteins, EspF (Quitard *et al.*, 2006), EspH (Dong *et al.*, 2010) and EspB (Iizumi *et al.*, 2007). Thus, J774A.1 macrophages were infected with pre-activated WT EPEC, *cfm-14*, DH10B (K12), TOB01 (K12/*pBFP_Per*) or TOB02 (K12/*pLEE* + *pBFP_Per*) strains for 1 h at an MOI of 100:1. Infections were stopped by the removal of bacteria and treated with chloramphenicol for 1 h, to prevent further effector protein delivery and ensure sufficient time for cell bound bacteria to be internalised. Following treatment, cells were fixed and stained using a differential fluorescence based antibody assay, distinguishing extracellular (Red) from total (Green) bacteria (Figure 17A), to determine the percentage of intracellular bacteria (see Materials and Methods). As reported (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006), the T3SS defective mutant (*cfm-14*) was readily internalised by J774A.1 macrophages with approximately 74.9% (±1.7%) of bacteria internalised (Figure 17A & B). In contrast, only

35.0% (± 6.2%) of WT EPEC bacteria were internalised, significantly less than the *cfm-14* mutant ($p<0.0001$; Figure 17B). Infection with LEE deficient K12 strains (DH10B & TOB01) led to a similar percentage uptake to the *cfm-14* mutant, with 77.8% (±6.5%; $p>0.9$) and 77.4% (±8.2%; $p>0.9$) of bacteria internalised respectively (Figure 17A & B). Like the WT EPEC strain, infection with the TOB02 (K12/*pLEE* + *pBFP_Per*) strain led to significantly fewer bacteria being internalised than the *cfm-14* mutant (46.8% ± 0.8%; $p<0.001$; Figure 17A & B). No significant difference in the percentage of uptake was identified between infections with the WT EPEC or TOB02 (K12/*pLEE* + *pBFP_Per*) strain. This work reveals, for the first time, the ability of the plasmid encoded LEE region to inhibit the uptake of *E. coli* by J774A.1 macrophages. This confirms the capacity of the TOB02 (K12/*pLEE* + *pBFP_Per*) strain to induce a rapid LEE dependent subversive event.

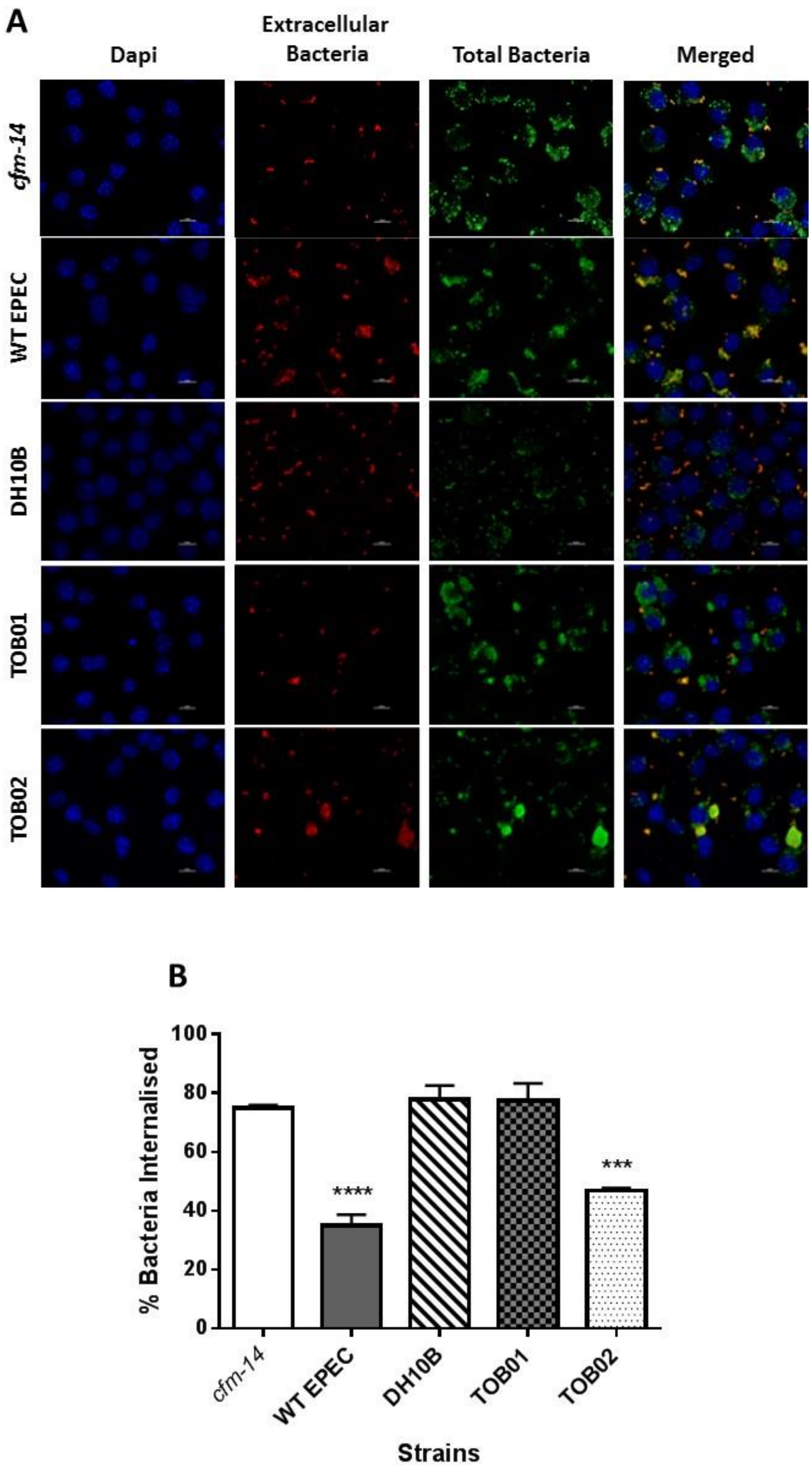

A
Dapi
Extracellular Bacteria
Total Bacteria
Merged
cfm-14
WT EPEC
DH10B
TOB01
TOB02
B
% Bacteria Internalised
0
20
40
60
80
100
****
***
cfm-14
WT EPEC
DH10B
TOB01
TOB02
Strains

Figure 17 LEE carrying TOB02 can inhibit PI3K mediated phagocytosis.

(**A**) J774A.1 macrophages were infected for 1 h with pre-activated WT EPEC, *cfm-14* (non-functional T3SS), DH10B (*E. coli* K12), TOBO1 (*E. coli* K12 carrying plasmid encoding the *bfp* and *perABC* regulatory genes) or TOBO2 (TOB01 strain carrying plasmid encoding the LEE region) bacteria. Infections were stopped by washing of cells to remove non-adherent bacteria before treating with chloramphenicol [25 µg/ml] for 1 h to prevent further effector delivery and allow adherent bacteria time to be internalised. Post incubation, cells were fixed and examined by differential fluorescence antibody labelling. Cells were probed with anti-EPEC or anti-*E. coli* antibodies and detected using Alexa Fluor 555 anti-rabbit (Red; Extracellular bacteria). These cells were then permeablised (1% Triton X-100), probed with anti-EPEC or anti-*E. coli* antibodies and detected using Alexa Fluor488 anti-rabbit (Green; Total bacteria). Bacterial and host DNA was labelled with Dapi (Blue). (**B**) A total of 50 randomly selected cells per experiment, with between 20-50 cell-associated bacteria, were quantified in a semi blind manner (strain identity revealed post-counting). Percentage of internalised bacteria was calculated by subtracting the number of extracellular bacteria (Red) from the total number of cell associated bacteria (Green), divided by the total (Green) and multiplied by 100. Data are from of three independent experiments, with values being the mean ± SD. Statistical analysis was performed by one-wave ANOVA followed by Dunnett's post-test (****$p < 0.0001$, ***$p < 0.001$).

3.2.5 *E. coli* K12 bacteria carrying the LEE region cannot induce the rapid loss of phosphorylated AKT

Having demonstrated the ability of the LEE carrying TOB02 strain to inhibit phagocytosis, the role of the LEE region to trigger the rapid loss of phosphorylated AKT was examined. Thus, J774A.1 macrophages were infected with pre-activated WT EPEC, TOB02 (K12/*pLEE* + *pBFP_Per*), TOB01 (K12/*pBFP_Per*) or DH10B (K12) strains at an MOI of 200:1, before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

As before (Figure 13B), infection with WT EPEC induced a transient increase in AKT phosphorylation (Figure 18A). This phosphorylation was visibly greater than uninfected control at 15 min post infection before rapidly decreasing to background levels after 60 min of infection (Figure 18A). In contrast, infection with strains DH10B (K12), TOB01 (K12/*pBFP_Per*) and TOB02 (K12/*pLEE* + *pBFP_Per*) led to an increase in AKT phosphorylation at 15 min post infection that was maintained at/or above the level of uninfected control over the 120 min infection period (Figure 18A-D), similar to the T3SS defective mutant *cfm-14* (Figure 13A). However, it should be noted that not in all cases was this increase in p-AKT$_{ser473}$ signal supported to be statistically significant by quantification.

Bacterial strain genotypes were supported by the absence (DH10B & TOB01) or presence (WT EPEC & TOB02) of LEE encoded effector proteins EspF and Tir (Figure 18A-D). Detection of the host kinase modified form of Tir (T'') supported a functional T3SS with the WT EPEC and TOB02 strain. Interestingly, detection of modified form of Tir (T'') coincided with the rapid loss of p-AKT$_{ser473}$ in cells infected with the WT EPEC (detected at 60 min post infection; Figure 18A), but not TOB02 (detected at 90 min post infection; Figure 18D), strain. Microcolony formation was evident with the TOB01 (K12/*pBFP_Per)*, TOB02 (K12/*pLEE* + *pBFP_Per*) and WT EPEC, but not DH10B (K12), strains supporting the functionality of plasmid encoding the adhesion protein BFP (Tobe and Sasakawa, 2001) (data not shown).

Quantification analysis confirms a transient p-AKT$_{ser473}$ profile induced by the WT EPEC strain, revealing the loss of significantly increased p-AKT$_{ser473}$ at or below the level of uninfected control after 60 min of infection (Figure 18E). By contrast, this rapid loss of p-AKT$_{ser473}$ signal was absent from cell extracts infected with the DH10B (K12), TOB01 (K12/*pBFP_Per*) or TOB02 (K12/*pLEE* + pBFP_*Per*) strain; revealing a sustained increase of p-

AKT_{ser473} signal with a subtle decline towards background levels similar to the *cfm-14* mutant (Figure 18E & Figure 13C). This decline of p-AKT_{ser473} levels was greater for DH10B (K12) than TOB01 (K12/pBFP_*Per*) strain at 90 and 120 min post infection, which may indicate a factor on TOB01 (BFP) to promote AKT phosphorylation. Comparison of results against the WT EPEC strain reveals significantly greater levels of p-AKT_{ser473} with the DH10B (60 min post infection) and TOB01 (60 & 90 min post infection), but not the TOB02, strain (Figure 18E). However, p-AKT_{ser473} levels induced by the TOB02 strain were significantly lower than those induced with the TOB01 strain at 90 min post infection (Figure 18E). Together this suggests the TOB02 strain may have a weak capacity to stimulate/reduce the levels of p-AKT_{ser473}.

This work suggests that *E. coli* strains with or without plasmids encoding the LEE region and/or BFP operon/perABC regulatory genes (DH10B, TOB01 & TOB02) do not stimulate a rapid loss of p-AKT_{ser473}, unlike the WT EPEC strain. Though able to inhibit phagocytosis (Figure 17), these results argue against the role for LEE encoded factors, alone, to induce the rapid loss of phosphorylated AKT. The latter finding implies that factors/effectors necessary to trigger the rapid decrease of p-AKT_{ser473} are encoded outside the LEE region (non-LEE).

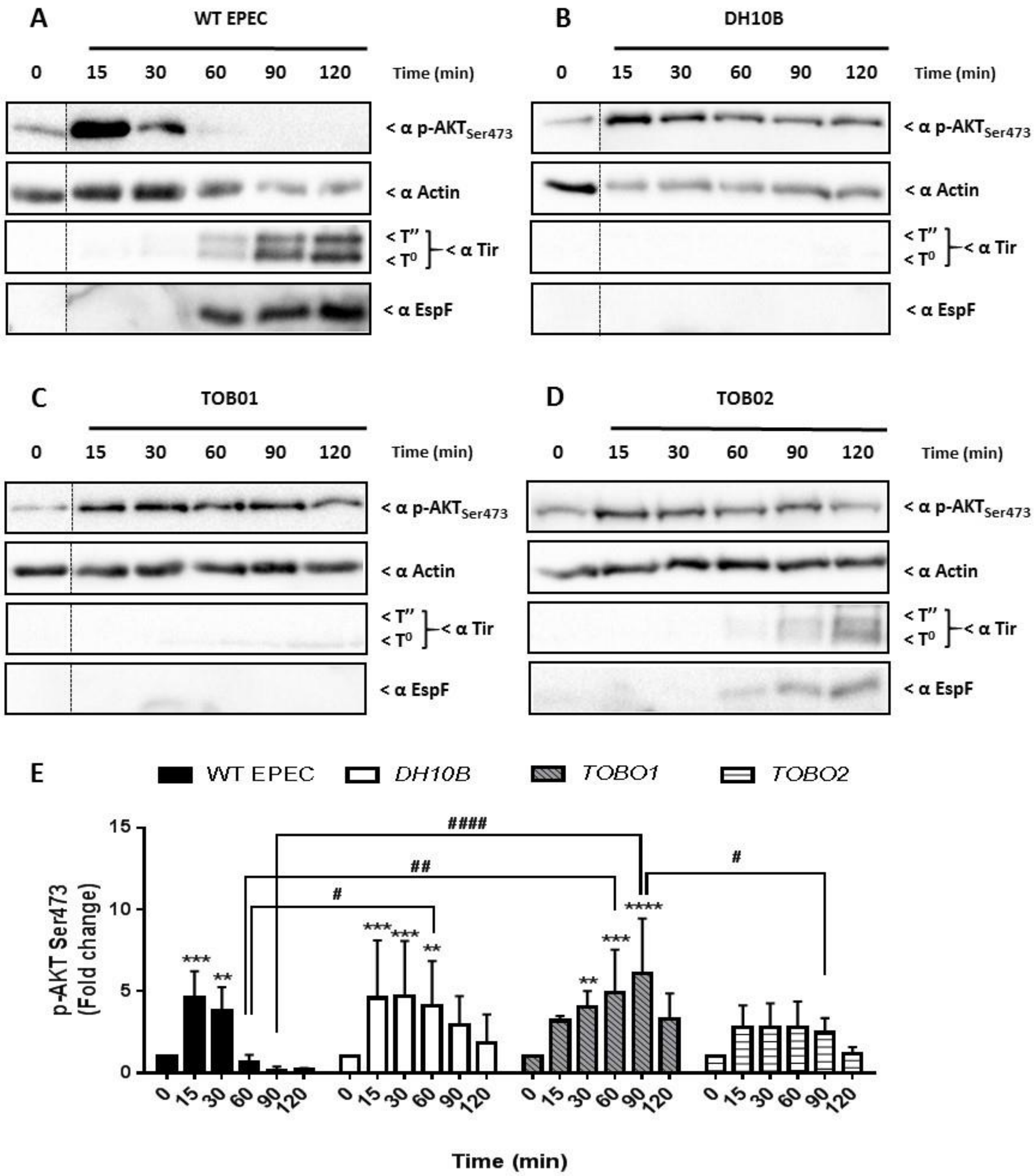

A
WT EPEC
0 15 30 60 90 120 Time (min)
< α p-AKT$_{Ser473}$
< α Actin
< T''
< T^{0}
< α Tir
< α EspF
B
DH10B
C
TOB01
D
TOB02
E
WT EPEC
DH10B
TOBO1
TOBO2
p-AKT Ser473 (Fold change)
Time (min)
####
##
#

Figure 18 The LEE region does not encode the necessary factors to induce a rapid decrease in AKT phosphorylation.

(**A-D**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated WT EPEC, DH10B (*E. coli* K12), TOBO1 (*E. coli* K12 carrying plasmid encoding the *bfp* and *perABC* regulatory genes) or TOB02 (TOB01 strain carrying plasmid encoding the LEE region) bacteria. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-EspF, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T’’) (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**E**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett’s post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak’s post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.2.6 Pre-infection of cells with EPEC can prevent AKT phosphorylation stimulated by T3SS mutant (*cfm-14*)

A possible problem identified with the established infection model was the unsynchronised infection of cells. This may lead to sub-populations of cells at early time points having none, few or many cell-associated bacteria to inhibit AKT phosphorylation, therefore accounting for subtle variations in the p-AKT_{ser473} profile. To resolve this, a two-wave infection protocol was designed to examine the capacity of cells pre-infected with EPEC to stimulate AKT phosphorylation following a second infection with the T3SS mutant (*cfm-14*). Thus, J774A.1 macrophages were left uninfected or infected with pre-activated WT EPEC or *cfm-14* mutant (1^{st} wave infection) strains for 1 h at an MOI of 200:1. Infections were stopped by treatment of cells with gentamicin (kill extracellular bacteria) for 1 h, followed by a 1 h recovery period in fresh DMEM. Cells were then infected post recovery with the *cfm-14* strain (2^{nd} wave infection) at an MOI of 100:1. Cell extracts were isolated after the initial EPEC infection (post recovery in DMEM) and after 15, 30 and 60 min of infection with the *cfm-14* mutant (2^{nd} wave infection). The T3SS defective mutant (*cfm-14*) was selected for the second-wave infection due to its robust stimulation of AKT phosphorylation (Figure 13A).

Analysis of 1^{st} wave infections only (post gentamicin/recovery) revealed the increase in AKT phosphorylation, above the level of uninfected control, after infection with the *cfm-14*, but not the WT EPEC, strain (Figure 19A). A second infection of cells (2^{nd} wave) pre-infected with the T3SS mutant (*cfm-14*) led to a robust increase in AKT phosphorylation after 15, 30 and 60 min of infection with the *cfm-14* strain (Figure 19A). In contrast, cells pre-infected with the WT EPEC strain led to only a weak increase of p-AKT_{ser473} signal after 15, 30 and 60 min of infection with the *cfm-14* mutant (Figure 19A). EPEC strain genotype was supported by the detection of the host kinase modified form of Tir (T'') in cell extracts infected with the WT EPEC, but not *cfm-14*, strain (Figure 19A). Quantification analysis supports the robust increase in p-AKT_{ser473} signal after a second infection with the *cfm-14* mutant in cells pre-infected with the *cfm-14*, but not WT EPEC, strain (Figure 19B). This reveals that the capacity of J774A.1 macrophages to induce AKT phosphorylation is prevented if cells are pre-infected with WT EPEC strain.

This work illustrates that pre-infection of cells for 1 h with EPEC can prevent the phosphorylation of AKT in a T3SS dependent manner, stimulated by a second infection with the *cfm-14* mutant.

3.2.7 Pre-infection of cells with LEE carrying *E. coli* K12 bacteria does not prevent AKT phosphorylation stimulated by T3SS mutant (*cfm-14*)

To interrogate the suggested weak capacity of the LEE carrying TOB02 strain to prevent or decrease the levels of p-AKT$_{ser473}$ (Figure 18D), the capacity to stimulate AKT phosphorylation was examined in cells pre-infected with the TOB02 (K12/*pLEE* + *pBFP_Per*) strain in a 2-wave infection model. Thus, J774A.1 macrophages were left uninfected or infected with the DH10B (*E. coli* K12), TOB01 (K12/ *pBFP_Per*) or TOB02 (K12/*pLEE* + *pBFP_Per*) strain for 2 h at an MOI of 200:1 (1st wave infection). Infections were stopped by treatment of cells with gentamicin (kill extracellular bacteria) for 1 h, followed by a 1 h recovery period in fresh DMEM. Cells were then infected post recovery with the *cfm-14* strain (2nd wave infection) at an MOI of 100:1. Cell extracts were isolated after the initial EPEC infection (post recovery in DMEM) and after 15, 30 and 60 min of infection with the *cfm-14* mutant (2nd wave infection).

The analysis of 1st wave infections (post gentamicin/recovery) revealed the level of p-AKT$_{ser473}$ is similar to that of uninfected control after infection with the DH10B (K12) or TOB02 (K12/*pLEE* + *pBFP_Per*) strain (Figure 20). In contrast, the 1st wave infection with TOB01 (K12/*pBFP_Per*) strain induced a notable increase in p-AKT$_{ser473}$ signal above background levels (Figure 20). A second infection of cells (2nd wave) pre-infected with strains DH10B (K12), TOB01 (K12/*pBFP_Per*) or TOB02 (K12/*pLEE* + *pBFP_Per*) led to an increase in p-AKT$_{ser473}$ signal above background levels after 15, 30 and 60 min of infection with the *cfm-14* mutant (Figure 20). However, quantification analysis reveals this increase of p-AKT$_{ser473}$ signal after the second-wave infection is, though subtle, overall greater in cells pre-infected with the DH10B (K12) and TOB01 (K12/*pBFP_Per*), than TOB02 (K12/*pLEE* + *pBFP_Per*) strain (Figure 20B). Again, this may tentatively suggest that the TOB02 (K12/*pLEE* + *pBFP_Per*) strain/LEE encoded factor has some capacity to decrease the level of phosphorylated AKT or a reduced capacity to induce AKT phosphorylation.

Bacteria strain genotype was supported by detection of the host kinase modified form of Tir (T'') in cells infected with the TOB02 but not DH10B or TOB01 strains (Figure 20A). The unmodified form of Tir (T^0) is detected in all cells infected with the *cfm-14* mutant as part of the 2-wave infection strategy (Figure 20A). As both the DH10B or TOB01 strain do not encode the effector Tir, detection of a band similar to the Mw of its unmodified form (T^0) in 1st wave infected cell extracts is likely due to a contamination of the sample or the non-specific detection of an *E. coli* K12 protein (Figure 20A).

This work suggests that pre-infection of cells with the TOB02 strain does not prevent the phosphorylation of AKT in a 2-wave infection model. However, the comparatively weak increase of p-AKT_{ser473} levels in cells pre-infected with the TOB02 strain may indicate a weak capacity to decrease phosphorylated AKT or that this strain poorly stimulates AKT phosphorylation. This would support findings that the critical factors necessary to prevent/trigger a rapid decline of phosphorylated AKT are encoded outside the LEE region.

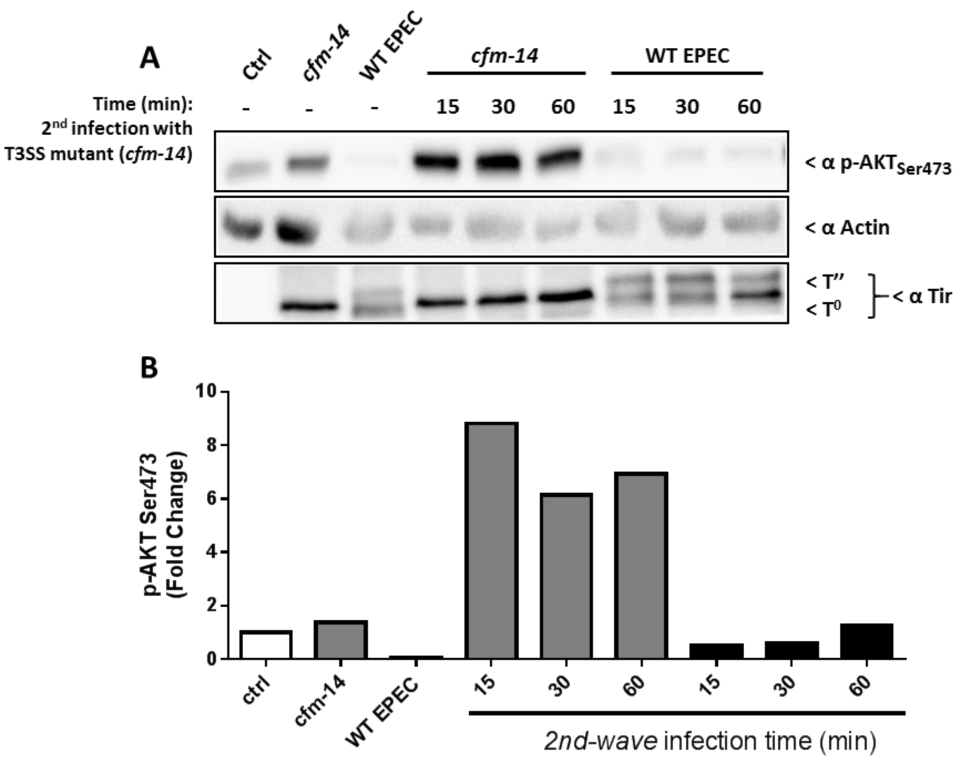


Figure 19 Infection triggered AKT phosphorylation is inhibited in a T3SS dependent manner in a two-wave infection model.

(**A**) J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 1 h with pre-activated WT EPEC or T3SS mutant (*cfm-14*) strains. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h to kill extracellular bacteria, followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2^{nd} infection; MOI 100:1) for 15, 30 and 60 min. Total cell extracts were isolated after the first infection (post gentamicin treatment and recovery) or following a 2^{nd} infection with the *cfm-14* strain. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT_{ser473}, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (Ctrl) cells. Data represent a single experiment.

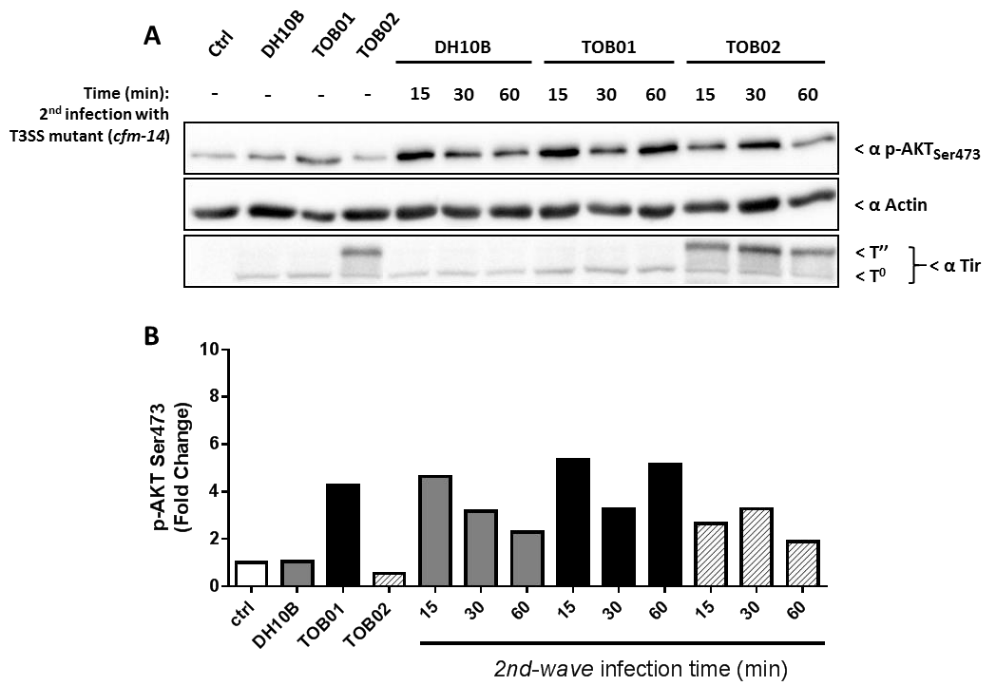


Figure 20 LEE expressing *E. coli* K12 strain (TOB02) cannot inhibit AKT phosphorylation stimulated by second-wave infection with the T3SS mutant (*cfm-14*).

(**A**) J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 2 h with pre-activated DH10B (*E. coli* K12), TOBO1 (*E. coli* K12 carrying plasmid encoding the *bfp* and *perABC* regulatory genes) or TOB02 (TOB01 strain carrying plasmid encoding the LEE region) bacteria. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h to kill extracellular bacteria, followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2^{nd} infection; MOI 100:1) for 15, 30 and 60 min. Total cell extracts were isolated after the first infection (post gentamicin treatment and recovery) or following a 2^{nd} infection with the *cfm-14* strain. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (Ctrl) cells. Data represent a single experiment.

3.2.8 The T3SS dependent loss of phosphorylated AKT is independent of 14 Nle effector proteins

The EPEC effector repertoire consists of up to 17 known Nle effector proteins, implicated to inhibit a number of cell signalling pathways including the innate inflammatory response (Iguchi *et al.*, 2009; Santos and Finlay, 2015). To investigate the requirement of 14 of the most studied Nle effector proteins to induce the rapid loss of p-AKT$_{ser473}$ signal, cells were infected with available EPEC strains TOEA1-A7 (Yen *et al.*, 2010), lacking subsets or all 14 Nle effectors (Table 7). EPEC strains TOEA1-A7 carry additive deletions of effector gene clusters from three prophages (PP2, PP4 & PP6), three integrative elements (IE2, IE4 & IE5) and the LEE region in a Nal^S EPEC (*WT EPEC Japan*; E2348/69) strain background (Yen *et al.*, 2010). EPEC Japan strain genotype was supported by PCR screening for the presence or absence of effector genes from each of the six genomic islands and/or LEE region (Figure 21), indicated in Table 7. The TOEA7 strain was the most complex mutant available, lacking functional genes for all effectors examined but the for LEE encoded *espF* gene (positive control; Figure 21). Thus, J774A.1 macrophages were left uninfected or infected with pre-activated WT EPEC Japan or strains TOEA1-A7 at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

Infection of cells with the WT EPEC Japan (Nal^S) strain induced a transient increase in AKT phosphorylation (Figure 22A), similar to that of our WT EPEC (Nal^R) strain (Figure 22B). Detection of the host-modified form of Tir (T'') was again correlated with decreasing p-AKT$_{ser473}$ signal at approximately 60 min post infection (Figure 22A). Quantification analysis supports this transient p-AKT$_{ser473}$ profile, revealing the significant increase of p-AKT$_{ser473}$ levels at 15 and 30 min post infection before declining to background levels after 60 min of infection. (Figure 22B). Importantly, no significant difference in p-AKT$_{ser473}$ levels stimulated by the WT EPEC Japan or WT EPEC strain was identified over the 120 min infection period (Figure 22B). Furthermore, this transient p-AKT$_{ser473}$ profile was also identified in cells infected with the TOEA7 and TOEA6-A1 strains, revealing maximal phosphorylation at 15 min post infection (Figure 22C-D). Again, quantification analysis supported this transient increase of p-AKT$_{ser473}$ levels, revealing no significant difference between strains TOEA2-A7 over the 120 min infection period (Figure 22D).

This work demonstrates that the 14 Nle effector proteins examined (Table 7) do not play critical roles (individual or cooperative) in the EPEC induced rapid decline of phosphorylated AKT. This would implicate the requirement for other non-LEE T3SS dependent factors, such as NleJ, LifA and/or EspC.

Pathogenicity Island	Gene	EPEC	TOEA1	TOEA2	TOEA3	TOEA4	TOEA5	TOEA6	TOEA7
PP2	nleH1	+	-	-	-	-	-	-	-
	cif	+	-	-	-	-	-	-	-
	espJ	+	-	-	-	-	-	-	-
IE2	nleB3	+	+	-	-	-	-	-	-
	nleE2	+	+	-	-	-	-	-	-
	espL1	+	+	-	-	-	-	-	-
PP4	nleD	+	+	+	-	-	-	-	-
	nleH3	+	+	+	-	-	-	-	-
	nleC	+	+	+	-	-	-	-	-
	nleB2	+	+	+	-	-	-	-	-
	nleG	+	+	+	-	-	-	-	-
IE6	nleE1	+	+	+	+	-	-	-	-
	nleB1	+	+	+	+	-	-	-	-
	espL2	+	+	+	+	-	-	-	-
PP6	nleF	+	+	+	+	+	-	-	-
	nleH2	+	+	+	+	+	-	-	-
	nleA	+	+	+	+	+	-	-	-
	nleO	+	+	+	+	+	-	-	-
IE5	espG 2/orf 3	+	+	+	+	+	+	-	-
LEE	espG	+	+	+	+	+	+	+	-
	espF	+	+	+	+	+	+	+	+

Table 7 Representation of the effector gene clusters deleted from TOEA1-A7 strains.

EPEC Japan strains TOEA1-A7 carry deletions of effector gene clusters present on three prophages (PP2, PP4 & PP6), three integrative elements (IE2, IE5 & IE6) and the LEE region from a Nalidixic Acid sensitive (Nal^s) EPEC strain (WT EPEC Japan; E2348/69 background). Effector gene clusters were deleted in additive fashion indicated by a red box with missing genes indicated with a (-). The 14 functional Nle-encoding and two of the LEE effector genes are indicated by Red and Blue text respectively.

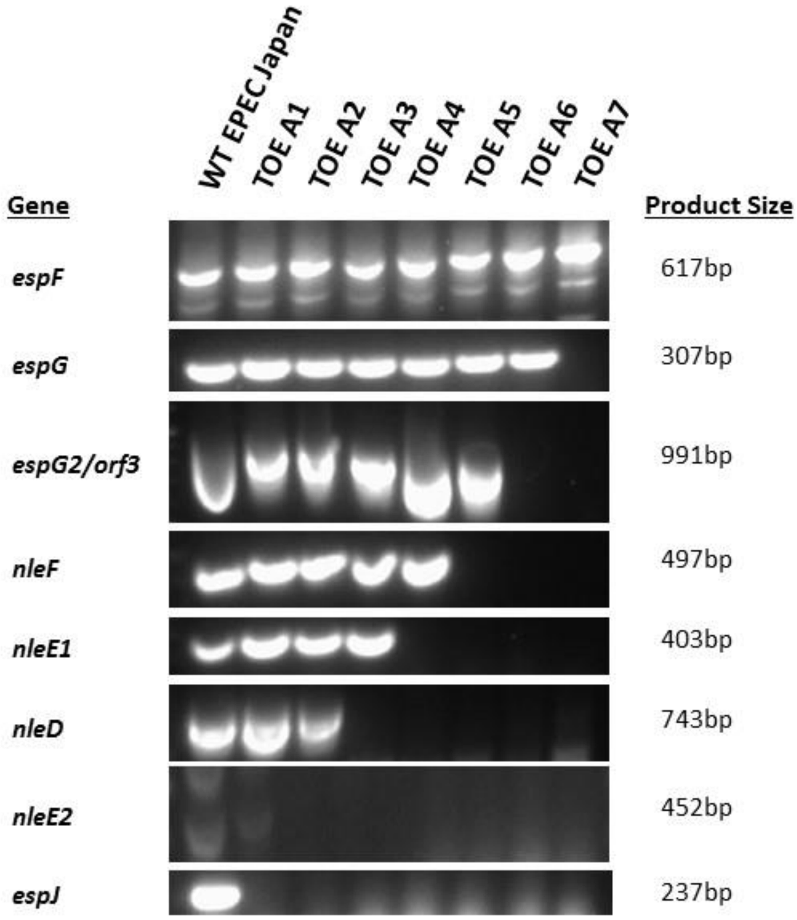
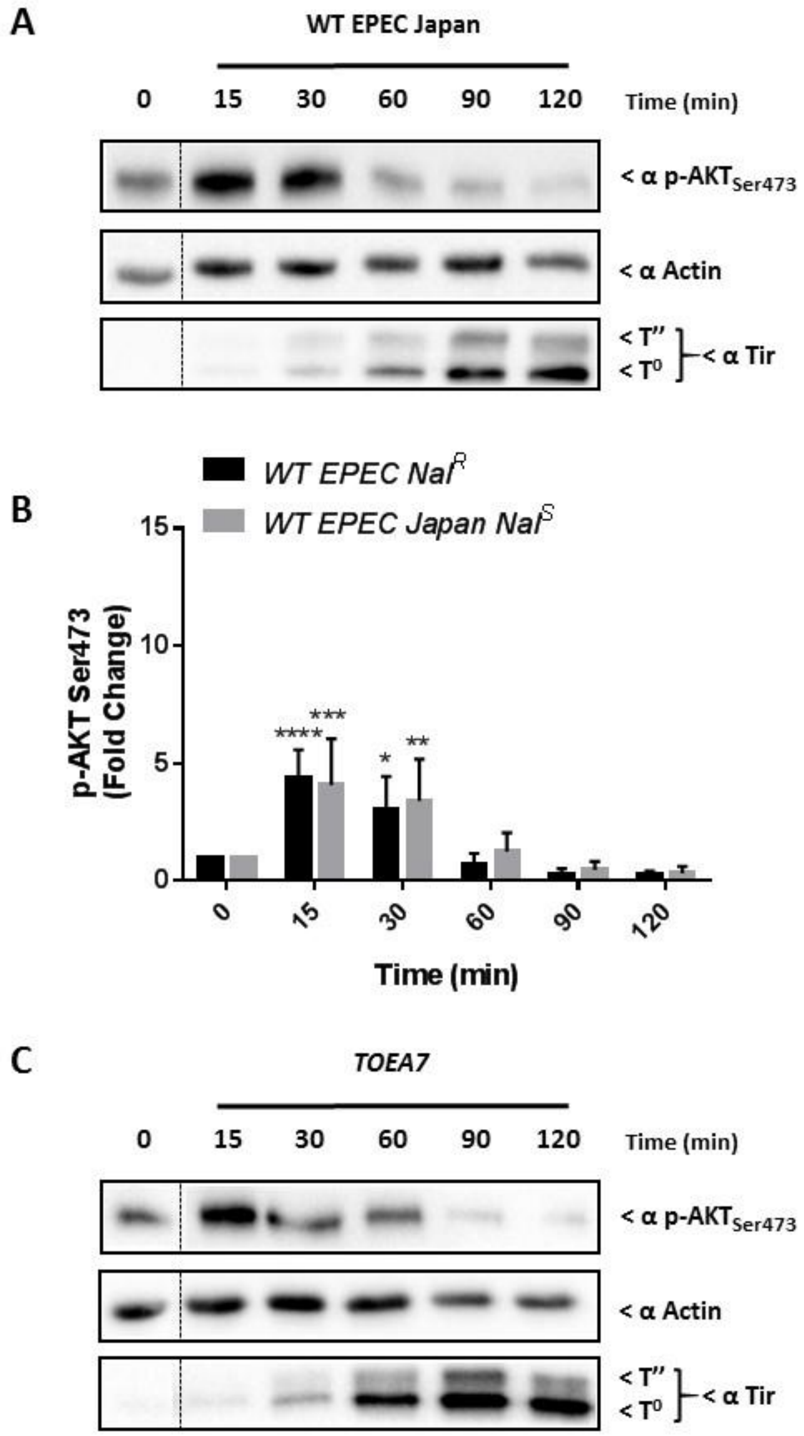


Figure 21 PCR analysis supports the strain genotype of TOEA1-A7 strains through absence of the appropriate effector genes.

EPEC Japan strains TOEA1-A7 and WT EPEC Japan were screened by colony PCR to confirm the presence or absence of appropriate effector genes (Table 7). The effector genes selected for analysis represent one of the genes from each of the gene clusters deleted from each of the 3 prophages (PP2, PP4 & PP6), 3 integrative elements (IE2, IE5 & IE6) and the LEE region. Genes selected were: *espF* (LEE), *espG* (LEE), *espG2/orf3* (IE5), *nleF* (PP6), *nleE1* (IE6), *nleD* (PP4), *nleE2* (IE2) and *espJ* (PP2). PCR products were separated by agarose gel (1%) electrophoresis, stained with Gel Red and imaged by UV illumination. Size of PCR products are indicated.


A
WT EPEC Japan
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T⁰
< α Tir
B
WT EPEC Nal^R
WT EPEC Japan Nal^S
p-AKT Ser473 (Fold Change)
15
10
5
0
****
***
*
**
0 15 30 60 90 120
Time (min)
C
TOEA7
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T⁰
< α Tir

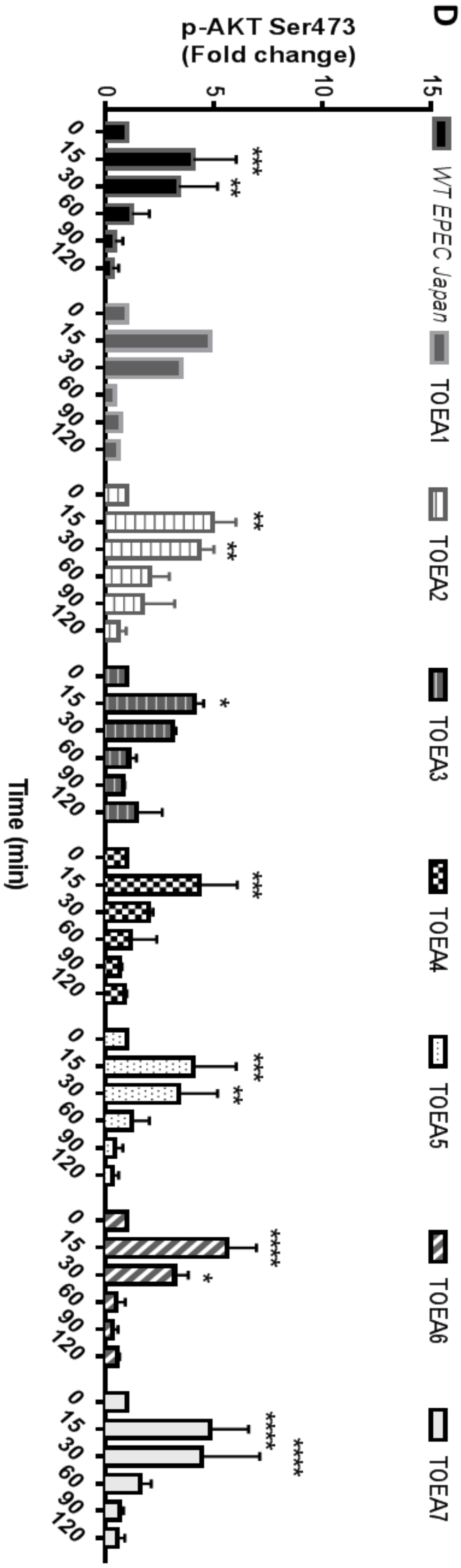

D
p-AKT Ser473
(Fold change)
0
5
10
15
WT EPEC Japan
TOEA1
TOEA2
TOEA3
TOEA4
TOEA5
TOEA6
TOEA7
0
15
30
60
90
120
Time (min)

Figure 22 The T3SS dependent decrease of phosphorylated AKT is independent of 14 Nle effectors and the LEE effector EspG.

(**A & C**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated WT EPEC Japan or its derivative strains (TOEA1-A7; lacking subsets or all 14 Nle effector proteins & LEE effector EspG). Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B & D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data is from 4 (WT EPEC Japan & TOEA7), 3 (TOEA6 & A4), 2 (TOEA3 & A2) and 1 (TOEA1) independent experiment, with values shown being the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC Nal^R infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05). Sidak's post-test for comparison of values at each corresponding time point did not identify any statistically significant differences (#p<0.05).

3.2.9 EPEC strains lacking the central 'core' LEE region display a defect in stimulating the rapid loss of phosphorylated AKT

The efficient translocation of most effector proteins (LEE & Nle) is dependent on the LEE encoded effector chaperones CesF and CesT (EspF, Tir, EspZ, EspH, Map, EspJ, NleG, NleH1, NleH2, NleB1, NleB2, NleC & NleA) (Mills *et al.*, 2013). To help identify the effector(s) responsible for the rapid loss of phosphorylated AKT, studies interrogated whether this subversive event requires the T3SS effector chaperones CesF and CesT. This was investigated using the available chaperone defective mutant, *Δcore::km* (lacks LEE region that encodes EspH, Map, Tir, Intimin, CesF & CesT; Figure 23). Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated EPEC *Δcore::km* strain at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

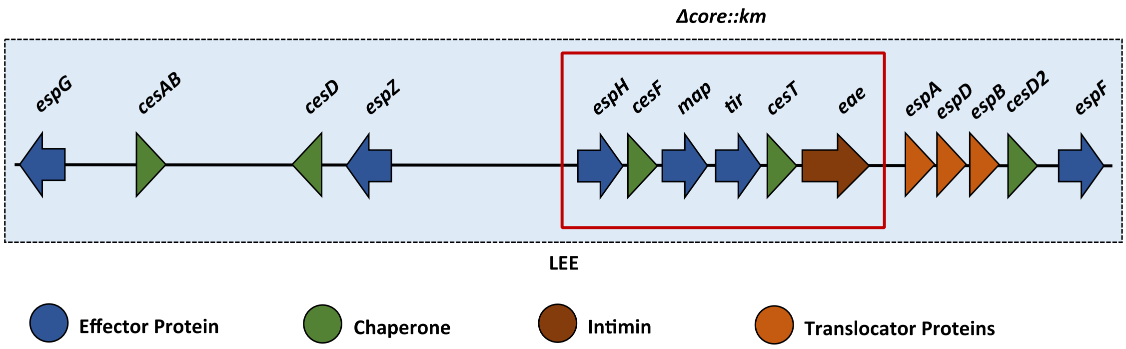


Figure 23 The EPEC *Δcore::km* mutant lacks the central core region of the LEE pathogenicity island.

Illustration of the LEE region encoding: (**1**) T3SS dependent effector proteins (Blue), (**2**) T3SS chaperones (Green), (**3**) translocator proteins (Orange) and (**4**) the outer membrane protein Intimin [*eae*] (Brown). The EPEC *Δcore:km* strain lacks the central 'core' region highlighted in a red box (replaced by the kanamycin antibiotic gene). This strain no longer has functional *espH*, *cesF*, *map*, *tir*, *cesT* and *eae* [Intimin] genes.

Infection with the *Δcore::km* mutant induced a robust increase in AKT phosphorylation at 15 min post infection (Figure 24A). This increase in p-AKT$_{ser473}$ signal was sustained above uninfected control cells before dramatically decreasing to background levels at the last (120 min) time point (Figure 24A); correlating with significant levels of cell detachment (data not shown). This prolonged increase in AKT phosphorylation was likened to that induced by the T3SS defective mutant, *cfm-14* (Figure 13A). The *Δcore::km* strain genotype was supported by the absence of Tir (T^0/T'') and the low levels of EspF in cell extracts (Figure 24A); the latter due to the absence of CesF chaperone (Elliott *et al.*, 2002). Quantification analysis confirms a significant increase in p-AKT$_{ser473}$ above the level of uninfected control at 15, 30, 60 and 90 min post infection before a dramatic decrease to background levels by 120 min post infection (Figure 24B). AKT phosphorylation induced by the *Δcore::km* mutant was significantly greater than that induced by the WT EPEC strain at 60 and 90 min post infection (Figure 24B). Similar results were obtained using an alternative *Δcore*-like strain, *gorf3Δcore::km* (lacks the LEE effector EspG, its Nle homologue EspG2/Orf3, as well as proteins encoded on the central core LEE region; see Figure 23) (Appendix Figure 3).

This work suggests that EPEC strains lacking the central 'core' LEE region are defective in their ability to induce a rapid loss of phosphorylated AKT; except for dramatic loss at the 120 min time point, linked to cell detachment. Having discounted a role for the outer membrane protein Intimin and three LEE effectors (EspH, Map & Tir; Figure 15 & Figure 16) lacking in the *Δcore::km* mutant, this work implicates a requirement for chaperones CesF and/or CesT.

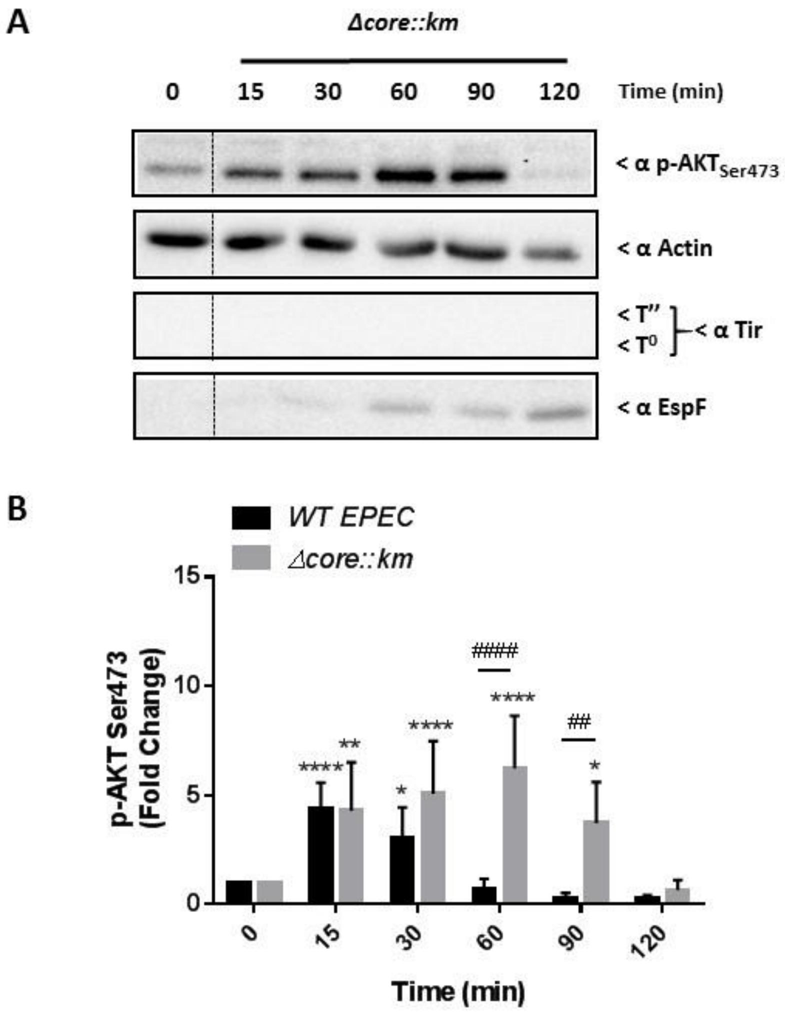

A
Δcore::km
0 15 30 60 90 120 Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T0
< α Tir
< α EspF
B
WT EPEC
Δcore::km
p-AKT Ser473 (Fold Change)
15
10
5
0
****
**
*
****
####
****
##
*
0 15 30 60 90 120
Time (min)

Figure 24 The EPEC *Δcore::km* strain displays a T3SS mutant-like phenotype, but for the dramatic loss of phosphorylated AKT at the final time point.

(**A**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated *Δcore:km* (lacking LEE genes *espH*, *cesF*, *map*, *tir*, *cesT* & *eae* [Intimin]) strain. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-EspF, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data is from a minimum of three independent experiments, with values shown being the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.2.10 EPEC strains unable to express or deliver EspZ induce increased host cell cytotoxicity

The LEE effector protein EspZ plays a critical role in maintaining cell viability by preventing host cell cytotoxicity, apoptosis and cell detachment (Shames *et al.*, 2011; Berger *et al.*, 2012; Roxas *et al.*, 2012). Translocation of the effector EspZ into host cells is dependent on the LEE encoded chaperone CesT (Mills *et al.*, 2013), which is absent form the *Δcore::km* strain. To support observations of significant cell detachment with the *Δcore::km* mutant and to help explain the dramatic loss of p-AKT$_{ser473}$ signal by 120 min post infection, cell viability was examined by the trypan blue exclusion assay (Strober, 2001). Thus, cells were left uninfected or infected with the WT EPEC, *cfm-14* (T3SS defective), or *Δcore::km* mutant (lacking genes of the central core LEE region) strains at an MOI of 200:1 for 2 h. Non-adherent bacteria were removed by washing cells before treating cells with trypan blue (0.2% in 1x PBS) and analysing the uptake of trypan blue by microscopy. Cells infected with the *Δcore::km* mutant revealed a significant reduction in cell density in comparison WT EPEC, *cfm-14* or uninfected cells (Figure 25). Moreover, comparison of strains reveals a notably greater number of cells permeable to the trypan blue following infection with the *Δcore::km* mutant than WT EPEC, *cfm-14* or uninfected cells (Figure 25). The latter findings suggest that a considerable number of cells infected with the *Δcore::km* mutant for 2 h are non-viable. Thus, the rapid decrease of p-AKT$_{ser473}$ after 2 h after of infection with the *Δcore::km* mutant may be due to a loss of cell viability.

The loss of cell viability and high levels of cell detachment with the *Δcore::km* mutant was hypothesised to be caused by increased host cell cytotoxicity, due to the absence of chaperone CesT; thus preventing delivery of EspZ into host cells (Mills *et al.*, 2013). To support the requirement of T3SS delivered EspZ to maintain host cell viability, J774A.1 macrophages were infected with pre-activated WT EPEC, *cfm-14* (T3SS defective), *Δcore::km* (lacks genes of the central core LEE region) or the *ΔespZ* (lacks a functional *espZ* gene) mutant at an MOI of 200:1. Cell culture supernatant was then sampled over a 120 min infection period for the release of cytosolic LDH (marker of cell toxicity) (Fotakis and Timbrell, 2006).

As reported (Shames *et al.*, 2010), infection with the *cfm-14* mutant induced minimal host cell cytotoxicity, with <10% ($p<0.05$) of LDH released into the cell culture media at 120 min post infection (Figure 26). In contrast, cells infected with the WT EPEC strain displayed a significant time dependent increase in LDH release at 60, 90 and 120 min post infection, with a maximal release of 27% (± 5%) at the final (120 min) time point (Figure 26). This increase in LDH release was significantly greater than that induced with the *cfm-14* mutant after 90 or 120 min of infection (Figure 26). Similar results were obtained with the *Δcore::km* and *ΔespZ* mutant, leading to a maximum release of 33% (± 9%) and 42% (± 9%) of total LDH respectively at 120 min post infection (Figure 26). Importantly, LDH release by the *Δcore::km* (90 & 120 min post infection) and *ΔespZ* (120 min post infection) mutant was significantly greater than that induced by the WT EPEC strain at corresponding times post infection (Figure 26).

This work suggests that increased cell detachment and loss of cell viability identified in cells infected with the *Δcore::km* mutant is linked to the loss of CesT chaperone dependent translocation of the effector EspZ. This may have important implications for the rapid loss of p-AKT_{ser473} signal at 120 min post infection.

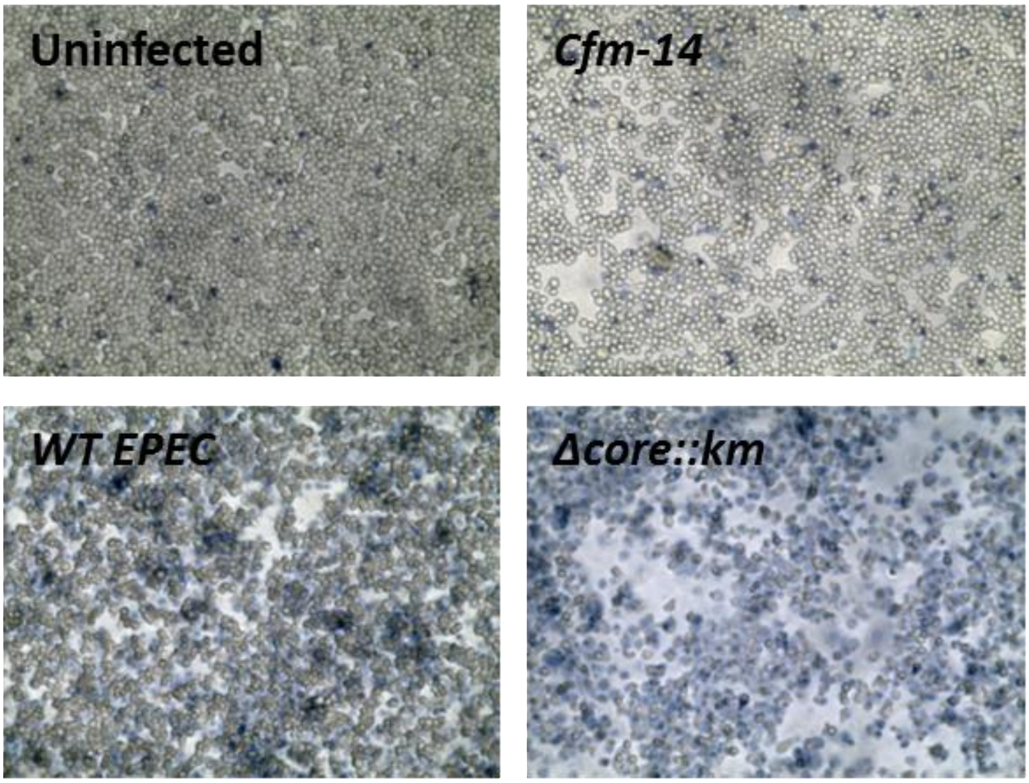


Figure 25 The EPEC *Δcore::km* strain induces increased cell detachment and permeability to trypan blue.

J774A.1 macrophages were left uninfected or infected (MOI 200:1) for 2 h with pre-activated WT EPEC, *cfm-14* (non-functional T3SS) or *Δcore::km* (lacking LEE genes *espH*, *cesF*, *map*, *tir*, *cesT* & *eae* [Intimin]) strains. Cell viability was assessed post infection by trypan blue exclusion assay, with uptake of dye indicating a damaged/permeable cell membrane. Images are representative of two independent experiments taken by light microscope at 10x the objective.

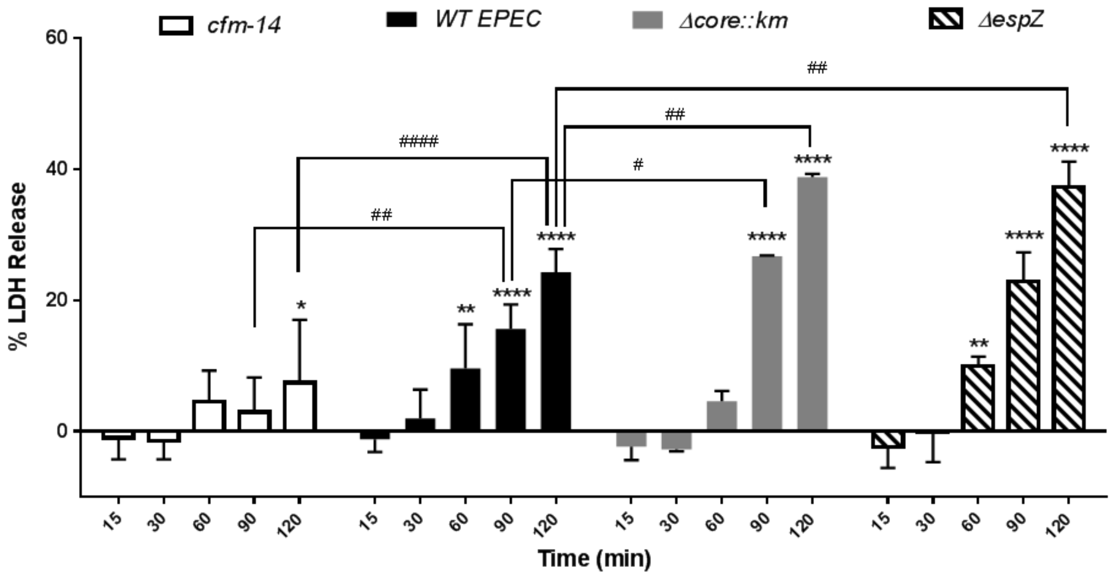


Figure 26 EPEC *Δcore::km* and *ΔespZ* mutants induce significantly greater host cell toxicity than WT EPEC strain.

J774A.1 macrophages were infected (MOI 200:1) with pre-activated WT EPEC, *cfm-14* (non-functional T3SS), *Δcore::km* (lacking LEE genes *espH*, *cesF*, *map*, *tir*, *cesT* & *eae* [Intimin]) or *ΔespZ* (lacking LEE effector EspZ) strains. Cell supernatants were isolated at 15, 30, 60, 90 and 120 min post infection and LDH release quantified (see Materials and Methods). Values shown are the mean ± SD from a minimum of three independent experiments. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against values at 15 min post infection (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001. ##p<0.01 and #p<0.05).

3.2.11 EPEC induced loss of phosphorylated AKT requires the LEE encoded CesT chaperone and/or effector Tir

To identify the gene products whose absence from the *Δcore::km* mutant compromised the strains ability to rapidly decrease phosphorylated AKT, complementation studies were undertaken using the available constructs *pHcFM* (encoding genes *espH, cesF* & *map*) and *pTcT* (encoding genes *tir* & *cesT*). Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated EPEC strains *Δcore::km/pHcFM, Δcore::km/pTcT* or *Δcore::km/pHcFM + pTcT* at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

Infection with the *Δcore::km/pHcFM* strain induced a *Δcore::km*-like p-AKT$_{ser473}$ profile (Figure 27A & Figure 24A), with a significant increase of p-AKT$_{ser473}$ signal detected at 15, 30 and 60 min post infection, before a dramatic decrease to background levels at the final (120 min) time point (Figure 27A & Figure 27D). In contrast, infection with both the *Δcore::km/pTcT* and *Δcore::km/pHcFM+pTcT* strains induced a transient increase in p-AKT$_{ser473}$ levels (Figure 27B & Figure 27C), similar to that of the WT EPEC strain (Figure 13B). Both the *Δcore::km/pTcT* and *Δcore::km/pHcFM+pTcT* strains stimulated the increase of p-AKT$_{ser473}$ signal above the level of uninfected control at 15 and 30 min post infection before decreasing rapidly to background levels thereafter (Figure 27B-D). Surprisingly, this infection induced increase of p-AKT$_{ser473}$ levels by the *Δcore::km/pHcFM+pTcT* strain was not found to be statistically significant (Figure 27D). Comparison of data against the WT EPEC strain revealed significantly greater levels of p-AKT$_{ser473}$ with the *Δcore::km/pHcFM* (60 min post infection) and *Δcore::km* mutant (60 & 90 min post infection) only (Figure 27D). Conversely, comparison against the *Δcore::km* mutant reveals significantly lower levels of p-AKT$_{ser473}$ at 60 min post infection with the *Δcore::km/pTcT* and *Δcore::km/pHcFM+pTcT* strains.

Strain complementation was supported by the presence of the *pHcFM* (encoding genes *espH, cesF* & *map*) and *pTcT* (encoding genes *tir* & *cesT*) plasmids being associated with greater levels of EspF (provides the EspF chaperone, CesF) and Tir (provides the Tir chaperone, CesT) respectively (Figure 27A-C). Detection of the host modified form of Tir (T'') with the *Δcore::km/pTcT* and *Δcore::km/pHcFM+pTcT* strains was correlated with decreasing p-AKT$_{ser473}$ signal at approximately 60 min post infection respectively (Figure 27B & Figure

27C). Significant cell detachment was evident for cells infected with the *Δcore::km* and *Δcore::km/pHcFM*, but not the *Δcore::km/pTcT* and *Δcore::km/pHcFM + pTcT*, strains over the 120 min infection period (data not shown). This supports the premise that the cell detachment phenotype is associated with the absence of the CesT chaperone, necessary for the translocation of EspZ into host cells.

This work suggests reintroducing genes for CesT chaperone and/or the effector Tir restores the capacity of the *Δcore::km* strain to induce the rapid loss of p-AKT_{ser473} signal and reduce significant cell detachment during infection. This suggests the effector(s) responsible for the rapid loss of p-AKT_{ser473} signal is/are likely to be CesT chaperone dependent.

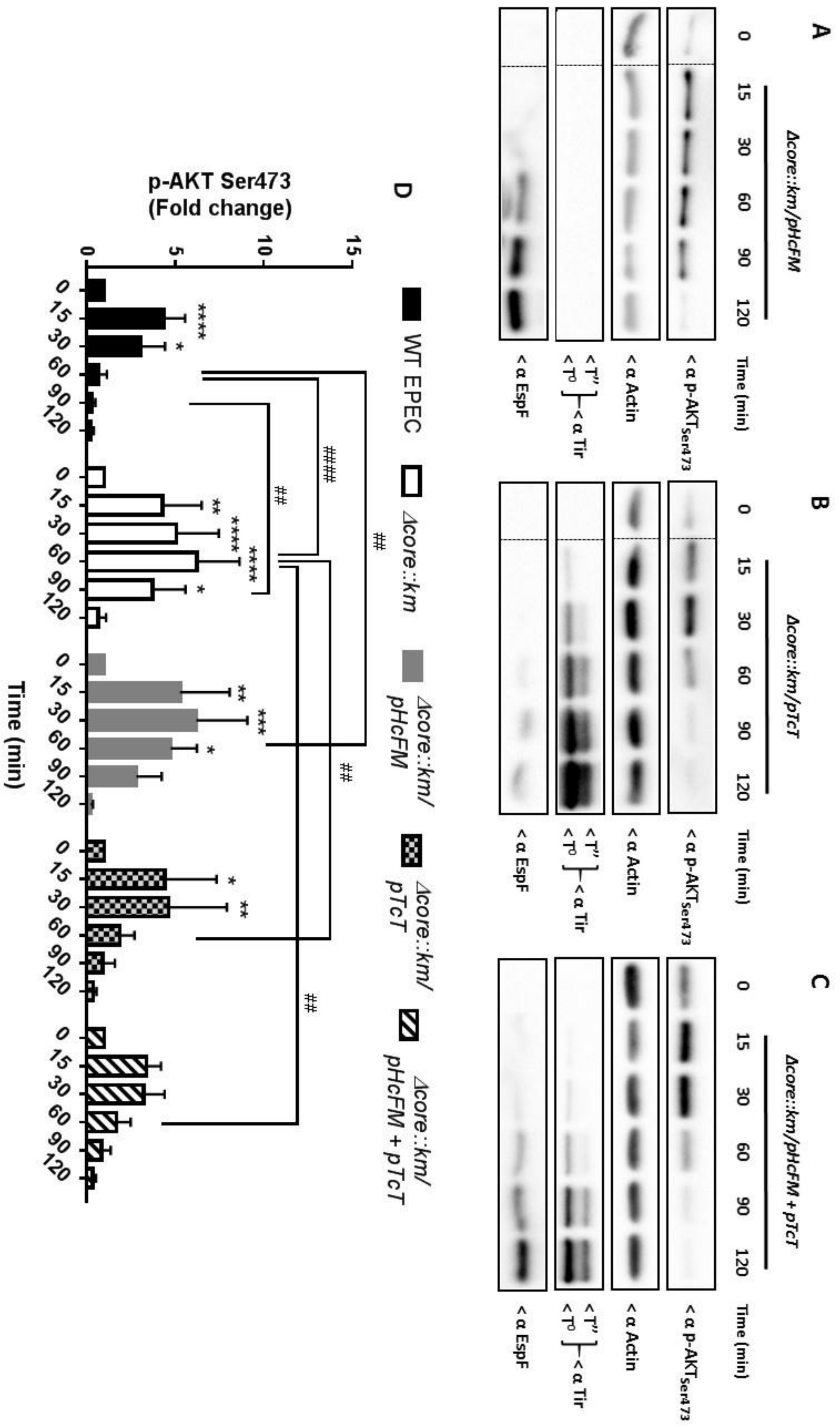

A
Δcore::km/pHcFM
B
Δcore::km/pTcT
C
Δcore::km/pHcFM + pTcT
Time (min)
0 15 30 60 90 120
< α p-AKT$_{Ser473}$
< α Actin
< T'' < T^{0} < α Tir
< α EspF
D
p-AKT Ser473 (Fold change)
WT EPEC
Δcore::km
Δcore::km/pHcFM
Δcore::km/pTcT
Δcore::km/pHcFM + pTcT
Time (min)

Figure 27 Re-introducing LEE genes *tir* and *cesT* restores the capacity of the *Δcore::km* mutant to rapidly decrease phosphorylated AKT.

(**A-C**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated EPEC *Δcore::km* strain carrying plasmids *pHcFM* (encoding LEE genes *espH*, *cesF* & *map*) and/or *pTcT* (encoding LEE genes *tir* & *cesT*). Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-EspF, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentational purposes, indicated by a dashed line. (**D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data is from a minimum of three independent experiments, with values shown being the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC and *Δcore::km* infected cell extracts are taken from Figure 13 and Figure 24 respectively. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.2.12 EPEC strain lacking the CesT chaperone induces a 'core' mutant-like sustained increase of phosphorylated AKT

To support this CesT dependent decrease of phosphorylated AKT, studies examined the impact of EPEC strains lacking only the CesT (*ΔcesT*) or CesF (*ΔcesF*) chaperones. Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated WT EPEC, *cfm-14*, *ΔcesF* or *ΔcesT* strains at an MOI of 200:1 before isolating total cell extracts after 15, 30, 45, 60 and 90 min of infection and processed for western blot analysis (see Materials and Methods). The infection time course was reduced from 120 min to limit complications of significant cell detachment identified with strains lacking the CesT chaperone (Figure 25 & Figure 26).

As expected, infection with the *cfm-14* mutant induced a sustained increase in p-AKT$_{ser473}$ levels over the 90 min infection period; contrasting with that of the transient p-AKT$_{ser473}$ profile triggered by the WT EPEC strain (Figure 28A, B & E). This transient increase of p-AKT$_{ser473}$ signal was also evident for the *ΔcesF*, but not *ΔcesT*, strain (Figure 28C & D). The latter illustrating a more sustained increase of p-AKT$_{ser473}$ signal before dramatically decreasing below background levels at 90 min post infection (Figure 28D & E) linked to increased host cell detachment (data not shown). Again, this cell detachment was associated with the absence of CesT chaperone dependent delivery of the effector EspZ into host cells. Crucially, these results were supported by quantification analysis indicating a transient (WT EPEC & *ΔcesF*) and sustained (*cfm-14* & *ΔcesT*) increase of phosphorylated AKT respectively (Figure 28E). However, it should be noted this increase of p-AKT$_{ser473}$ signal stimulated by the *ΔcesF* strain was not found to be significantly greater than uninfected controls throughout the infection period. Furthermore, this work was unable to identify any statistically significant differences in the levels of phosphorylated AKT induced by *ΔcesF* or *ΔcesT* strains, or when compared with WT EPEC. Intriguingly, the p-AKT$_{ser473}$ profile induced by the *ΔcesT* strain was similar to that induced by *Δcore::km* mutant (Figure 24). EPEC strain genotype was supported by the absence (*cfm-14* & *ΔcesT*) or presence (WT EPEC & *ΔcesF*) of the host modified form of Tir (T''), in addition to the strong (*cfm-14,* WT EPEC & *ΔcesT*) and weak (*ΔcesF*) detection of the effector EspF in respective cell extracts (Figure 28A-E). These findings support the requirement for an unknown CesT dependent effector to induce the rapid T3SS dependent decrease of phosphorylated AKT.

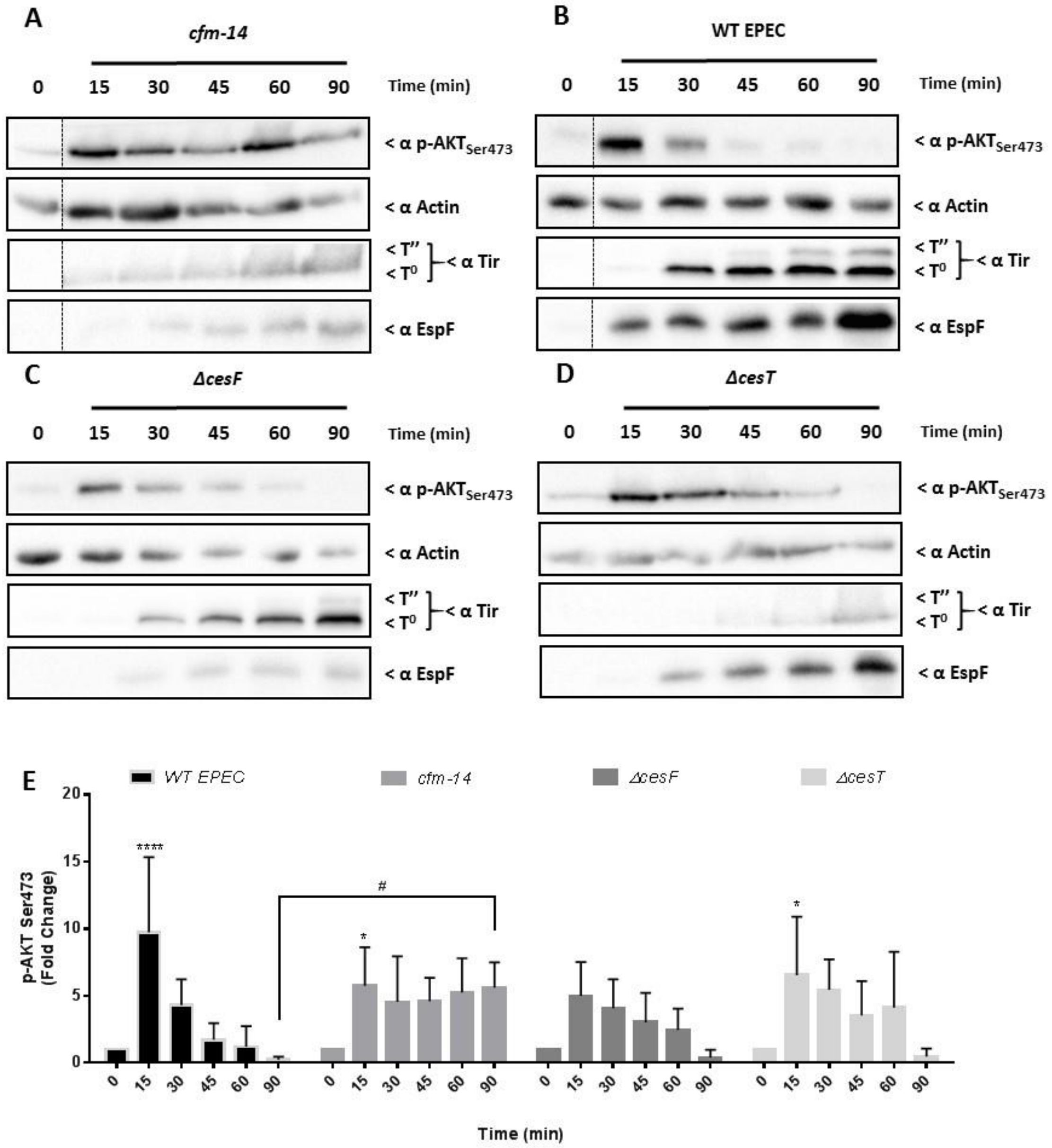

A
cfm-14
0 15 30 45 60 90 Time (min)
< α p-AKTSer473
< α Actin
< T''
< T0
< α Tir
< α EspF
B
WT EPEC
0 15 30 45 60 90 Time (min)
< α p-AKTSer473
< α Actin
< T''
< T0
< α Tir
< α EspF
C
ΔcesF
0 15 30 45 60 90 Time (min)
< α p-AKTSer473
< α Actin
< T''
< T0
< α Tir
< α EspF
D
ΔcesT
0 15 30 45 60 90 Time (min)
< α p-AKTSer473
< α Actin
< T''
< T0
< α Tir
< α EspF
E
WT EPEC
cfm-14
ΔcesF
ΔcesT
p-AKT Ser473 (Fold Change)
20
15
10
5
0
****
#
*
*
0 15 30 45 60 90
Time (min)

Figure 28 EPEC strains lacking the CesF, but not CesT, chaperone induce a WT EPEC-like decrease of phosphorylated AKT.

(**A-D**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated WT EPEC, T3SS mutant (*cfm-14*), *ΔcesF* (lacking LEE chaperone CesF) or *ΔcesT* (lacking LEE chaperone CesT) strains. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 45, 60 and 90 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-EspF, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**E**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data is from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****$p<0.0001$ and *$p<0.05$) or by Sidak's post-test for comparison of values at each corresponding time point (#$p<0.05$).

3.2.13 The EPEC TOEA7*Δcore::km* mutant does not induce a rapid loss of phosphorylated AKT

The recently generated TOEA7*Δcore::km* mutant was the most complex EPEC mutant available (lacks 14 Nle effectors [Table 7] and the central 'core' LEE genes [Figure 23]); defective in the efficient delivery of up to 20 of the 24 known T3SS dependent effector proteins. This strain was examined with the expectation of a defect in the loss of phosphorylated AKT, similar to the *Δcore::km* mutant. Thus J774A.1 macrophages were infected with the pre-activated EPEC TOEA7*Δcore::km* strain at an MOI of 200:1 before isolating total protein extracts over a 120 min infection period and processed for western blot analysis (see Materials and Methods).

Infection with the TOEA7*Δcore::km* mutant induced a robust and sustained increase in AKT phosphorylation (Figure 29A). This increase of p-AKT_{ser473} levels was significantly greater than uninfected control throughout the 120 min infection period (Figure 29B), similar to that of the T3SS mutant, *cfm-14* (Figure 13A). Furthermore, the levels of p-AKT_{ser473} stimulated by the TOEA7*Δcore::km* mutant was significantly greater than corresponding values triggered by the WT EPEC strain at 15, 30, 60, 90 and 120 min post infection (Figure 29B). In contrast to the *Δcore::km* mutant (Figure 24), no dramatic decrease of p-AKT_{ser473} levels was evident by 120 min post infection (Figure 29A). Furthermore, no significant cell detachment was observed over the 120 min infection period (data not shown); linked to loss of CesT chaperone dependent delivery of EspZ into target cells. Strain genotype was supported by the absence of Tir (T^0/T'') and only weak detection of EspF in cell extracts infected with the TOEA7*Δcore::km* mutant (Figure 29A).

This work supports the requirement for the LEE encoded chaperone CesT to trigger the rapid loss of phosphorylated AKT. Furthermore, these findings suggest that the cell detachment identified with strains lacking CesT and/or EspZ requires one or more of the 14 Nle effectors lacking in the TOEA7*Δcore::km* mutant.

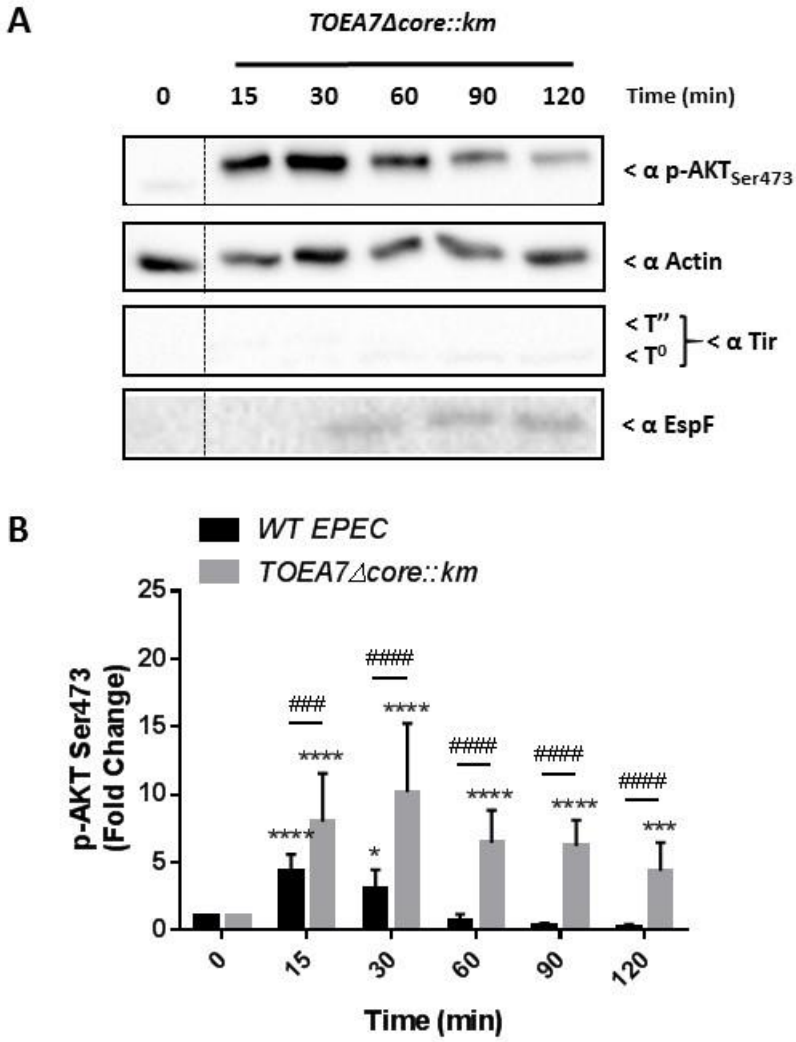

A
TOEA7Δcore::km
0 15 30 60 90 120 Time (min)
< α p-AKTSer473
< α Actin
< T''
< T0
< α Tir
< α EspF
B
WT EPEC
TOEA7Δcore::km
p-AKT Ser473 (Fold Change)
Time (min)

Figure 29 The TOEA7*Δcore::km* mutant displays a T3SS mutant-like (*cfm-14*) p-AKT$_{ser473}$ profile.

(**A**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated TOEA7*Δcore:km* (lacking 14 Nle effectors [EspG2, NleA, NleB1, NleB2, NleC, NleD, NleE1, NleE2, NleF, NleG, NleH1, NleH2, EspJ & EspL], LEE effector EspG & proteins of the central core LEE region [EspH, Map Tir, CesF, CesT & Intimin]) strain. Total cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis probing for anti-p-AKT$_{ser473}$, anti-EspF, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data is from a minimum of three independent experiments, with values shown being the mean ± SD. Values for p-AKT$_{ser473}$ levels from WT EPEC infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

3.3 Discussion

EPEC inhibition of PI3K mediated phagocytosis was previously correlated with the rapid loss of infection induced phosphorylated AKT in J774A.1 macrophages (Celli *et al.*, 2001). This relationship has been uncoupled by the discovery of EspF deficient EPEC strains unable to inhibit PI3K dependent phagocytosis, yet inducing an EPEC-like rapid decline of phosphorylated AKT (Quitard *et al.*, 2006). This study aimed to identify the T3SS dependent effector(s) responsible for EPEC to trigger the rapid decrease of phosphorylated AKT (Ser473). Screening of bacterial strains lacking or expressing subsets of EPEC effector proteins suggests that EPEC induced T3SS dependent decrease of phosphorylated AKT (Ser473) is independent of 7 LEE and 14 examined Nle effectors; implicating roles for other Nle effectors EspC (Guignot *et al.*, 2015), NleJ, LifA (Deng *et al.*, 2012), six putative effectors (experimentally supported effectors; Dean *et al.*, unpublished) and/or other effectors not yet identified. Furthermore, this study suggests the effector(s) responsible for the rapid decline of phosphorylated AKT is dependent on the effector chaperone CesT.

The initial aim of this chapter was to re-establish a robust J774A.1 macrophage cell model to study the impact of EPEC infection on AKT activity. Using this model, this study confirmed an EPEC induced T3SS dependent transient increase of phosphorylated AKT (Ser473) over an extended 120 min infection period; consistent with the literature over a 90 min infection time course (Celli *et al.*, 2001; Quitard *et al.*, 2006). The quantification of p-AKT$_{ser473}$ levels by densitometry analysis of western blots confirmed the reproducibility of these results for the first time. This analysis supported the rapid T3SS dependent decrease of infection-induced AKT phosphorylation (Ser473) to background levels after 60 min of infection with the WT EPEC strain, but not T3SS mutant (*cfm-14*). This defect in capacity to trigger a rapid decrease of phosphorylated AKT was also evident with strains lacking either of the three translocator proteins, EspA, EspB and EspD, which are critical for effector translocation into target cells. Thus, this work confirmed the previously assumed roles for translocator proteins to induce the rapid T3SS dependent decrease of p-AKT$_{ser473}$ signal, implicating the requirement for one or more T3SS/Esp translocon-dependent EPEC effector proteins.

In this study, the effector protein Tir was used as a marker for cell adherence in total cell extracts, with detection of its host modified form (T'') supporting a functional T3SS (Kenny *et al.*, 1997). Indeed, Tir is the first and most abundantly delivered effector into target cells (Mills *et al.*, 2008; Mills *et al.*, 2013). Interestingly, detection of the host-modified form of Tir

(T'') was correlated with the rapid T3SS dependent decrease of p-AKT_{ser473} signal in this model at 60 min post infection with the WT EPEC strain. Since the requirement of Tir to trigger a rapid decrease of p-AKT_{ser473} signal has previously been discounted (Celli *et al.*, 2001; Quitard *et al.*, 2006), the decline of phosphorylated AKT was therefore linked to the T3SS translocation of other effectors into host cells.

The mechanism of EPEC induced AKT phosphorylation in this macrophage model has not previously been examined, though this presumably requires the engagement of cell surface receptors, such as toll-like receptors (TLR). Whilst this mechanism was not investigated, it is interesting to note that infection induced AKT phosphorylation was evident before detection of the effector Tir (T^0/T'') in WT EPEC infected cell extracts, at 15 and 60 min post infection respectively. This suggests that EPEC induced AKT phosphorylation in this model is triggered by either: 1) a transient/early interaction of EPEC with macrophages (too few bacteria bound to detect with sensitive anti-Tir antibodies), likely involving cell surface proteins such as BFP and/or flagella; or 2) a diffusible factor such as LPS, a known stimulant of AKT phosphorylation in macrophages (McGuire *et al.*, 2013). This infection induced increase of p-AKT_{ser473} is consistent with previous reports, indicating that EPEC cell contact and not cell uptake is responsible for PI3K dependent AKT phosphorylation (Celli *et al.*, 2001). Future experiments could look to identify the responsible factor(s) by the treatment of cells with sterile EPEC culture supernatant, purified BFP, flagella and/or LPS.

To investigate the role of LEE-encoded effectors in the rapid loss of phosphorylated AKT, studies re-examined a *Δquad* mutant (lacks 3 LEE effectors [Map, EspF & Tir] & Intimin) and a more recently generated complex mutant *Δmfzgorf3eh::km* (lacks all LEE effectors except for Tir [marker of T3SS functionality] & EspB [critical for effector delivery]). Consistent with studies by Quitard *et al.,* using the *Δquad* strain (Quitard *et al.*, 2006), this study confirmed the infection induced transient increase of phosphorylated AKT, revealing the loss of p-AKT_{ser473} signal by 60 min post infection. Furthermore, this rapid decline of p-AKT_{ser473} signal was also triggered by infection with the *Δmfzgorf3eh::km* mutant. Collectively, this work discounted a role (individual or cooperative) for all LEE encoded effectors, except for EspB individually or in cooperation with Tir.

EspB is the only translocator protein with known effector function (Iizumi *et al.*, 2007); however, distinguishing this activity from its critical role in effector protein translocation is not possible using a gene deletion strategy. To interrogate the requirement for EspB effector activity (plus other LEE encoded factors), this study took an indirect approach to examine the ability of the LEE carrying *E. coli* K12 strain (TOB02/pLEE + pBFP_Per) to trigger the rapid decrease of infection induced phosphorylated AKT. Importantly, this strain was found to encode the necessary information to enable *E. coli* K12 to inhibit its uptake by macrophages; a rapid T3SS dependent phenotype linked to the activity of LEE effectors EspF (Quitard *et al.*, 2006), EspH (Dong *et al.*, 2010) and EspB (Iizumi *et al.*, 2007). This finding demonstrates, for the first time, the ability of the LEE region to inhibit phagocytosis in a manner linked to the inhibition of PI3K activity (Celli *et al.*, 2001); an event upstream of AKT phosphorylation. However, whether this inhibiton of EPEC phagocytosis is indeed LEE sufficient, or dependent on the presence of the plasmid encoding BFP and Per regulatory genes (pBFP_Per) cannot be confirmed here. Resolving this question would require additional work, preferably using a TOB02 strain without plasmid encoding BFP and Per. Nevertheless, this work thus expands the number of known LEE dependent subversive events (McDaniel and Kaper, 1997; Simonovic *et al.*, 2000).

Analysing the infection with TOB02 (K12/pLEE + pBFP_Per) strain revealed the host modified form of Tir (T’’) at 90 min post infection; however, unlike the WT EPEC strain, this was not correlated with a dramatic loss of p-AKT$_{ser473}$ signal. Whilst a transient p-AKT$_{ser473}$ profile was not evident with the TOB02 (K12/pLEE + pBFP_Per) strain over the 120 min infection period, the levels of p-AKT$_{ser473}$ were notably weaker in comparison to the LEE negative controls TOB01 (K12/pBFP_Per) and DH10B (K12). This suggested two possibilities: 1) TOB02 is a poor stimulant of AKT phosphorylation, caused by poor cell binding (linked to weak detection of Tir [T^0/T’’]) or reduced levels of p-AKT$_{ser473}$ stimulant (e.g. BFP); or 2) TOB02 has a weak capacity to reduce p-AKT$_{ser473}$ levels, implicating the need for other key factors encoded outside the LEE region (e.g. Nle effector proteins) to induce the rapid decline of phosphorylated AKT. Intriguingly, the greater levels of phosphorylated AKT with the TOB01 (K12/pBFP_Per) than DH10B (K12) strain at 90 and 120 min post infection, supports the requirement for BFP to sustain the infection induced increase of p-AKT$_{ser473}$ signal. However, it should be noted that TOB0 strains were created using an *E. coli* K12 derivative of DH10B, known as EPI300 (unavailable at the time of experiment) (Yen *et al.*, 2010). Further work is therefore required to confirm the p-AKT$_{ser473}$ profile with this plasmid free TOB0 strain (cured of pBFP_Per plasmid) to ensure there is no notable difference in ability to stimulate/sustain AKT phosphorylation.

The ability of the LEE region to induce the rapid decrease of p-AKT_{ser473} signal was further interrogated in this study through the development of a two-wave infection model; an approach previously used to illustrate the capacity of EPEC to inhibit innate immune signalling (Ruchaud-Sparagano *et al.*, 2007). This strategy illustrated, for the first time, the ability of EPEC to prevent AKT phosphorylation stimulated by second-wave infection with T3SS mutant (*cfm-14*). Importantly, this inhibition of AKT phosphorylation was dependent on a functional T3SS and was stable for at least 3 h (time between 1^{st} & 2^{nd} wave infections). Crucially, this capacity to inhibit AKT phosphorylation was not identified in cells pre-infected with the DH10B (K12), TOB01 (K12/pBFP_Per) or TOB02 (K12/pLEE + pBFP_Per) strain. However, it is interesting to note the levels of p-AKT_{ser473} stimulated in cells pre-infected with the TOB02 (K12/pLEE + pBFP_Per) strain were again comparatively reduced. These results support the premise of a weak capacity of LEE region to prevent/reduce AKT phosphorylation. Though, as this work is from a single experiment, further work is required to confirm these observations and support the reproducibility of these results.

It is important to note that the LEE, BFP and Per loci carried by the TOB0 strains were cloned from the alternative prototypic EPEC strain, B171-8 (Yen *et al.*, 2010). Whilst there exists some divergence between the LEE effectors of EPEC B171-8 and our EPEC E2348/69 strain (e.g. 66% Tir, 66% EspB & 77% EspF protein identity), both LEE regions have demonstrated the capacity to translocate effector proteins (EspB dependent), induce intimate cell attachment (Tir dependent) (McDaniel and Kaper, 1997; Simonovic *et al.*, 2000; Yen *et al.*, 2010) and inhibit phagocytosis (EspF, EspB & EspH dependent; this study); thus supporting the comparative function of both sets of LEE effectors. Nevertheless, whether this EPEC B171-8 strain has the capacity to induce a rapid loss of phosphorylated AKT has not previously been examined. Additional work is therefore required to determine the ability of the EPEC B171-8 strain and/or whether the sufficiency of the EPEC E2348/69 LEE region expressed in an *E. coli* K12 bacteria can prevent/reduce infection induced AKT phosphorylation.

The examination of EPEC strains lacking most LEE effectors and the LEE carrying TOB02 (K12/pLEE + pBFP_Per) strain, had argued the need for additional factors encoded outside the LEE region to trigger the dramatic loss of p-AKT_{ser473} signal. In addition to the LEE region, the effector repertoire of EPEC includes up to 17 known Nle effector proteins (E2348/69). The requirement for 14 of the most studied Nle effectors was interrogated in this study using the available strains TOEA1-A7. Created from a Nal^S EPEC background (WT EPEC Japan), assessment of this parental strain revealed the near identical loss p-AKT_{ser473} signal as our WT EPEC Nal^R strain over the 120 min infection period. This decrease was characterised by the rapid decline of p-AKT_{ser473} signal to background levels by 60 min post infection. Investigations with the complex Nle effector deficient strains (TOEA1-A7) supported this transient increase of phosphorylated AKT, with no obvious defect in capacity to decrease the p-AKT_{ser473} signal. Crucially, this work discounted a key role (individual or cooperative) for the 14 examined Nle effectors (NleA, NleB1, NleB2, NleC, NleD, NleE1, NleE2, NleF, NleG NleH1, NleH2, EspJ, EspL & EspG2) and the LEE effector EspG. This implied that other T3SS dependent substrates, such as EspC (Guignot *et al.*, 2015), LifA, NleJ (Deng *et al.*, 2012), 6 experimentally supported effectors (Dean *et al.,* unpublished) and/or other effectors yet to be identified are required to trigger the rapid decline of phosphorylated AKT.

The LEE encoded chaperones CesF and CesT are critical for the efficient translocation of up to 13 effector proteins (5 LEE & 8 Nle); with EspG, NleD and NleF the only known effectors translocated independently of both chaperones (Mills *et al.*, 2013). To gain insight into the T3SS dependent factors responsible for inducing the rapid loss of phosphorylated AKT, an alternative approach was taken to examine the requirement for LEE encoded effector chaperones, CesF and CesT. This was initially investigated using the available, complex double chaperone mutant *Δcore::km* (lacks 3 LEE effectors: EspH, Map & Tir; 2 chaperones: CesF & CesT; and Intimin), revealing a T3SS mutant-like increase of p-AKT_{ser473} levels, except for a dramatic loss of p-AKT_{ser473} signal at 120 min post infection. This loss of p-AKT_{ser473} signal was associated with increased cell detachment, linked to the absence of the effector EspZ translocation into target cells (delivery is CesT dependent) (Shames *et al.*, 2010; Berger *et al.*, 2012; Roxas *et al.*, 2012). This premise was supported by the significantly greater levels of LDH release (marker of cell cytotoxicity) at 90 and 120 min post infection with *Δcore::km* and *ΔespZ* (lacks EspZ) mutant, than WT EPEC and *cfm-14* strains. These results are consistent with previous reports (Shames *et al.*, 2010; Berger *et al.*, 2012). Importantly, re-introduction of genes encoding CesT and Tir, but not EspH, Map and CesF, on a plasmid restored the capacity of the *Δcore::km* mutant to trigger the rapid decline of p-AKT_{ser473} signal at 60 min post infection, similar to the WT EPEC strain. Furthermore, this had the added effect of preventing the significant increase of cell detachment identified at latter time points with the *Δcore::km* mutant when carrying plasmid encoding CesT and Tir (data not shown). Having previously discounted a role for Tir and other LEE encoded CesT dependent effectors (EspZ, EspH & Map); this work suggested a key role for CesT chaperone to mediate the rapid decline of phosphorylated AKT. Thus, this suggests the effector(s) responsible is/are likely to be a CesT dependent Nle effector other than the 14 previously discounted. Indeed, the finding that infection with EPEC strains lacking the CesF, but not CesT, chaperone led to the WT EPEC-like decrease of phosphorylated AKT supported this assumption.

Interestingly, studies using the recently generated complex effector mutant TOEA7*Δcore::km* (lacks all known Nle effectors except EspC, LifA & NleJ, in addition to being theoretically unable to express or deliver all LEE effectors, except EspB), revealed a T3SS mutant-like (*cfm-14*) sustained increase of phosphorylated AKT throughout the 120 min infection period. Importantly, the absence of a 'core' mutant-like (*Δcore::km* & *gorf3Δcore::km*) dramatic loss of p-AKT_{ser473} signal at 120 min post infection was associated with the lack of significant cell detachment with this complex mutant (data not shown). This suggests that increased cell detachment/cytotoxicity identified with the *Δcore::km* mutant is dependent on one or more of the 14 Nle effector proteins absent from the TOEA7*Δcore::km* strain. This may include one of the several Nle effector proteins with pro-apoptotic activities, such as NleD (Baruch *et al.*, 2011), NleB (Pearson *et al.*, 2013), NleH (Hemrajani *et al.*, 2010) and NleF (Blasche *et al.*, 2013). Deletion of the central core LEE region from the TOEA1-A6 strains should enable identification of the responsible effector(s) to mediate cell detachment and how/if they promote cell cytotoxicity associated loss of p-AKT_{ser473} signal. Crucially, this work supports requirement for the CesT chaperone to trigger the rapid decline of phosphorylated AKT. However, this would require confirmation by the re-introduction of genes (CesT; CesT & Tir; and/or the core LEE region) on a plasmid to determine the capacity to restore the rapid loss of p-AKT_{ser473} signal, similar to WT EPEC strain.

As a consequence of increased host cell detachment triggered by EPEC strains lacking the CesT chaperone, there was a greatly reduced population of cells at the end of the experiment available for protein extraction and subsequent analysis. Whilst it is assumed all cells are infected equally (MOI 200:1) in this assay, it is possible that those cells remaining after infection with CesT deficient EPEC strains are not representative of the whole cell population, due to low and/or no evidence of infection. To corroborate our findings of a T3SS and CesT dependent decrease of p-AKT_{ser473} signal, additional work could thus look to examine the level of phosphorylated AKT in both the detached and adherent cells post infection by western blot analysis. Furthermore, antibody labelling of EPEC on remaining cells post-infection could support or not whether these cells are indeed infected and to what level. Alternatively, single cell analysis of those cells remaining could provide a more accurate interpretation of the level of AKT phosphorylation in those cells that are left post infection.

In summary, this study has demonstrated the development of a reproducible and quantifiable model to examine the impact of EPEC infection on AKT phosphorylation. This work supports the literature of a T3SS dependent decrease of phosphorylated AKT and extends these observations to discount a key role for all known LEE and the 14 examined Nle effectors. Importantly, whilst the LEE region was shown for the first time to encode the necessary factors to inhibit PI3K mediated phagocytosis, it does not contain the necessary factors to trigger the rapid loss of p-AKT$_{ser473}$ signal; though it may have some capacity to prevent/reduce infection induced AKT phosphorylation. Furthermore, this study provides evidence to suggest the T3SS dependent loss of phosphorylated AKT requires a functional CesT chaperone, implicating roles for an unknown CesT dependent effector(s). These may include effectors EspC, LifA, NleJ, 6 putative effectors (identified T3SS substrates by bioinformatic analysis; Dean *et al*., unpublished) and/or other unknown Nle effectors. Though, whether there exists cooperative activity between LEE and Nle effectors to reduce phosphorylated AKT cannot be discounted here. This would indicate a need to examine strains lacking all LEE (except translocation critical EspB) and all 14 Nle effectors, in addition to EspC, NleJ, LifA and other candidate effector proteins.

Crucially, further work is required to support and extend these findings to enable identification of the responsible CesT dependent effector(s) needed promote the rapid loss of p-AKT$_{ser473}$ signal. Future studies should therefore continue to optimise this J774A.1 macrophage cell model to overcome the identified limitations; such as the infection induced EspZ linked cell detachment/cytotoxicity and the associated loss of p-AKT$_{ser473}$ signal. This could include the transfection of the EspZ effector into host cells prior to EPEC infection, though this is notably difficult in the macrophage cell model.

Chapter 4 Investigating the T3SS dependent decrease of phosphorylated AKT in a HeLa cell model

4.1 Introduction

AKT signalling is stimulated downstream of receptor tyrosine kinase (RTK) and G-protein coupled receptor (GPCR) in a class I PI3K dependent manner. RTK family proteins include the cell surface epidermal growth factor receptor (EGFR), activated following the detection of the ligand epidermal growth factor (EGF). Although PI3K is the predominant mode of AKT activation, several studies have reported alternative PI3K independent mechanisms of AKT activation. These include the direct phosphorylation of AKT by host kinases such as activated CDC42 kinase 1 (ACK1) (Mahajan and Mahajan, 2010), Src kinase (Jiang and Qiu, 2003), IκB kinase ε (IKKε) (Guo *et al.*, 2011), and TANK-binding kinase (TBK1) (Joung *et al.*, 2011). Indeed, the activation of these kinase and AKT signalling has been demonstrated downstream of TNFR and TLR activation, stimulated by detection of agonists TNFα and PAMPs (e.g. LPS) respectively (Jiang and Qiu, 2003; Pincheira *et al.*, 2008; Guo *et al.*, 2011; Cheung *et al.*, 2015).

EPEC infection targets the epithelial cells of the small intestine (enterocytes and M-cells). These cells are polarised with distinct apical and basolateral membranes, and are routinely studied *in vitro* using cell lines such as Caco-2 (polarised intestinal cell model), sometimes in co-culture with Raji B-lymphocytes (phagocytic M-cell model), or using J774A.1 macrophages as a simplistic model of phagocytic cells (M-cell like). However, non-polarised epithelial cell lines (HeLa cells) have also been extensively used to study EPEC pathogenesis (Law *et al.*, 2013). For example, HeLa cells have proved useful to characterise and dissect many aspects of EPEC infection, including pedestal formation (Knutton *et al.*, 1989), T3SS dependent effector translocation hierarchy (Mills *et al.*, 2013), as well as the functional analysis of delivered effector proteins such as Tir (Nieto-Pelegrin and Martinez-Quiles, 2009).

In addition to macrophages (J774A.1), EPEC infection has previously been shown to reduce AKT phosphorylation in epithelial cell models, including non-polarised HeLa (Shames *et al.*, 2010) and polarised Caco-2 (Ruchaud-Sparagano *et al.*, 2007) cells. HeLa cells offer particular advantages over polarised epithelial cell models (e.g. Caco-2, TC7 & T84) and J774A.1 macrophages, including their ease of transfection and greater resistance to EPEC induced cell detachment (Kenny *et al.*, unpublished data). HeLa cells may therefore provide an alternative, robust, stable and more amenable (transfection) model to interrogate the effectors responsible for EPEC to trigger the rapid decline of phosphorylated AKT; avoiding some of the limitations identified in J774A.1 macrophages.

Using HeLa cells, it was therefore hypothesised this model would support observations of a T3SS dependent decrease of phosphorylated AKT, aiding identification of the virulence factors required to subvert the AKT signalling pathway. The aim of this chapter was thus to develop a HeLa cell model to investigate the impact of EPEC infection on AKT phosphorylation.

4.2 Results

4.2.1 EPEC does not stimulate AKT phosphorylation in HeLa cells under the conditions examined

EPEC is reported to influence AKT phosphorylation in a number of cell models, including macrophages (J774A.1 & RAW 264.7), polarised (MDCK & Caco-2) and non-polarised epithelial (HeLa) cells (Roxas *et al.*, 2007; Ruchaud-Sparagano *et al.*, 2007; Sason *et al.*, 2009; Shames *et al.*, 2010; Roxas *et al.*, 2012). Indeed, EPEC infection has been shown to decrease phosphorylated AKT in a HeLa cell model; however, previous work within the Kenny group has failed to recapitulate this result (Kenny *et al*., unpublished). To determine the capacity of EPEC to stimulate AKT phosphorylation in HeLa cells, cells were left uninfected or infected with the EPEC T3SS defective mutant (*cfm-14*) or WT EPEC strain at an MOI of 100:1 over a standard 120 min infection period before isolating total cell extracts at indicated times for western blot analysis. Samples infected with the *cfm-14* mutant revealed no induction of AKT phosphorylation above the levels of uninfected control cells at all time points (Figure 30A). Lack of infection induced AKT phosphorylation with *cfm-14* mutant was not due to notable changes in levels of total AKT or Actin (loading control; Figure 30A). Similar results were found following infection with WT EPEC strain, revealing no induction of p-AKT$_{ser473}$ signal or changes in total AKT and Actin (Figure 30B). As expected (Kenny *et al.*, 1997), EPEC strain genotype was supported by the detection of the host kinase modified form of Tir (T'') in WT EPEC but not *cfm-14* mutant infected cells (Figure 30). This work indicates that EPEC strains do not induce AKT phosphorylation in this HeLa cell model under the conditions examined. Thus, to examine the impact of EPEC infection on AKT activity would require a robust stimulus of AKT phosphorylation.

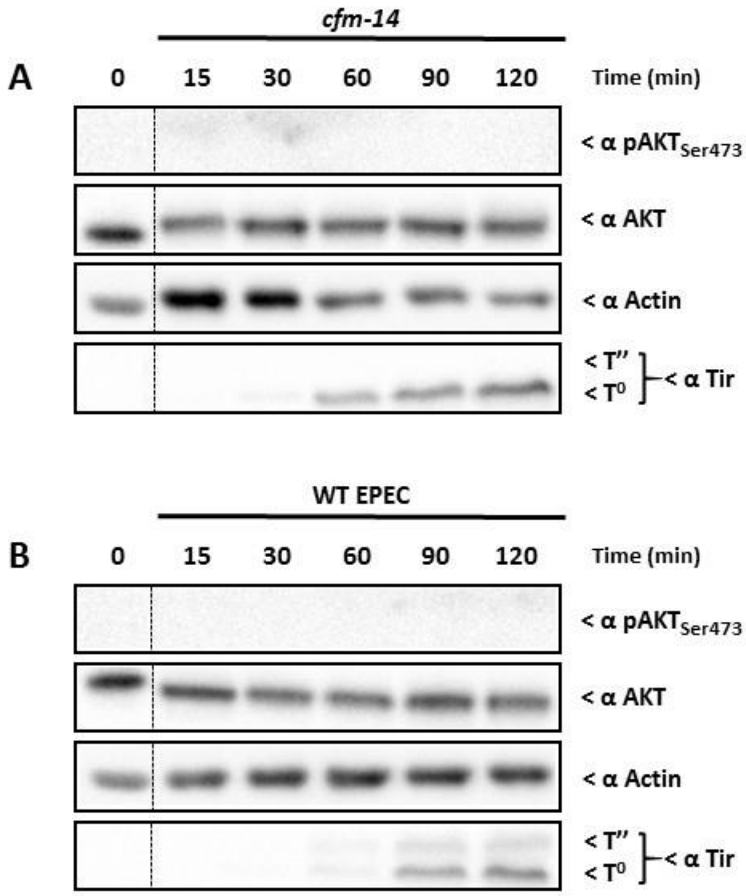


Figure 30 EPEC infection does not induce AKT phosphorylation in HeLa cells.

(**A & B**) HeLa cells were left uninfected (0) or infected (MOI 100:1) with the pre-activated T3SS defective mutant (*cfm-14*) or WT EPEC strain for indicated times (min) over a 2 h infection before infections were stopped. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-pan-AKT (detect isoforms 1, 2 & 3), anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a signle experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.

4.2.2 TNFα induces AKT phosphorylation in HeLa cells

AKT phosphorylation at residue Ser473 in response to treatment with TNFα has previously been reported in a number of cell models, including HeLa cells (Ozes *et al.*, 1999; Osawa *et al.*, 2001; Kettritz *et al.*, 2002; Wang *et al.*, 2013). To re-establish the appropriate conditions for TNFα induced AKT phosphorylation, HeLa cells were left untreated or treated with TNFα using a range of concentrations (10, 15 & 50 ng/ml) over a 20 min time course before isolating total cell extracts at indicated times for western blot analysis. Untreated HeLa cells reveal weak background levels of p-AKT_{ser473}. Treatment with TNFα induced a time dependent increase in AKT phosphorylation for all concentrations examined (Figure 31A). Maximum stimulation of p-AKT_{ser473} was achieved at concentrations of 15 ng/ml and 50 ng/ml, reaching an approximate 5 fold increase in comparison to uninfected control cells after 10 and 5 min of stimulation respectively (Figure 31B). This work supports TNFα as a stimulus of AKT phosphorylation in HeLa cells, indicating a weak transient p-AKT_{ser473} profile at the concentrations examined (Figure 31). To achieve a stronger induction of AKT phosphorylation in comparison to basal levels of untreated cells, alternative agonists were examined.

4.2.3 EGF is a strong inducer of AKT phosphorylation in HeLa cells

EGF stimulation of the EGFR/PI3K/AKT signalling pathway induces a rapid and transient increase in AKT phosphorylation in HeLa cells (Steele-Mortimer *et al.*, 2000; Cooper *et al.*, 2011). To confirm these findings in our HeLa cell clone, cells were left untreated or treated with EGF (100 ng/ml) over a 20 min time course before isolating total cell extracts at indicated times for western blot analysis. Untreated HeLa cells displayed very week (poorly detected) basal levels of phosphorylated AKT (Figure 32A). Treatment with EGF induced a strong increase in AKT phosphorylation, detected after treatment for 1, 2 and 5 min with EGF before decreasing rapidly to background levels after 10 and 20 min (Figure 32A). This observed increase in p-AKT_{ser473} signal with EGF treatment, at all time points, was not due to notable changes in total AKT (Figure 32A). Quantification analysis of the p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as a relative change to untreated cells, reveals a clear transient increase in AKT phosphorylation, indicating an approximate 600 fold increase in p-AKT_{ser473} after 2 min of EGF treatment (Figure 32B). This work supports the use of EGF as a suitable agonist for the strong induction of AKT phosphorylation in HeLa cells.

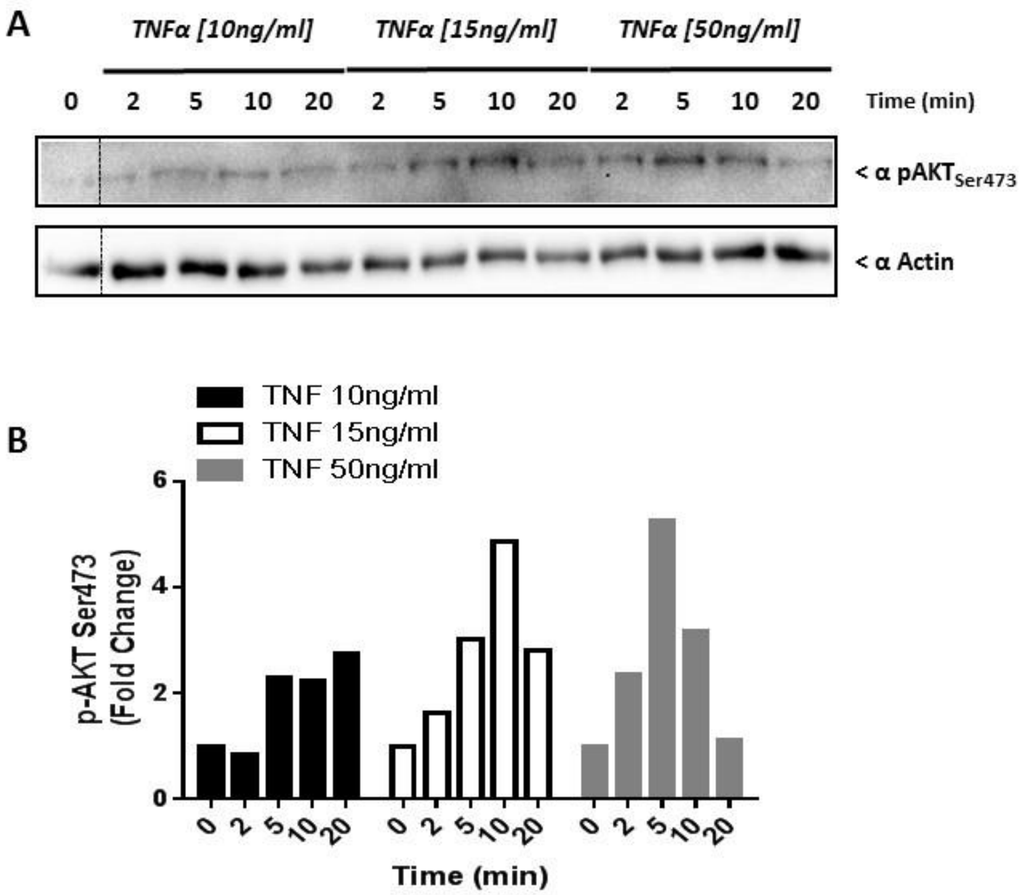


Figure 31 TNFα stimulates AKT phosphorylation in HeLa cells.

(**A**) HeLa cells were left untreated (0) or treated with TNFα over a range of concentrations (10, 15 & 50 ng/ml) for the indicated times (min) before isolating total cell extracts (1x SDS sample buffer). Protein samples were separated by SDS-PAGE (10%) for western blot analysis; probing for anti-p-AKT_{ser473} and anti-Actin (as loading control) antibodies. Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**B**) Densitometry analysis was performed using ImageLab software, with values representing a fold change in p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as a relative change to uninfected control (0) cells.

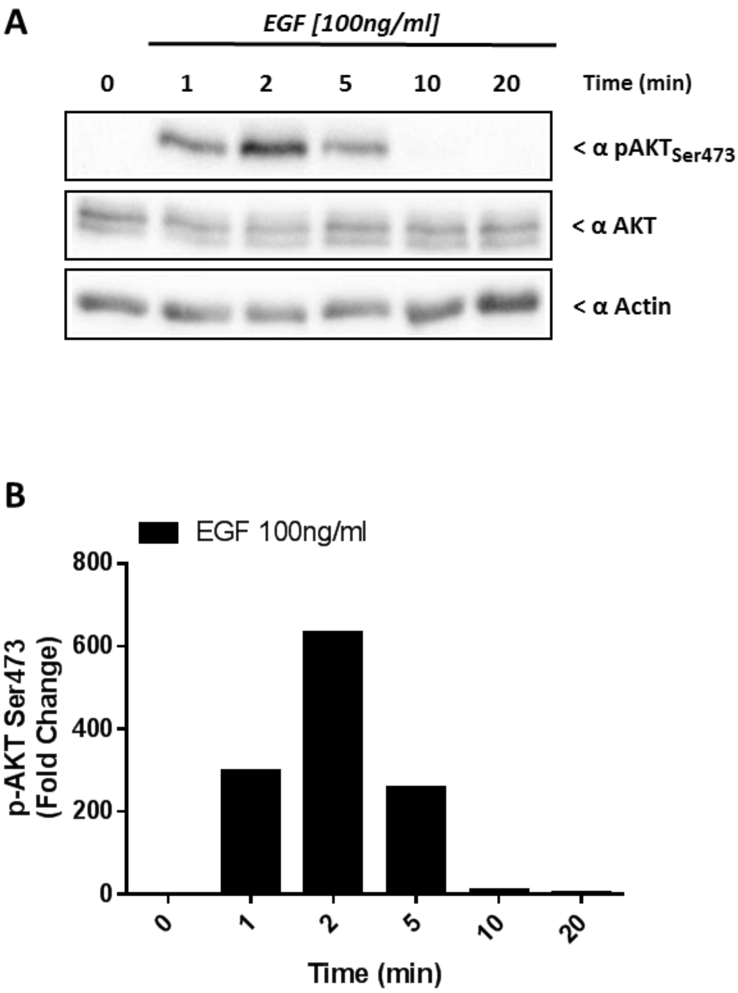


Figure 32 EGF induces a strong transient increase in AKT phosphorylation.

(**A**) HeLa cells were left untreated (0) or treated with EGF (100 ng/ml) for the indicated times (min) before isolating total cell extracts (1x SDS sample buffer). Protein samples were separated by SDS-PAGE (10%) for western blot analysis; probing for anti-p-AKT$_{ser473}$, anti-pan-AKT (detect isoforms 1, 2 & 3) and anti-Actin (as loading control) antibodies. Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes. (**B**) Densitometry analysis was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to uninfected control (0) cells.

4.2.4 EPEC infection prevents EGF induced AKT phosphorylation in a T3SS dependent manner

Having established EGF as an appropriate agonist for the strong induction of AKT phosphorylation, studies were undertaken to examine the ability of EPEC to prevent, in a T3SS dependent manner, EGF induced AKT phosphorylation. Thus, HeLa cells were left uninfected or infected with the EPEC T3SS defective mutant (*cfm-14*) or WT EPEC strain at an MOI of 100:1 for 1, 2 or 3 h prior to stimulating cells with EGF (100 ng/ml) for 2 min before isolating total cell extracts for western blot analysis. As expected, infection with the T3SS mutant (*cfm-14*) did not induce AKT phosphorylation at 3 h post infection (Figure 33A). In contrast, stimulation of uninfected cells with EGF induced strong levels of p-AKT$_{ser473}$ (Figure 33A). Furthermore, EGF treatment of cells pre-infected with the *cfm-14* mutant for 1, 2 or 3 h led to the robust stimulation of AKT phosphorylation (Figure 33A). Like the *cfm-14* mutant, infection with WT EPEC alone did not induce AKT phosphorylation (Figure 33A). However, treatment of EGF to WT EPEC infected cells led to a strong induction of AKT phosphorylation for cells pre-infected for 1 h, but not 2 or 3 h (Figure 33A). Observed changes in AKT phosphorylation, post infection, with the *cfm-14* mutant or WT EPEC strain (± EGF) were not linked to alterations in levels of total AKT (Figure 33A). Quantification analysis of p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to uninfected EGF treated cells, demonstrated the clear capacity of EGF to induce strong levels of AKT phosphorylation in cells pre-infected with the *cfm-14* mutant, but not WT EPEC strain, after 2 h of infection (Figure 33B). Effector protein delivery in WT EPEC infected cells was supported by the detection of the host modified form of Tir (T'') after 1, 2 and 3 h post infection, which was absent from samples infected with the T3SS defective mutant, *cfm-14* (Figure 33A). These results illustrate the ability of the WT EPEC strain to prevent EGF from inducing AKT phosphorylation in a T3SS dependent manner.

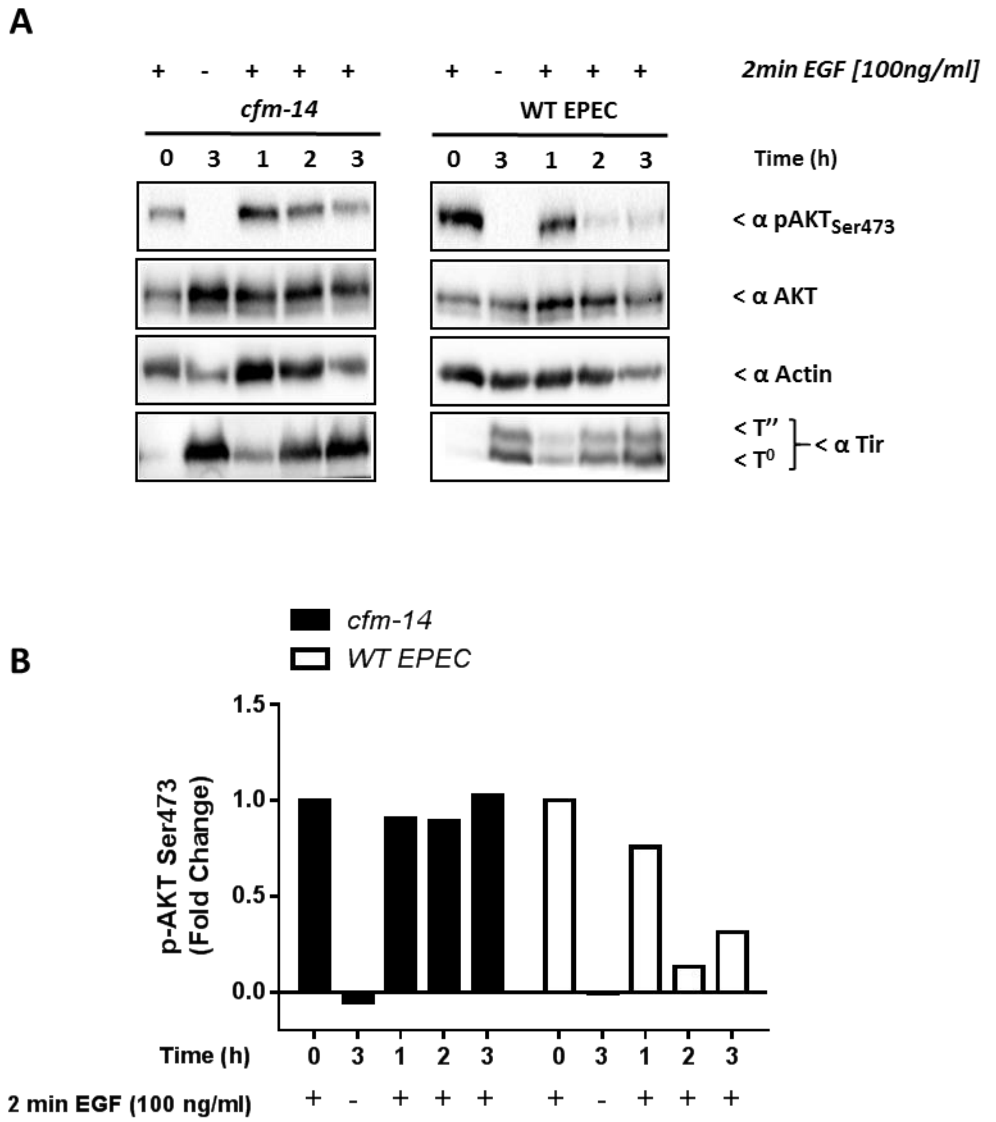


Figure 33 EPEC prevents EGF induced AKT phosphorylation in a T3SS dependent manner.

(**A**) HeLa cells were left uninfected (0) or infected (MOI 100:1) with the pre-activated T3SS defective mutant (*cfm-14*) or WT EPEC strain for 1, 2 & 3 h, before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT_{ser473}, anti-pan-AKT (detect isoforms 1, 2 & 3), anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes. (**B**) Densitometry analysis was performed using ImageLab software, with values representing a fold change in p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as a relative change to uninfected EGF treated (0) cells.

4.2.5 The outer membrane protein Intimin is not essential to prevent EGF induced AKT phosphorylation in HeLa cells

Although crucial for EPEC mediated intimate attachment with host cells and other host cell subversive events (Knutton *et al.*, 1989; Dean and Kenny, 2004; Dean *et al.*, 2006), Intimin has previously been discounted in EPEC induced loss of phosphorylated AKT in macrophages (Quitard *et al.*, 2006) and polarised Caco-2 (Ruchaud-Sparagano *et al.*, 2007) cells. To examine the requirement of Intimin to prevent EGF induced AKT phosphorylation in HeLa cells initial experiments focused on the EPEC *Δeae* mutant over an extended 5 h infection. Thus, HeLa cells were left uninfected or infected with the EPEC T3SS defective mutant (*cfm-14*), WT EPEC strain or *Δeae* mutant at an MOI of 100:1 for 2, 3, 4 and 5 h prior to stimulating cells with EGF (100 ng/ml) for 2 min and isolating total cell extracts for western blot analysis. Stimulation of uninfected cells with EGF demonstrated the robust induction of AKT phosphorylation (Figure 34A). Moreover, EGF treatment of *cfm-14* infected cells revealed the strong stimulation of AKT phosphorylation at 2, 3, 4 and 5 h post infection (Figure 34A). In contrast, infection with the WT EPEC strain revealed reduced levels of EGF induced AKT phosphorylation at all time points post infection (2, 3, 4 & 5 h; Figure 34B), consistent with earlier findings (Figure 33). Similar results were obtained following EGF treatment of EPEC *Δeae* mutant pre-infected cells, revealing low levels of p-AKT$_{ser473}$ at 2, 3, 4 and 5 h post infection (Figure 34C); though notably greater than p-AKT$_{ser473}$ levels in WT EPEC infected samples over the same time course (Figure 34B). Quantification analysis of p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to uninfected EGF treated cells, supports the capacity of EGF to induce strong levels of AKT phosphorylation in cells pre-infected with the *cfm-14* mutant, but not WT EPEC or the *Δeae* mutant (Figure 34D). EPEC strain genotype was supported by the detection of the host kinase modified form of Tir (T'') in WT EPEC and the *Δeae* strain, but not *cfm-14* mutant infected cell extracts (Figure 34A-C). It should be noted some cell detachment was observed with the WT EPEC and Δeae mutant, but not *cfm*-14 strain, at approximately 5 h post infection in this model. These results suggest that Intimin (*Δeae*) is not essential for EPEC to prevent EGF induced AKT phosphorylation.

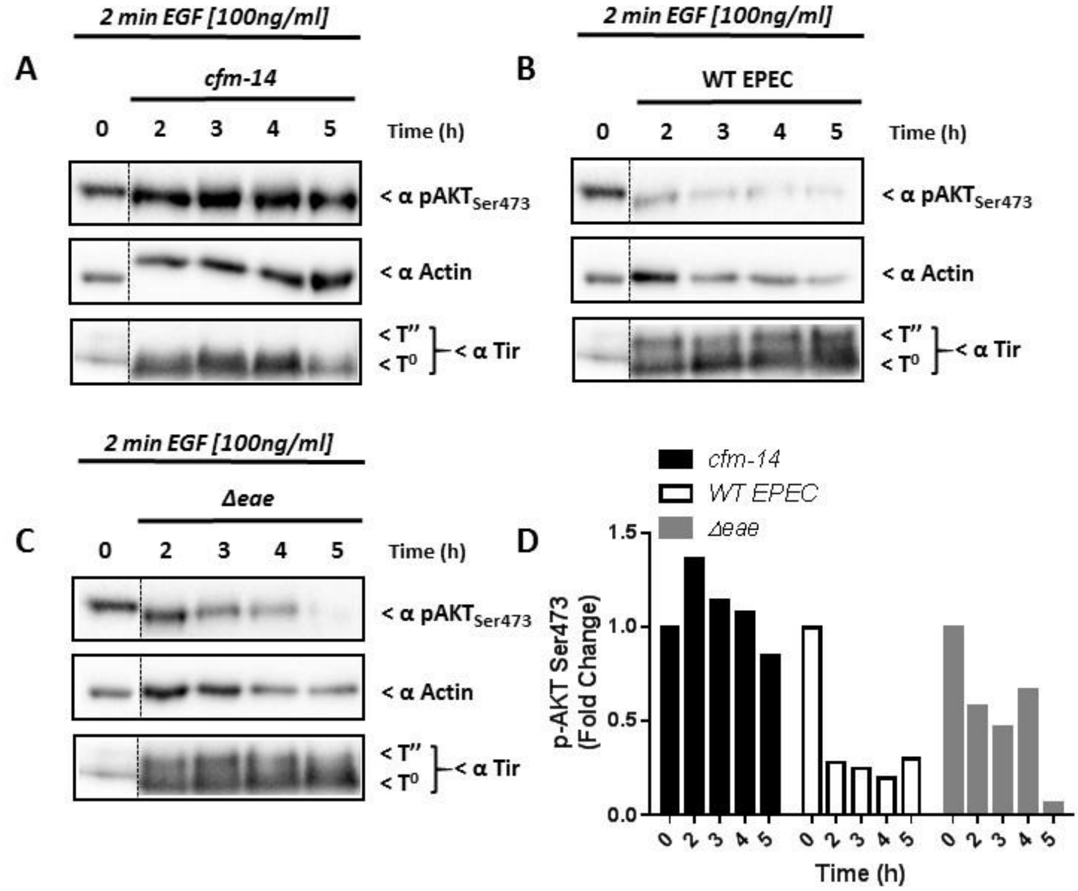


Figure 34 The EPEC Intimin mutant has a delayed capacity to prevent EGF induced AKT phosphorylation.

(**A-C**) HeLa cells were left uninfected (0) or infected (MOI 100:1) with the pre-activated T3SS defective mutant (*cfm-14*), WT EPEC, or *Δeae* (lacking outer membrane protein Intimin) strains for 2, 3, 4 & 5 h, before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**D**) Densitometry analysis was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to uninfected EGF treated (0) cells.

4.2.6 The capacity of EPEC to prevent EGF induced AKT phosphorylation is independent of Intimin, 5 LEE effectors (EspZ, EspH, Map, EspF & EspG) and the Nle effector EspG2/Orf3

To investigate the virulence factor(s) required for EPEC to prevent EGF induced AKT phosphorylation in HeLa cells, initial studies focused on examining the role of LEE effector proteins using the available complex EPEC *Δmfzgorf3eh::km* mutant (lacking Intimin, 5 LEE effectors [EspZ, EspH, Map, EspF & EspG] and Nle effector EspG2/Orf3). This *Δmfzgorf3eh::km* strain still encodes LEE effectors Tir and EspB. Thus, HeLa cells were left uninfected or infected with the *Δmfzgorf3eh::km* strain at an MOI of 100:1 for 2, 3, 4 and 5 h prior to stimulating cells with EGF (100 ng/ml) for 2 min and isolating total cell extracts for western blot analysis. Treatment with EGF in uninfected cells demonstrated the robust stimulation of AKT phosphorylation (Figure 35). In contrast, treatment with EGF to *Δmfzgorf3eh::km* infected cells failed to induce AKT phosphorylation at all time points post infection (2, 3, 4 & 5 h), with the p-AKT_{ser473} signal below the level of detection (Figure 35). It should be noted infection with this complex mutant (*Δmfzgorf3eh::km*) resulted in high levels of cell detachment after 3 h of infection (data not shown), supported by decreasing levels of actin over the infection period (Figure 35); linked to the absence of EspZ delivery into host cells (Shames *et al.*, 2010; Berger *et al.*, 2012; Roxas *et al.*, 2012). EPEC strain genotype was supported by the detection of the host kinase modified form of Tir (T''; Figure 35) and PCR analysis for the appropriate disruption of genes (A. Madkour *et al.*, data not shown). This work suggests the outer membrane protein Intinim, 5 LEE effectors (EspZ, EspH, Map, EspF & EspG) and the Nle effector EspG2/Orf3 are not required to prevent EGF induced AKT phosphorylation, similar to results identified in the J774A.1 macrophage cell model.

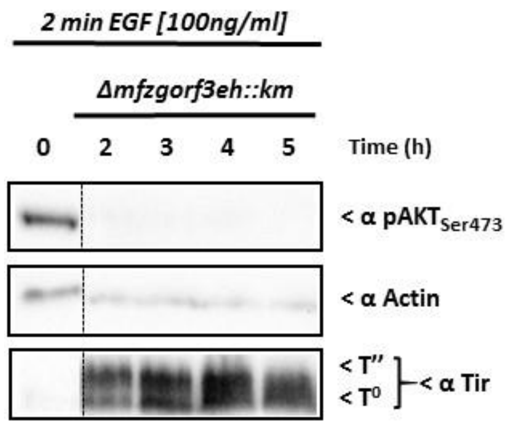


Figure 35 EPEC's capacity to prevent EGF induced AKT phosphorylation is independent of 5 LEE effectors (Map, EspH, EspZ, EspG & EspH), Intimin and the Nle effector EspG2/Orf3.

HeLa cells were left uninfected (0) or infected (MOI 100:1) with the pre-activated EPEC mutant *Δmfzgorf3eh::km* (lacking the outer membrane protein Intimin, LEE effectors EspH, Map, EspF, EspZ, EspG and its Nle homolog EspG2/Orf3) for 2, 3, 4 & 5 h before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.

4.2.7 The EPEC strain lacking the central 'core' LEE region prevents EGF induced AKT phosphorylation

LEE encoded chaperones CesF and CesT are required for the efficient translocation of up to 13 known LEE and Nle effector proteins into host cells (Mills *et al.*, 2013). To examine whether the virulence factor(s) required to prevent EGF induced AKT phosphorylation are dependent on these chaperones (CesF & CesT) and to determine the requirement of the effector protein Tir, p-AKT_{ser473} levels were examined in cells infected with the available EPEC *Δcore::km* mutant. This *Δcore::km* strain lacks the central 'core' LEE region encoding Intimin; 3 LEE effectors, EspH, Map and Tir; and 2 chaperones, CesF and CesT. HeLa cells were left uninfected or infected with the *Δcore::km* strain at an MOI of 100:1 for 2, 3, 4 and 5 h prior to stimulating cells with EGF (100 ng/ml) for 2 min and isolating total cell extracts for western blot analysis. As expected, EGF treatment of uninfected cells revealed strong levels of p-AKT_{ser473} (Figure 36). However, EGF treatment of cells infected with the *Δcore::km* mutant revealed weak stimulation of AKT phosphorylation at 2 and 3 h post infection, with p-AKT_{ser473} signal undetected at 4 and 5 h post infection (Figure 36). Again, high levels of cell detachment were observed with this strain after 3 h of infection (data not shown), supported by decreasing levels of actin over the infection period (Figure 36). Absence of host kinase modified (T'') and unmodified (T^0) forms of Tir (Figure 36), in addition weak levels of EspF (data not shown) supports EPEC *Δcore::km* strain genotype. This result tentatively suggests that the EPEC virulence factor(s) necessary to prevent EGF induced AKT phosphorylation in HeLa cells do not require the effector protein Tir or effector chaperones CesF and/or CesT; thus differing from a CesT dependent phenotype identified in J774A.1 macrophages. However, due to notable levels of infection induced cell detachment, linked to increased cell cytotoxicity (Shames *et al.*, 2010; Berger *et al.*, 2012; Roxas *et al.*, 2012), it remains to be determined whether these cells are capable of responding to EGF induced AKT phosphorylation.

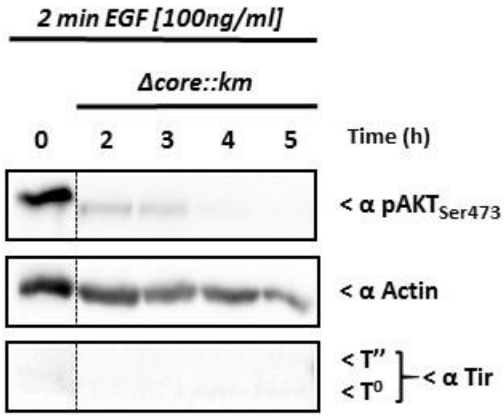


Figure 36 The EPEC *Δcore::km* mutant prevents EGF induced AKT phosphorylation.

HeLa cells were left uninfected (0) or infected (MOI 100:1) with pre-activated EPEC mutant *Δcore::km* (lacking LEE proteins EspH, Map, CesF, Tir, CesT and Intimin [Figure 23]) for 1, 2, 3, 4 & 5 h before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.

4.2.8 EPEC inhibition of EGF induced AKT phosphorylation is independent of 6 LEE and 14 known Nle effector proteins

Using the most recently generated complex EPEC effector mutant, TOEA7*core::km*, studies were undertaken to examine the ability of this strain prevent EGF-induced AKT phosphorylation. Crucially, this strain is unable to express and/or deliver all known LEE effectors (except EspB), all Nle effectors (except EspC, LifA & NleJ), two effector chaperones (CesF & CesT) and the outer membrane protein Intimin. Thus, this strain is theoretically unable to translocate up to 20 of 24 known effector proteins into host cells. HeLa cells were left uninfected or infected at an MOI of 100:1 for 2, 3, 4 and 5 h prior to stimulating cells with EGF (100 ng/ml) for 2 min and isolating total cell extracts for western blot analysis. EGF treatment of uninfected HeLa cells induced the robust stimulation of AKT phosphorylation (Figure 37). In contrast, cells infected with TOEA7*Δcore::km* demonstrated no detectable levels of AKT phosphorylation following EGF treatment at 2, 3, 4 and 5 h post infection (Figure 39). Similar to the *Δcore::km* mutant infected cells, high levels of cell detachment were observed with TOEA7*Δcore::km* mutant after 3 h of infection (data not shown), supported by decreasing levels of actin over the infection period (Figure 37). Again, absence of host kinase modified (T'') and unmodified (T^0) forms of Tir (Figure 38), in addition weak levels of EspF (data not shown) supports EPEC TOEA7*Δcore::km* strain genotype. This work tentatively suggests that Intimin, 6 LEE effectors, 14 Nle effectors and the two effector chaperones (CesF & CesT) do not inhibit EGF induced AKT phosphorylation in HeLa cells. Again, this result differs from observations identified in the J774A.1 macrophage cell model

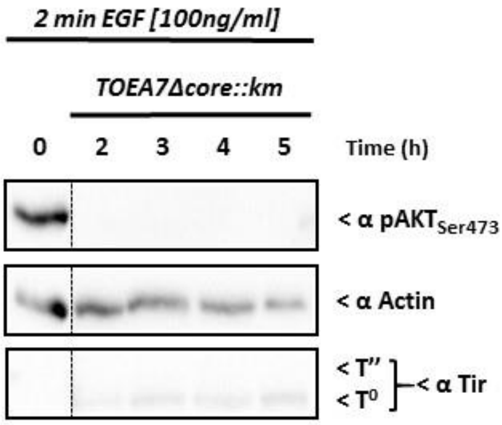


Figure 37 The EPEC TOEA7*Δcore::km* mutant prevents EGF induced AKT phosphorylation.

HeLa cells were left uninfected (0) or infected (MOI 100:1) with pre-activated EPEC mutant TOEA7*Δcore::km* (lacking core LEE genes [Figure 23] in addition to 14 Nle effectors [Table 7]) for 2, 3, 4 & 5 h before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.

4.2.9 EPEC induces the loss of EGFR in a T3SS dependent manner in HeLa cells

The ability of the *Δcore::km* and TOEA7*Δcore::km* mutant strains to prevent agonist-induced AKT phosphorylation in HeLa cells differed from findings made in the J774A.1 macrophage cell model. This suggested that 1) there is an intrinsic difference between the two cell models, 2) EPEC infected HeLa cells are too unhealthy to respond to the agonist EGF (note cell detachment), or 3) EPEC induced degradation of the EGFR prevents EGF induced AKT phosphorylation; the latter dependent on the effector EspF and promoted by EspZ deficient EPEC strains (Roxas *et al.*, 2014). To examine EPEC induced changes to the EGFR, HeLa cells were left uninfected or infected with the EPEC T3SS defective mutant (*cfm-14*), WT EPEC strain or the TOEA7*Δcore::km* mutant (does not deliver EspF and EspZ effectors) at an MOI of 100:1 for 2, 3, 4 and 5 h prior to stimulating cells with EGF (100 ng/ml) for 2 min and isolating total cell extracts for western blot analysis. EGF treatment of uninfected cells revealed the robust detection of the EGFR signal, correlating with a strong p-AKT$_{ser473}$ signal (Figure 38A). EGF treatment of *cfm-14* mutant infected cells revealed a strong signal for EGFR and p-AKT$_{ser473}$ at 2, 3, 4 and 5 h post infection (Figure 38A). In contrast, cells pre-infected with the WT EPEC strain induced a time dependent decrease of the EGFR signal at 3, 4 and 5 h post infection (Figure 38B). Although the detection of EGFR signal was comparable with the uninfected EGF treated cells (control) at 2 h of infection, p-AKT$_{ser473}$ signal was poorly detected at 2, 3, 4 and 5 h post infection with the WT EPEC strain (Figure 38B). This suggests the loss of p-AKT$_{ser473}$ signal at 2 h post infection with the WT EPEC strain is not linked to the degradation of the EGFR, detected at 3 h post infection. Interestingly, EGF treatment of TOEA7*Δcore::km* mutant infected cells reveals the presence of two bands associated with EGFR (additional band of lower Mw) after 2, 3, 4 and 5 h of infection, which is absent from uninfected EGF treated cells (Figure 38C). As expected, TOEA7*Δcore::km* infection resulted in loss of EGF induced AKT phosphorylation at 2, 3, 4 and 5 h post infection (Figure 38C). Again, infection of cells with the TOEA7*Δcore::km* mutant was associated with high levels of cell detachment after 3 h of infection (data not shown). Taken together, these results reveal the EPEC induced loss of EGFR in a T3SS dependent manner in HeLa cells. In addition, this work suggests that the EPEC mediated inhibition of EGF induced AKT phosphorylation is not associated with loss in detection of EGFR at 2 h post infection. However, with evidence of EGFR modification at 2 h post infection with the TOEA7*Δcore::km* mutant, this raises the possibility that the EGFR is inactivated and/or non-functional.

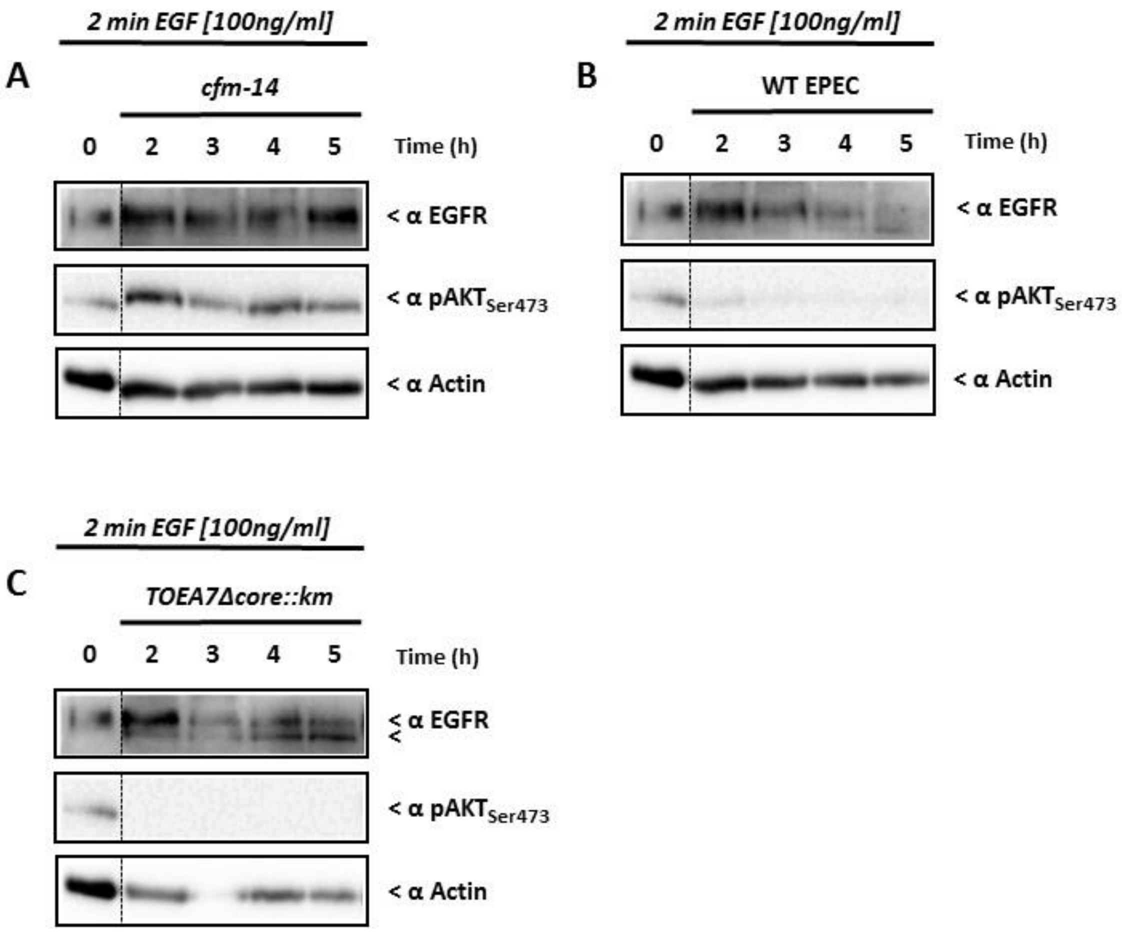


Figure 38 EPEC triggers the T3SS dependent loss of EGFR.

(**A-C**) HeLa cells were left uninfected (0) or infected (MOI 100:1) with the pre-activated T3SS defective mutant (*cfm-14*), WT EPEC or TOEA7*Δcore::km* mutant (lacking 'core' LEE genes [Figure 23] in addition to 14 Nle effectors [Table 7]) strains for 2, 3, 4 & 5 h before treating cells with EGF agonist (100 ng/ml) for 2 min. Total cell extracts were isolated (1x SDS sample buffer) and protein samples separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-EGFR and anti-Actin (loading control) antibodies. Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.

4.3 Discussion

The effector proteins required and the mechanism by which they mediate EPEC induced loss of phosphorylated AKT remains inconclusive, with few observations reported in HeLa cells. This study aimed to develop a simple (non-polarised) HeLa cell model to examine the impact of EPEC infection on AKT phosphorylation; avoiding limitations of greater cell detachment (over short infection periods; Kenny *et al*., unpublished) and poor transfection efficiency in J774A.1 macrophages (Van De Parre *et al.*, 2005) and polarised epithelial (e.g. Caco-2, TC7 & T84) cells (Dean *et al.*, 2013). This study demonstrates for the first time that EPEC infection can prevent AKT phosphorylation stimulated by the EGF agonist in a HeLa cell model, in a T3SS dependent manner. Furthermore, this work suggests that the inhibition of EGF induced AKT phosphorylation is independent of the T3SS effector chaperone CesT, thus differing from observations made in the J774A.1 macrophage cell model. However, this study has uncovered important limitations of the HeLa cell model including infection induced cell detachment and EPEC triggered degradation/modification of the EGFR, thus undermining these observations. Since these findings were made from a single experiment, additional work is required to confirm these observations. Thus, further development of the HeLa cell model is needed to support, or not, these findings to provide an alternative and more transfectable cell model to investigate the impact of EPEC infection on AKT phosphorylation.

PI3K/AKT signalling is activated downstream of a number of receptor pathways, including TLR's (Brown *et al.*, 2011). However, whether the activation of TLR's (TLR1-TLR11) plays a key role in EPEC infection induced AKT phosphorylation is unknown. In this study we demonstrate that infection with T3SS defective mutant (*cfm-14*) or WT EPEC strain does not stimulate AKT phosphorylation in our HeLa cell model. This presumably reflects the lack of LPS induced TLR4 signalling (Shimazu *et al.*, 1999) or the absence of TLR receptors, such as TLR5, in HeLa cells (Gewirtz *et al.*, 2001; Yu *et al.*, 2006; Deng *et al.*, 2012). Indeed, EPEC flagella is the major antigen responsible for inducing pro-inflammatory signalling via the TLR5 receptor in polarised epithelial cell models (Gewirtz *et al.*, 2001; Ruchaud-Sparagano *et al.*, 2007). Furthermore, activation of the TLR5 receptor by flagella is also known to activate the PI3K/AKT signalling pathway (Yu *et al.*, 2006). This premise could be supported by the transfection of TLR5 (and/or other receptors) into HeLa cells to determine the capacity of EPEC to stimulate, and then decrease, infection induced AKT phosphorylation.

To examine the impact of EPEC infection on AKT phosphorylation it was important to identify an appropriate agonist to stimulate the robust increase of p-AKT$_{ser473}$ in HeLa cells. Using the agonists EGF and TNFα, this study demonstrates the robust and transient increase of AKT phosphorylation following treatment over a short 20 min time course. This transient increase of AKT phosphorylation is consistent with similar observations reported in the literature using comparable conditions (Steele-Mortimer *et al.*, 2000; Kettritz *et al.*, 2002). As EGF was found to be the most potent stimulator of AKT phosphorylation in our HeLa cell model, EGF was selected for treatment in subsequent studies.

In this study we provide a model whereby the pre-infection of cells with WT EPEC, but not the *cfm-14* mutant, leads to the T3SS dependent inhibition of EGF induced AKT phosphorylation. Indeed, this result is consistent with similar strategies revealing the T3SS dependent inhibition of TNFα induced AKT phosphorylation in polarised epithelial cells pre-infected with EPEC (Ruchaud-Sparagano *et al.*, 2007). In contrast to J774A.1 macrophages, detection of the host modified form of Tir (T'') was not correlated to the loss of p-AKT$_{ser473}$ signal, with Tir modification and inhibition of AKT signalling detected at 1 h and 2 h post infection respectively; in contrast to 1 h for both in J774A.1 macrophages. Since Tir modification (T'') was used as a marker of effector protein translocation into host cells (Kenny *et al.*, 1997), this might suggest inhibitory activity of effector proteins is possibly slower in the HeLa cell model. Alternatively, this may suggest that insufficient levels of inhibitory effector protein(s) have been translocated into HeLa cells at 1 h post infection.

It is important to note that infection with the WT EPEC strain, but not the T3SS mutant, was associated with low levels of cell detachment by the end of the 3 h infection period. These observations are consistent with studies by Shames *et al*., revealing the increase of cell lifting in a HeLa cell model with the WT EPEC (E2348/69) strain after 4 h of infection (Shames *et al.*, 2010). Interestingly, previous studies within the Kenny Lab had suggested that infection with Intimin deficient strains displayed a reduced capacity to cause cell detachment (Kenny *et al*., unpublished). However, these observations were not supported in this study revealing comparable levels of cell detachment to the WT EPEC strain with the *Δeae* mutant (lacks Intimin).

To gain insight into the factor(s) necessary for EPEC to inhibit EGF induced AKT phosphorylation, cells were pre-infected with EPEC strains lacking one or more virulent proteins. Examining the role of Intimin, using the *Δeae* mutant, revealed the clear inhibition of EGF stimulated AKT phosphorylation in a time dependent manner. These results support previous observations of an Intimin independent inhibition of AKT phosphorylation in a polarised epithelial cell model (Ruchaud-Sparagano *et al.*, 2007). Comparison against the WT EPEC strain suggested this inhibition was delayed with the *Δeae* mutant, revealing maximum inhibition of AKT phosphorylation at 2 h and 5 h post infection respectively. Importantly, this slow decline of p-AKT$_{ser473}$ signal was not associated with a delay or reduced detection of the host modified form of Tir (T''); marker of effector translocation (Kenny *et al.*, 1997). Although Intimin does not appear to be critical to inhibit AKT signalling, this result may suggest that Intimin is necessary to promote the rapid inhibition of AKT phosphorylation, possibly through mediating intimate host cell attachment (Tir-Intimin dependent) (Kenny *et al.*, 1997), which is necessary for the efficient translocation of effectors (Mills *et al.*, 2013).

Investigations using more complex effector deficient multiple mutants, *Δmfzgorf3eh::km* (lacks Map, EspF, EspZ, EspG, EspH & EspG2/Orf3 effectors), *Δcore::km* (lacks LEE EspH, Map & Tir effectors; CesF & CesT chaperones; and Intimin) and TOEA7*Δcore::km* (as per *Δcore::km*, and lacks LEE EspG and 14 Nle effectors: NleA, NleB1, NleB2, NleC, NleD, NleE1, NleE2, NleF, NleG, EspG2/Orf3, NleH1, NleH2, EspJ & EspL), revealed that pre-infection with each strain inhibited the capacity of cells to respond to EGF stimulated AKT phosphorylation. Importantly, this inhibition of AKT phosphorylation was evident at 2 h post infection, prior to the detection of increased cell detachment after 3 h of infection (data not shown). This tentatively suggested the inhibition of EGF stimulated AKT phosphorylation is not associated with the onset of host cell detachment. However, whether HeLa cells are indeed functional at 2 h post infection prior to host cell detachment remains to be determined. Increased cell detachment is linked to the absence of CesT chaperone dependent translocation of EspZ into host cells; implicated to inhibit apoptosis (Roxas *et al.*, 2012), promote survival signalling (Shames *et al.*, 2010) and prevent excessive translocation of effector proteins (cytotoxic) (Berger *et al.*, 2012).

It is interesting to note the apparent delay in inhibition of AKT phosphorylation, previously identified with the *Δeae* mutant, was not evident in cells pre-infected with Intimin deficient mutants *Δmfzgorf3eh::km*, *Δcore::km* or TOEA7*Δcore::km*. This may suggest an effector missing from these latter strains is necessary to promote the phosphorylation of AKT, thus leading to the rapid loss of p-AKT$_{ser473}$ signal, counteracting the need for Intimin. Indeed, similar antagonistic activities of EPEC effectors have previously been reported (Schmidt, 2010; Shames and Finlay, 2012). For example, studies by Shames *et al*., have suggested that the EspZ effector can promote the phosphorylation of AKT in a HeLa cell model (Shames *et al.*, 2010). Importantly, the translocation of EspZ effector into target cells is lacking with the *Δmfzgorf3eh::km*, *Δcore::km* and TOEA7*Δcore::km* mutant strains.

Studies by Roxas *et al*., have shown that the EGFR is degraded in a T3SS dependent manner in a polarised epithelial cell model (Roxas *et al.*, 2014). Given this association it was important to determine impact of EPEC infection on the EGFR in our model. This work reveals the T3SS dependent loss EGFR signal at 3 h post infection in HeLa cells. Importantly, this observation tentatively uncouples the inhibition of EGF induced AKT phosphorylation, at 2 h post infection, from the loss of EGFR. However, whether the EGFR is indeed still functional or present at the cell surface, due to receptor internalisation (Kirisits *et al.*, 2007), is unknown and remains to be investigated.

EPEC induced caspase dependent degradation of the EGFR has been reported to be stimulated by the effector protein EspF and curtailed by the activity of EspZ in polarised cells (Roxas *et al.*, 2014). Although the TOEA7*Δcore::km* mutant is unable to translocate these effector proteins, due to absence of chaperones CesF (EspF dependent) and CesT (EspZ dependent), changes to the EGFR were detected throughout the 5 h infection period. Whilst infection with the TOEA7*Δcore::km* mutant had no obvious impact on total EGFR levels at 2 h post infection, extracts revealed an unexpected second band of lower apparent molecular mass. This band is also evident in studies by Roxas *et al*., supporting the EPEC induced degradation of the EGFR (Roxas *et al.*, 2014). This suggested that additional T3SS dependent effector protein(s) other than EspF and EspZ may play a key role in disrupting EGFR function. Further work should look to identify the EGFR specific changes in cells infected with the EspF and EspZ single/double mutants to better define the capacity of the TOEA7*Δcore::km* mutant to alter the EGFR in this model. Alternatively, the re-introduction of missing genes from the TOEA7*Δcore::km* mutant by complementation could support the requirement of EspF, and/or other effectors, to degrade the EGFR similar to WT EPEC levels. Importantly, this work may suggest the loss of EGF induced AKT phosphorylation in the HeLa cell model is caused by the disruption/degradation of EGFR signalling following the infection with EPEC strains lacking the central 'core' LEE region (*Δcore::km* & TOEA7*Δcore::km*).

Collectively, this work suggested that the capacity of EPEC to inhibit EGF induced AKT phosphorylation is independent of the outer membrane protein Intimin, 6 LEE effectors (Tir, Map, EspH, EspZ, EspF & EspG), 14 Nle effectors (NleA, BleB1, NleB2, NleC, NleD, NleE1, NleE2, NleF, NleG, EspG2/Orf3, NleH1, NleH2, EspJ & EspL) and two effector chaperones (CesF & CesT). However, this interpretation is undermined by significant levels of cell detachment and the ability of EPEC to induce the degradation/modification of the EGFR. This latter finding likely explains the differing observations of CesT dependent and CesT independent inhibition of AKT phosphorylation in the J774A.1 macrophage and HeLa cell model respectively, following infection with the *Δcore::km* and TOEA7*Δcore::km* mutants.

It is important to note the observations in this study were made from one experiment and therefore requires further work to support, or not these findings. Taking into consideration the complexity of EPEC mediated T3SS dependent loss of the EGFR; it would be inappropriate to continue to select EGF as an agonist to examine EPEC induced inhibition of AKT phosphorylation. One possibility could be to develop a receptor independent AKT phosphorylation model, such as the transfection of constitutively activated AKT (Andjelkovic *et al.*, 1997) to examine the effectors necessary to inhibit AKT phosphorylation. As discussed earlier, the HeLa cell model offers important advantages over J774A.1 macrophages to examine EPEC inhibition of AKT phosphorylation, such as greater transfection efficiency. Developing this HeLa cell model further, to overcome the identified limitations, could provide an alternative and more amenable model to interrogate the effector(s) and mechanism responsible for terminating AKT signalling.

Chapter 5 Investigating the mechanism of EPEC induced T3SS dependent decrease of phosphorylated AKT

5.1 Introduction

Class I PI3K are activated downstream of receptor tyrosine kinase (RTK) and G-protein coupled receptors (GPCR) (Vanhaesebroeck *et al.*, 2012). Once activated, class I PI3K's phosphorylate the membrane phosphoinositide PI[4,5]P2, at the 3' hydroxyl group, generating the second messenger PI[3,4,5]P3 (Vanhaesebroeck and Alessi, 2000). Increased availability of PI[3,4,5]P3 stimulates the recruitment of AKT to the inner membrane, dependent on its N-terminal PH domain (Frech *et al.*, 1997). On binding of PI[3,4,5]P3, AKT undergoes a conformational change (Calleja *et al.*, 2007), exposing its activation loop for phosphorylation by PDK1; the latter co-localised with AKT at the membrane in a PH dependent manner (Alessi *et al.*, 1997b). In addition, AKT undergoes a second phosphorylation at residue Ser473 within its hydrophobic motif resulting in the full activation of AKT (Alessi *et al.*, 1996; Alessi *et al.*, 1997b). This second modification step is thought to be regulated by the kinase mTORC2 (Sarbassov *et al.*, 2005), though additional kinases have also been reported to play a role, including DNA-dependent protein kinase (DNA-PK) and TANK binding kinase 1 (TBK1) (Feng *et al.*, 2004; Ou *et al.*, 2011).

Regulation of the PI3K/AKT pathway is a tightly controlled multistep process; however, the precise mechanism by which AKT signalling is regulated is not well understood (Gao *et al.*, 2011). Phosphatase and tensin homolog (PTEN) is a negative regulator of AKT activity that opposes the action of PI3K (Hers *et al.*, 2011). PTEN acts by dephosphorylating the second messenger PI[3,4,5]P3 to PI[4,5]P2, thereby preventing membrane recruitment and co-localisation of AKT and PDK1 (Leslie *et al.*, 2008; Hers *et al.*, 2011). The importance of PTEN in regulating AKT activity is highlighted by its frequent mutation in cancer (Chalhoub and Baker, 2009). In addition, dephosphorylation of PI[3,4,5]P3 by the inositol-phosphatase SHIP, to PI[3,5]P2 is also known to terminate cell signalling downstream of PI3K (Liu *et al.*, 1999b; Carver *et al.*, 2000).

AKT activity and phosphorylation at residues Thr308 and Ser473 are also negatively regulated through dephosphorylation by protein phosphatases (Bayascas and Alessi, 2005). Protein phosphatases PP2A and PHLPP are reported to demonstrate selectivity for residues Thr308 and Ser473 of AKT respectively (Bayascas and Alessi, 2005; Brognard *et al.*, 2007; Liao and Hung, 2010). Subsequent dephosphorylation of both residues restores AKT to an inactive conformation (Liao and Hung, 2010). Though, how these protein phosphatases are themselves regulated and targeted to specific AKT residues is not entirely clear.

The ability of bacteria to manipulate the PI3K/AKT signalling pathway to promote bacterium colonisation and survival is not uncommon, being subverted by *Salmonella enterica* (Steele-Mortimer *et al.*, 2000; Roppenser *et al.*, 2013), *Shigella flexneri* (Pendaries *et al.*, 2006), *Yersinia pseudotuberculosis* (Uliczka *et al.*, 2009) and UPEC (Wiles *et al.*, 2008). These bacteria employ a diverse set of strategies to target the PI3K/AKT pathway; however, in many cases the precise mechanism of action remains to be fully defined.

AKT phosphorylation, and thus its activation, can be regulated at three principal stages including: 1) PI[3,4,5]P3 mediated recruitment and colocalisation to the membrane of AKT and PDK1, 2) phosphorylation of AKT at residues Thr308 and Ser473, or 3) dephosphorylation of AKT and/or PI[3,4,5]P3 by protein and lipid phosphatases. It was therefore hypothesised that EPEC infection may target and subvert one or several of these events to induce the rapid T3SS dependent decrease of phosphorylated AKT. The aim of this chapter was thus to interrogate the mechanism by which EPEC disrupts AKT signalling in the J774A.1 macrophage cell model.

5.2 Results

5.2.1 EPEC infection is associated with subtle T3SS dependent differences in key events associated with the AKT signalling pathway

As discussed earlier (Chapter 3), EPEC infection induces a rapid T3SS dependent decrease of phosphorylated AKT in the J774A.1 macrophage cell model (Celli *et al.*, 2001; Quitard *et al.*, 2006). To investigate the possible mechanism(s) of inhibition, cell extracts were fractionated into cytoplasmic and membrane proteins using a well-established protocol (Kenny *et al.*, 1997; Kenny, 1999; Warawa and Kenny, 2001), to identify potential T3SS dependent changes in key AKT signalling associated events. Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated T3SS mutant (*cfm-14*) or WT EPEC strain at an MOI of 200:1, before isolating cell extracts over a 120 min infection period. J774A.1 macrophages were then fractionated into cytosolic and membrane fractions for western blot analysis. This protocol was known as the standard 'one-wave' infection strategy.

Prior to interrogating the impact of EPEC infection on the PI3K-AKT signalling pathway, it was first necessary to confirm the T3SS dependent decrease of phosphorylated AKT (Ser473) in this fractionated model. As predicted, infection with the WT EPEC strain triggered a rapid infection induced increase of p-AKT$_{Ser473}$ signal followed by a swift decline to or below background levels in both the cytosolic (Figure 39A & Figure 39B) and membrane (Figure 39C & Figure 39D) fraction at 60 min post infection. In contrast, infection with the T3SS mutant (*cfm-14*) revealed a more sustained increase of p-AKT$_{Ser473}$ signal over the 120 min infection period, decreasing to background levels after 90 min of infection in both the cytosolic (Figure 39A & Figure 39B) and membrane fractions (Figure 39C & Figure 39D). As discussed in Chapter 3, this T3SS dependent decrease of phosphorylated AKT was correlated with the detection of the host kinase modified form of Tir (T''). As expected (Warawa and Kenny, 2001), host modified Tir (T'') was detected in the membrane but not cytosolic fraction of WT EPEC infected cells only; supporting the insertion of EPEC effector Tir into the host cell membrane. Thus, this work supports the development of a fractionated cell model and corroborates findings of a rapid T3SS dependent decrease of phosphorylated AKT in EPEC infected cell extracts (cytosolic & membrane fraction).

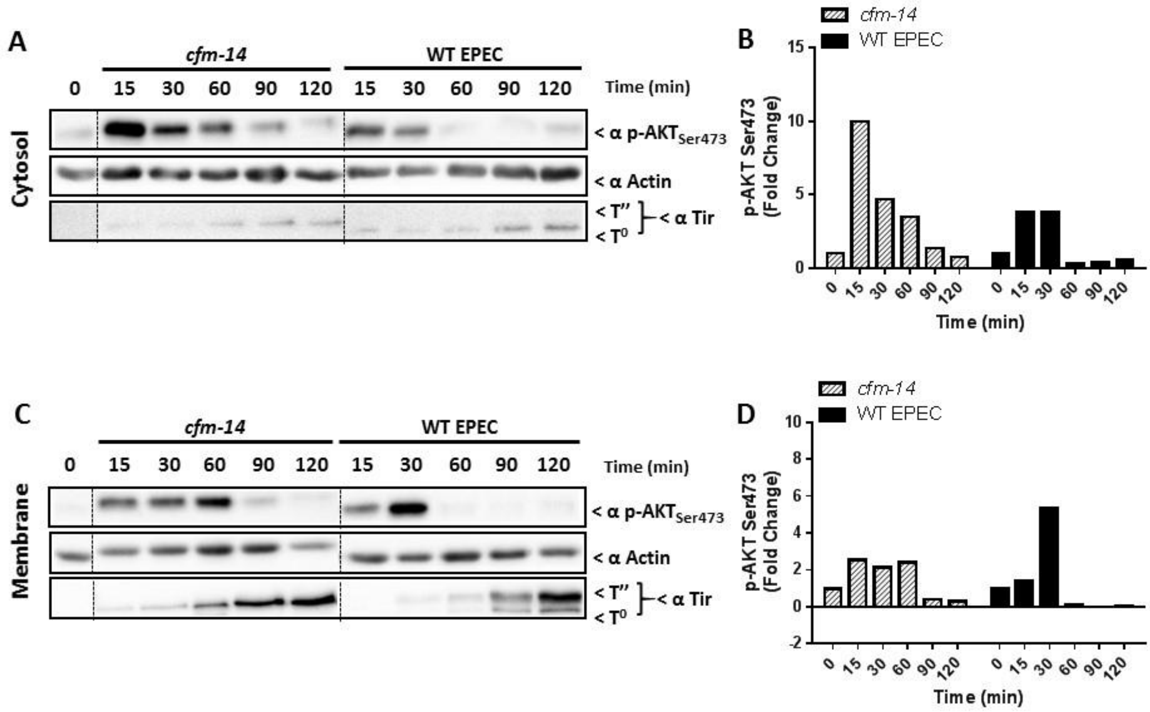


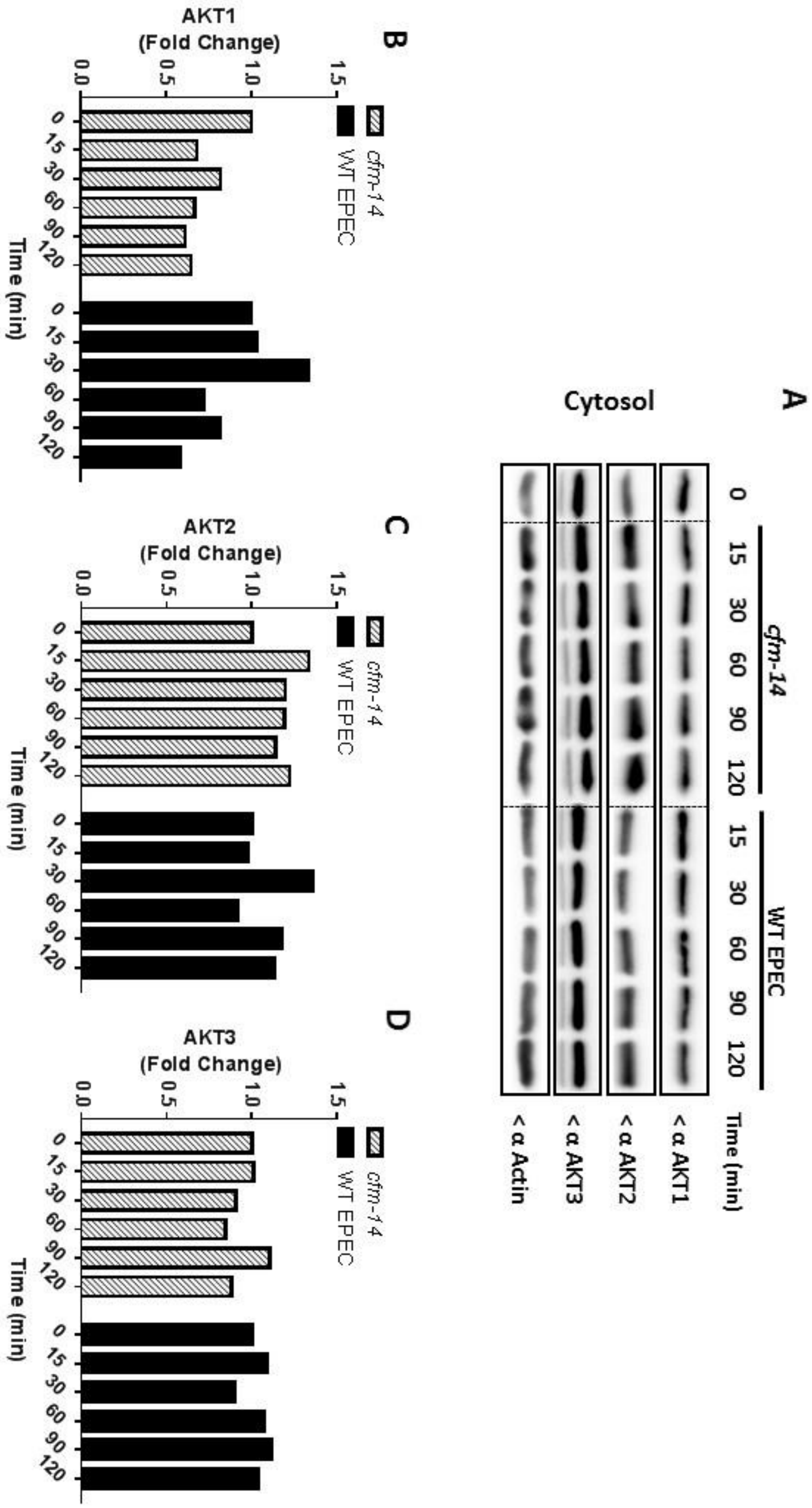
Figure 39 EPEC induces a rapid T3SS dependent decrease of phosphorylated AKT in cytoplasmic and membrane extracts.

J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated WT EPEC or T3SS mutant (*cfm-14*) strains. Cells were washed after 15, 30, 60, 90 and 120 min of infection and J774A.1 macrophages fractionated into host cytosolic and membrane proteins. (**A & C**) Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{Ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**B & D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{Ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to uninfected (0) cells.

To investigate the impact of EPEC infection on the localisation of AKT, cell extracts were probed for AKT isoforms 1, 2 and 3 (Hers *et al.*, 2011). Analysis of the cytoplasmic fractions of both the WT EPEC and T3SS mutant (*cfm-14*) infected cells revealed very little, if any, change in the levels of all AKT isoforms (1, 2 & 3) from that of uninfected control (Figure 40A-D). Though, probing for AKT1 did reveal a subtle decrease below background levels for both strains (Figure 40B). By contrast, infection with the WT EPEC or T3SS mutant (*cfm-14*) led to the transient increase in AKT isoforms (1, 2 & 3) above background levels in the membrane fraction (Figure 40E-H). This transient increase of AKT (isoforms 1, 2 & 3) at the membrane peaked after 30 and 60 min of infection with the WT EPEC and *cfm-14* mutant respectively. Interestingly, peak membrane localisation of AKT (isoforms 1, 2 & 3) was correlated with the maximal phosphorylation of AKT (Ser473) in the membrane fraction of WT EPEC, but not T3SS mutant (*cfm-14*) infected cells (Figure 39C & Figure 40E).

The phosphorylation of PDK1 at Ser241 is reported to increase its activity, which is necessary to phosphorylate AKT at Thr308 (Casamayor *et al.*, 1999). Conversely, the phosphorylation of PTEN at Ser380 is thought to promote its stability and decrease its activity, thereby preventing the dephosphorylation of PI[3,4,5]P3 to PI[4,5]P2 (Hopkins *et al.*, 2014; Chia *et al.*, 2015). Thus, active PDK1 and PTEN have an important role in regulating AKT phosphorylation. Probing of the cytoplasmic fractions revealed similar profiles of p-$PDK1_{Ser241}$ and p-$PTEN_{Ser380}$ for both the WT EPEC and *cfm-14* mutant infected cells (Figure 41A-C). This was characterised by the apparent decrease of p-$PDK1_{Ser241}$ and transient increase of p-$PTEN_{Ser380}$ in the cytosolic extract for both strains (Figure 41A-C). In contrast, analysis of the membrane fractions revealed subtle T3SS dependent differences in the localisation of both proteins. Infection with the WT EPEC strain led to a sustained increase of p-$PDK1_{Ser241}$ in the membrane fraction, which was less apparent for the T3SS mutant (*cfm-14*) (Figure 41D & Figure 41E). Furthermore, probing for p-$PTEN_{Ser380}$ revealed the short transient increase of signal in membrane extract of WT EPEC infected cells, decreasing to or below background levels after 60 min of infection (Figure 41D & Figure 41F). Whilst this increase of p-$PTEN_{Ser380}$ was also transient for the *cfm-14* mutant, this decline of signal was notably more gradual; returning to background levels at 120 min post infection (Figure 41D & Figure 41F).

Collectively, this work suggests that EPEC infection is linked with subtle T3SS dependent differences in the subcellular localisation (cytoplasmic & membrane) of key proteins associated with regulating the phosphorylation of AKT. However, this one-wave infection model provides little insight into the cause and/or mechanism behind these T3SS dependent alterations. Whilst these subtle T3SS dependent differences may reflect an inhibitory mechanism, it was predicted these findings were likely caused by an unsynchronised (one-wave) infection strategy, resulting in variable populations of cells at early time points with sufficient cell-adherent bacteria to interfere with AKT phosphorylation.


A
Cytosol
Time (min)
0 15 30 60 90 120 15 30 60 90 120
cfm-14
WT EPEC
< α AKT1
< α AKT2
< α AKT3
< α Actin
B
AKT1 (Fold Change)
C
AKT2 (Fold Change)
D
AKT3 (Fold Change)
cfm-14
WT EPEC
Time (min)
0.0 0.5 1.0 1.5
0 15 30 60 90 120

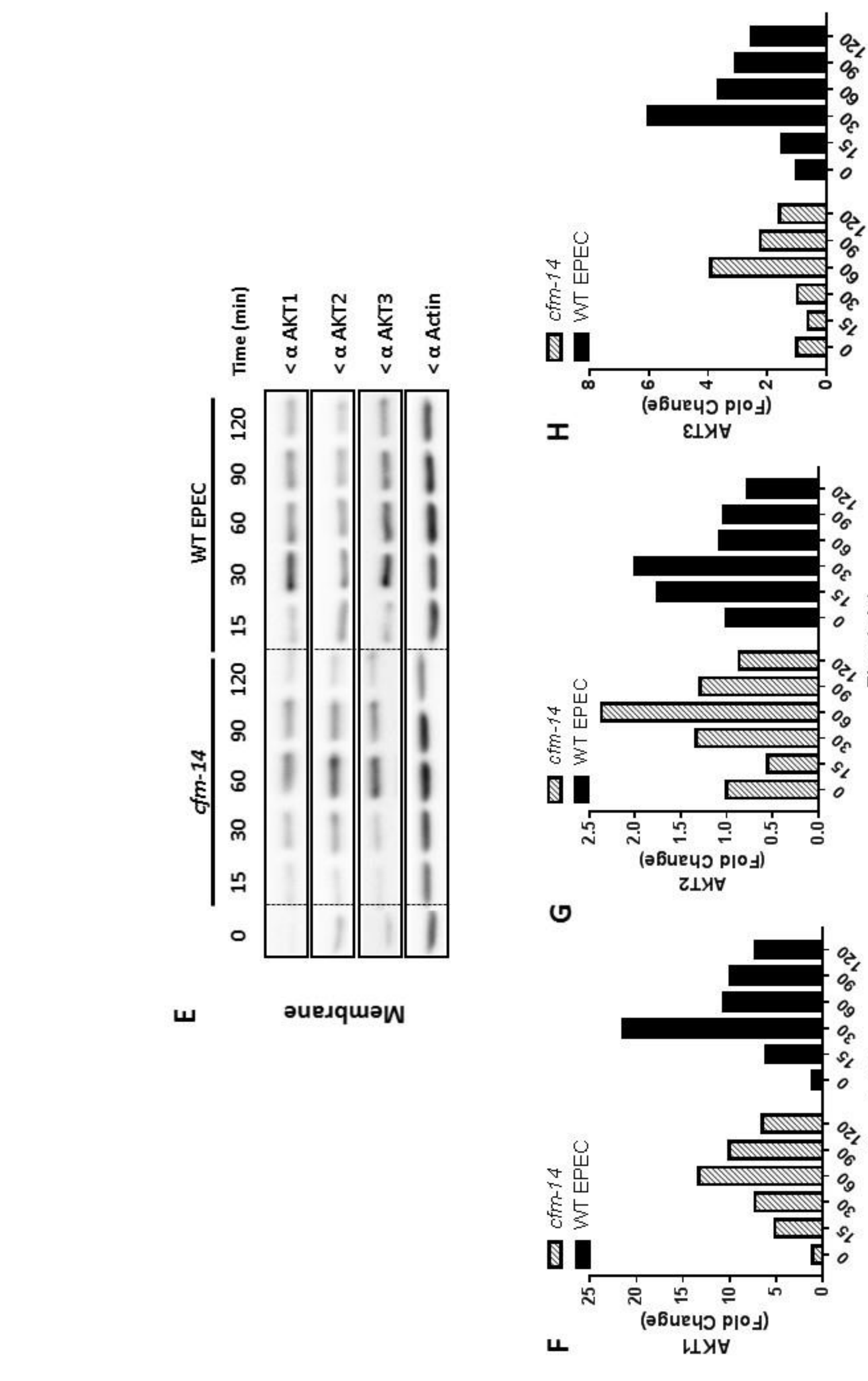

E
Membrane
cfm-14
WT EPEC
Time (min)
0
15
30
60
90
120
< α AKT1
< α AKT2
< α AKT3
< α Actin
F
AKT1 (Fold Change)
G
AKT2 (Fold Change)
H
AKT3 (Fold Change)
Time (min)

Figure 40 EPEC induces the transient localisation of AKT (isoforms 1, 2 & 3) to the membrane.

J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated WT EPEC or T3SS mutant (*cfm-14*) strains. Cells were washed after 15, 30, 60, 90 and 120 min of infection and J774A.1 macrophages fractionated into host cytosolic and membrane proteins. (**A & E**) Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-AKT1, anti-AKT2, anti-AKT3 and anti-Actin (loading control) antibodies. Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, D, F, G & H**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in AKT1, AKT2 or AKT3 signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (0) cells.

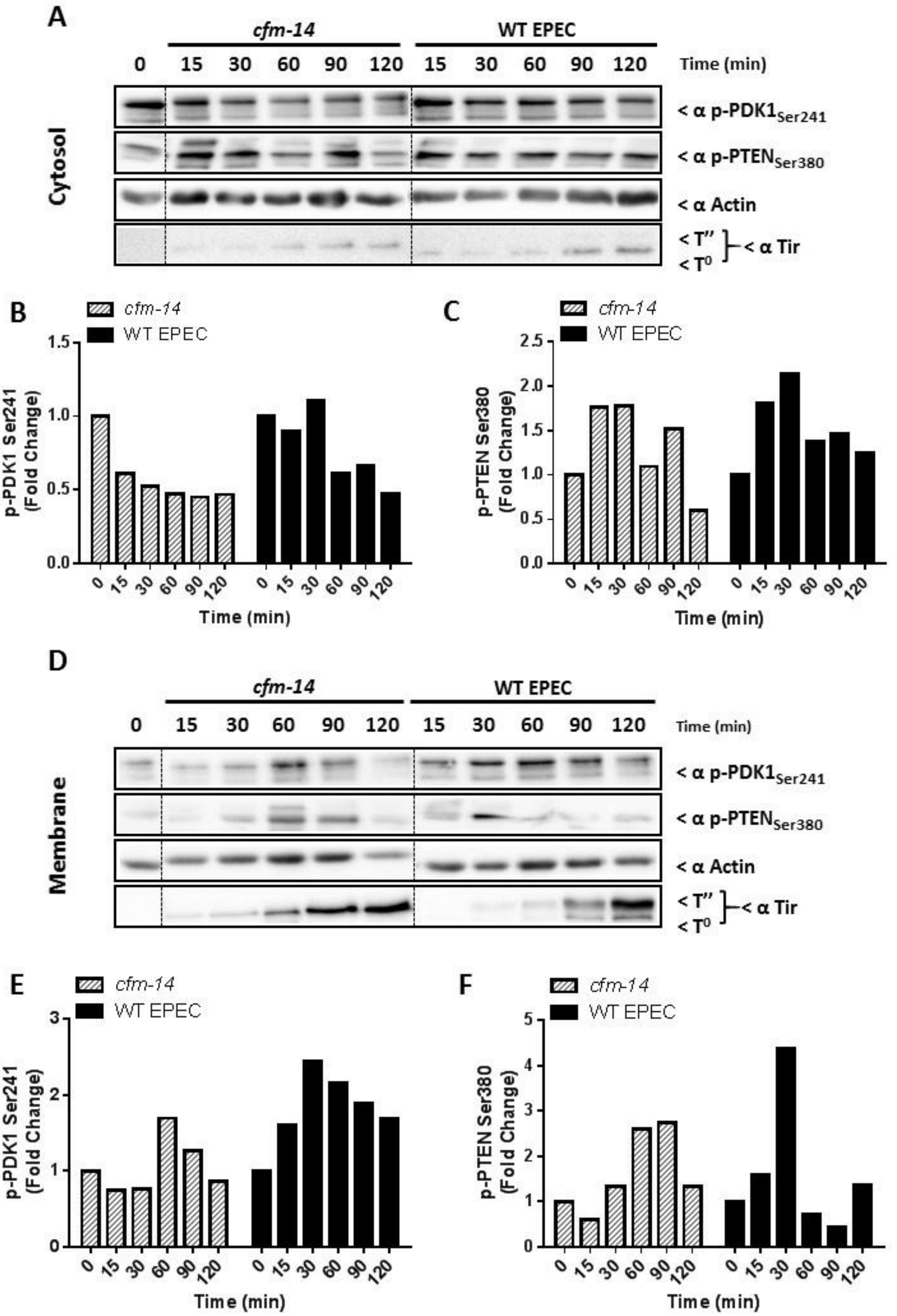

A
cfm-14
WT EPEC
0 15 30 60 90 120 15 30 60 90 120
Time (min)
Cytosol
< α p-PDK1 Ser241
< α p-PTEN Ser380
< α Actin
< T''
< T0
< α Tir
B
cfm-14
WT EPEC
p-PDK1 Ser241 (Fold Change)
Time (min)
C
cfm-14
WT EPEC
p-PTEN Ser380 (Fold Change)
Time (min)
D
cfm-14
WT EPEC
0 15 30 60 90 120 15 30 60 90 120
Time (min)
Membrane
< α p-PDK1 Ser241
< α p-PTEN Ser380
< α Actin
< T''
< T0
< α Tir
E
cfm-14
WT EPEC
p-PDK1 Ser241 (Fold Change)
Time (min)
F
cfm-14
WT EPEC
p-PTEN Ser380 (Fold Change)
Time (min)

Figure 41 EPEC induces subtle T3SS dependent changes in the localisation of phosphorylated PDK1 (Ser241) and PTEN (Ser380).

J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated WT EPEC strain or T3SS mutant (*cfm-14*). Cells were washed after 15, 30, 60, 90 and 120 min of infection and J774A.1 macrophages fractionated into host cytosolic and membrane proteins. (**A & D**) Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-PDK1$_{ser241}$, anti-p-PTEN$_{ser380}$ and anti-Actin (loading control) antibodies. Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, E & F**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-PDK1$_{ser241}$ or p-PTEN$_{ser380}$ signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (0) cells.

5.2.2 EPEC induced loss of phosphorylated AKT is not readily reversible in a fractionated two-wave infection model

To overcome the limitations of a one-wave infection model, this study switched to a two-wave infection strategy. This strategy examines the ability of cells pre-infected with EPEC to phosphorylate AKT in response to a second-wave infection with the T3SS mutant (*cfm-14*). Thus, J774A.1 macrophages were left uninfected or infected with pre-activated EPEC strains (WT EPEC, *cfm-14* or *Δquad* [lacking LEE encoded Map, EspF, Tir & Intimin] mutant) at an MOI of 200:1 for 1 h. After infection, cells were washed and then treated with gentamicin (kill extracellular bacteria) for 1 h, before leaving cells to recover in fresh cell culture media for 1 h. Post recovery, cells were then left uninfected or infected with the T3SS mutant (*cfm-14*) for 15, 30 and 60 min at an MOI of 100:1 to trigger AKT phosphorylation. Cell extracts were isolated after treatment with gentamicin/recovery (1st wave) and after a second infection with the *cfm-14* strain (2nd wave). As described earlier, cell extracts were then fractionated into cytosolic and membrane fractions for western blot analysis (Kenny *et al.*, 1997; Kenny, 1999; Warawa and Kenny, 2001).

As expected, analysis of the first-wave infection (post gentamicin/recovery) with the T3SS mutant (*cfm-14*), but not the WT EPEC or *Δquad* strain, revealed the robust increase of phosphorylated AKT (Ser473 & Thr308) above the level of uninfected control in the cytosolic fraction (Figure 42A-C). Furthermore, subsequent challenge with the *cfm-14* mutant (2nd wave) induced the significant time dependent increase of phosphorylated AKT (Ser473 & Thr308) in cells pre-infected with the T3SS mutant (*cfm-14*), but not the WT EPEC or *Δquad* strain (Figure 42A-C). Similar results were evident in the membrane fraction for cells pre-infected T3SS mutant (*cfm-14*) or WT EPEC strain as part of a first-wave and second-wave infection strategy (Figure 42D-F). However, in contrast to the cytosolic fraction, cells pre-infected with the *Δquad* mutant revealed an increase in the levels of membrane p-AKT$_{Thr308}$, but not p-AKT$_{Ser473}$, after the first-wave (post gentamicin/recovery) and second-wave (*cfm-14*) infections (Figure 42D-F). Though not statistically significant, this increase of membrane p-AKT$_{Thr308}$ signal after infection with the *Δquad* mutant was similar to that induced in cells pre-infected with the T3SS mutant (*cfm-14*). Whilst not verified statistically, it is also interesting to note the detection of phosphorylated AKT (Ser473 & Thr308) was notably greater in the cytosol than membrane fraction following infection with all strains, with the levels of p-AKT$_{Ser473}$ typically higher than that of p-AKT$_{Thr308}$ (Figure 42).

Fractionation of host cells into cytoplasmic and membrane proteins were examined by probing for the host cytosolic protein lactate dehydrogenase (LDH) and the insertion modification of the EPEC effector Tir (T'') into the host cell membrane (Warawa and Kenny, 2001). As expected, greater levels of LDH were detected in the cytosolic fraction of all samples (Figure 42A & Figure 42D), whilst detection of the host kinase modified form of Tir (T'') was primarily detected in the membrane fraction of WT EPEC infected cells only (Figure 42D & Figure 42A). EPEC strain genotype was supported by the absence of Tir (*Δquad*), detection of the unmodified form of Tir (T^0; *cfm-14*) or the host modified form of Tir (T''; WT EPEC) in samples from infected cells (Figure 42A & Figure 42D).

This work reveals that EPEC infection can prevent AKT phosphorylation in a T3SS dependent manner, which is stable for up to 3 h (time between 1st & 2nd wave infections) and thus not readily reversible. Furthermore, these findings show that EPEC effectors Map, EspF, Tir and the outer membrane protein Intimin do not play a role in the loss of p-AKT$_{Ser473}$ signal (cytosolic & membrane), though may be necessary to prevent/decrease the levels of p-AKT$_{Thr308}$ at the membrane. Taken together, this work supports the development of a robust two-wave (fractionated) infection model to examine the T3SS dependent loss of phosphorylated AKT.

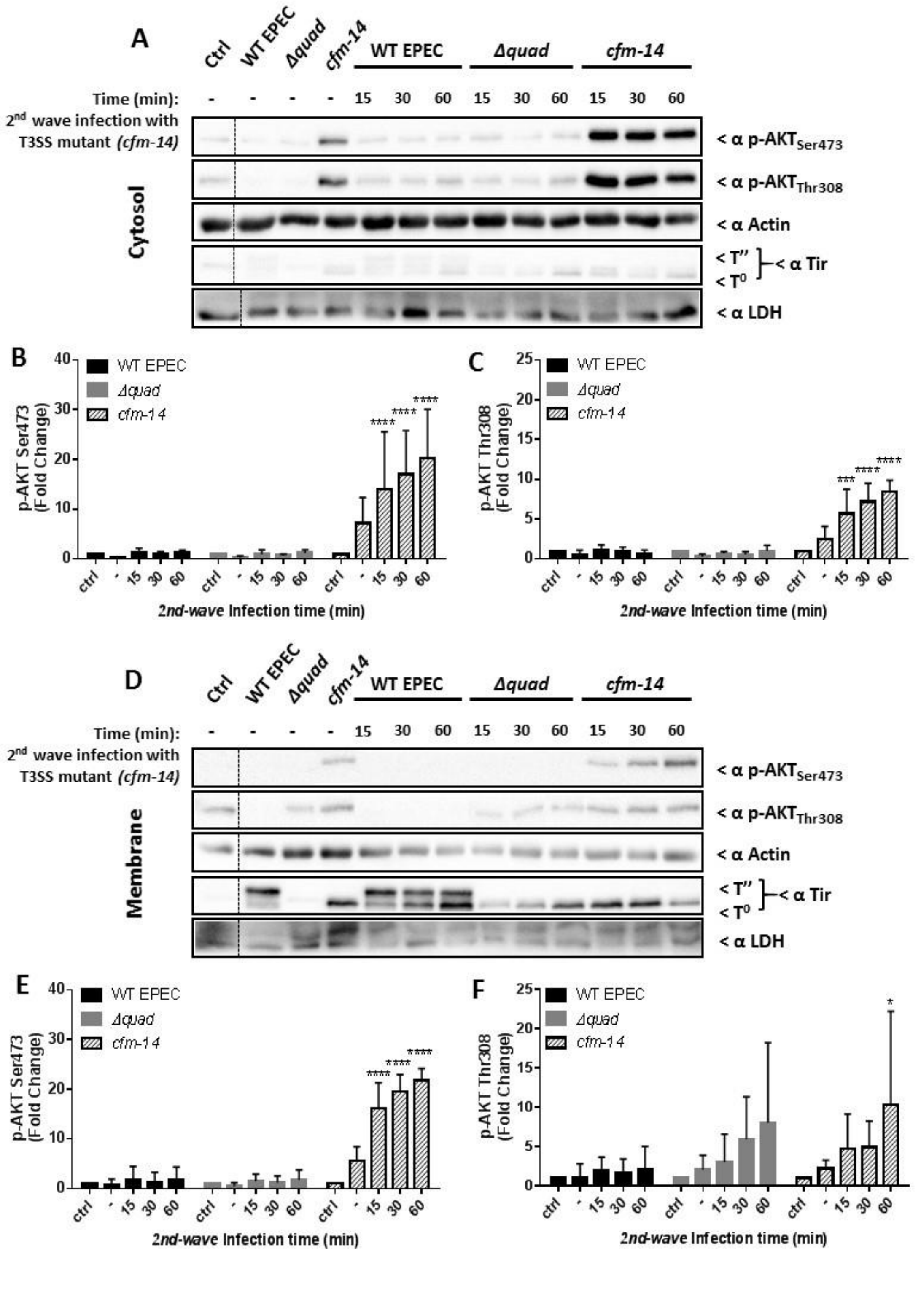

A
Ctrl
WT EPEC
Δquad
cfm-14
WT EPEC
Δquad
cfm-14
Time (min):
- - - - 15 30 60 15 30 60 15 30 60
2nd wave infection with T3SS mutant (cfm-14)
Cytosol
< α p-AKTSer473
< α p-AKTThr308
< α Actin
< T''
< T0
< α Tir
< α LDH
B
WT EPEC
Δquad
cfm-14
p-AKT Ser473 (Fold Change)
2nd-wave Infection time (min)
ctrl - 15 30 60 ctrl - 15 30 60 ctrl - 15 30 60
C
p-AKT Thr308 (Fold Change)
D
Membrane
E
F

Figure 42 EPEC pre-infection inhibits *cfm-14* induced AKT phosphorylation in a T3SS dependent manner in a two-wave infection model.

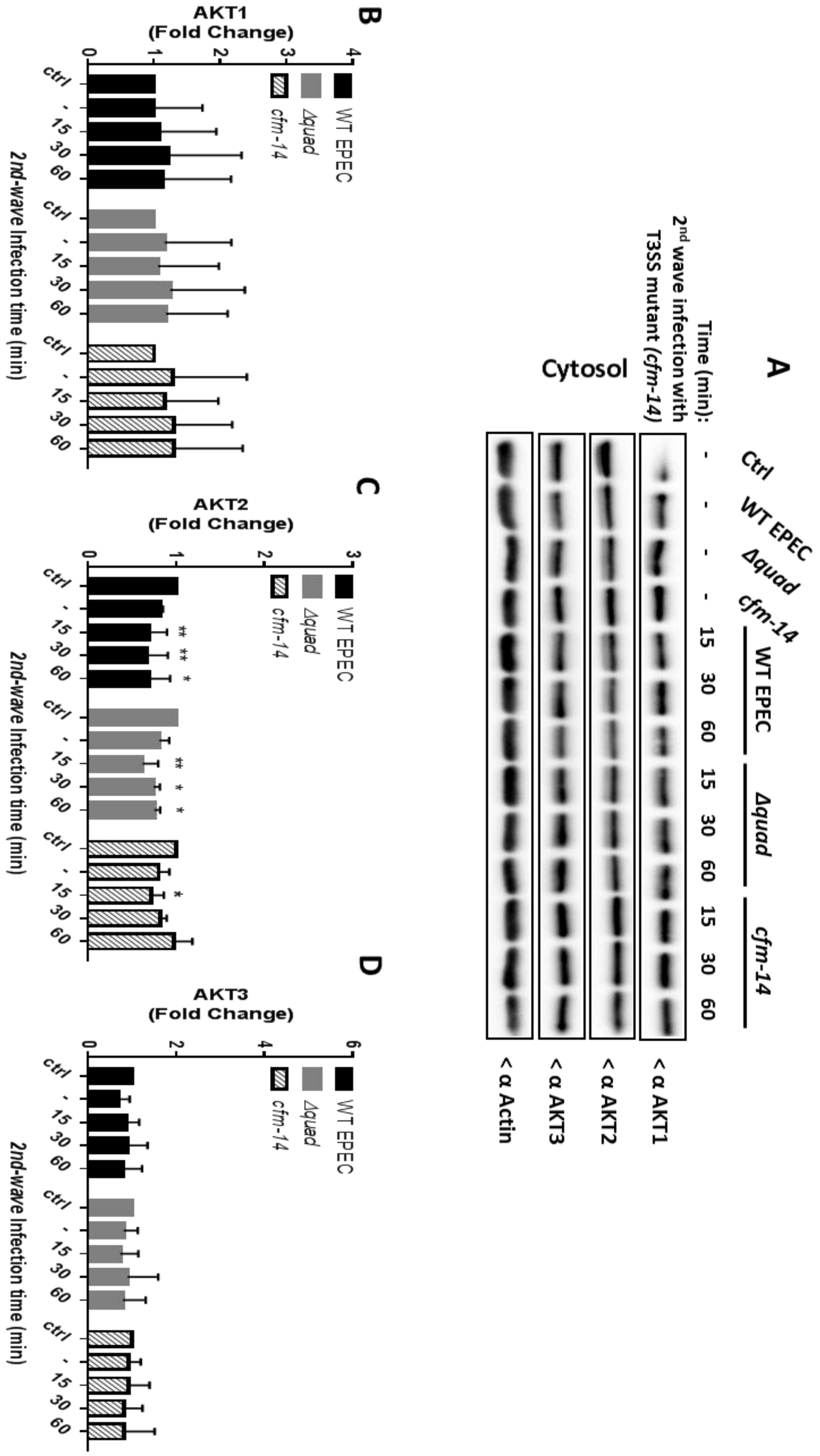
J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 1 h with the pre-activated WT EPEC, *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) or the T3SS mutant (*cfm-14*) strains. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h (kill extracellular bacteria), followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2^{nd} infection; MOI 100:1) for 15, 30 and 60 min. Cell extracts were isolated after the first-wave infection ('-'; post gentamicin treatment & recovery) or following a second-wave infection with the *cfm-14* strain. (**A & D**) J774A.1 macrophages were fractionated into cytosolic and membrane proteins, before separating by SDS-PAGE (10%) for western blot analysis; probing for anti-p-AKT_{Thr308}, anti-p-AKT_{ser241}, anti-Tir (membrane marker), anti-LDH (cytosolic marker) and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, E & F**) Densitometry analysis of western blot was performed using ImageLab software, with values representing a fold change in p-AKT_{ser241} or p-AKT_{Thr308} signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (Ctrl) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (Ctrl; ****p<0.0001, ***p<0.001, **P<0.01 and *p<0.05).

5.2.3 EPEC infection does not decrease AKT membrane localisation

To examine if the capacity of EPEC to prevent AKT phosphorylation (Ser473 & Thr308) in a T3SS dependent manner was due to a decrease in the localisation of AKT to the membrane, cell extracts were probed for the AKT isoforms 1, 2 and 3. Analysis of the cytosolic fractions revealed the robust detection AKT isoforms 1, 2 and 3 in uninfected cells, with no notable change after the first-wave infection (post gentamicin/recovery) with the *cfm-14*, WT EPEC or *Δquad* mutant; or following a second-wave infection with the *cfm-14* strain (Figure 43A-D). Though not immediately obvious by western blot analysis, quantification of cytosolic AKT2 revealed a small but significant decrease in levels for cells pre-infected with the WT EPEC, *Δquad* and T3SS mutant (*cfm-14*), stimulated after a second infection with the *cfm-14* strain (2^{nd} wave; Figure 43C).

Similar to the cytosolic fraction, detection of AKT1 and AKT2 was largely unaltered from background levels in membrane extract after the first-wave infection (post gentamicin/recovery) with the *cfm-14*, WT EPEC or *Δquad* mutant; or following a second infection with the *cfm-14* strain (2^{nd} wave; Figure 43E-H). However, infection with the *cfm-14*, WT EPEC or *Δquad* mutant (1^{st} wave infection; post gentamicin/recovery) was associated with the increase of AKT3 at the membrane, though not always statistically significant (Figure 43H). Interestingly, this increase of AKT3 was sustained after a second infection with the *cfm-14* strain (2^{nd} wave) in cells pre-infected with the WT EPEC and *Δquad* strain. Whilst this increase in AKT3 signal was also evident for cells pre-infected with the T3SS mutant (*cfm-14*) this was not supported to be statistically significant by quantification (Figure 43H).

These findings suggest that EPEC infection does not prevent AKT (isoforms 1, 2 & 3) localisation to the membrane fraction in a T3SS dependent manner. This implies that the capacity of EPEC to prevent AKT phosphorylation (Ser473 & Thr308) is likely mediated by an alternate mechanism. In addition, this work suggests that increased AKT phosphorylation in T3SS mutant (*cfm-14*) pre-infected cells, stimulated by second-wave infection (*cfm-14*), may be linked to the increase of AKT3 at the membrane.


A
Time (min):
2nd wave infection with T3SS mutant (cfm-14)
Cytosol
Ctrl
WT EPEC
Δquad
cfm-14
15 30 60 15 30 60 15 30 60
< α AKT1
< α AKT2
< α AKT3
< α Actin
B
AKT1 (Fold Change)
C
AKT2 (Fold Change)
D
AKT3 (Fold Change)
WT EPEC
Δquad
cfm-14
ctrl
15
30
60
2nd-wave Infection time (min)

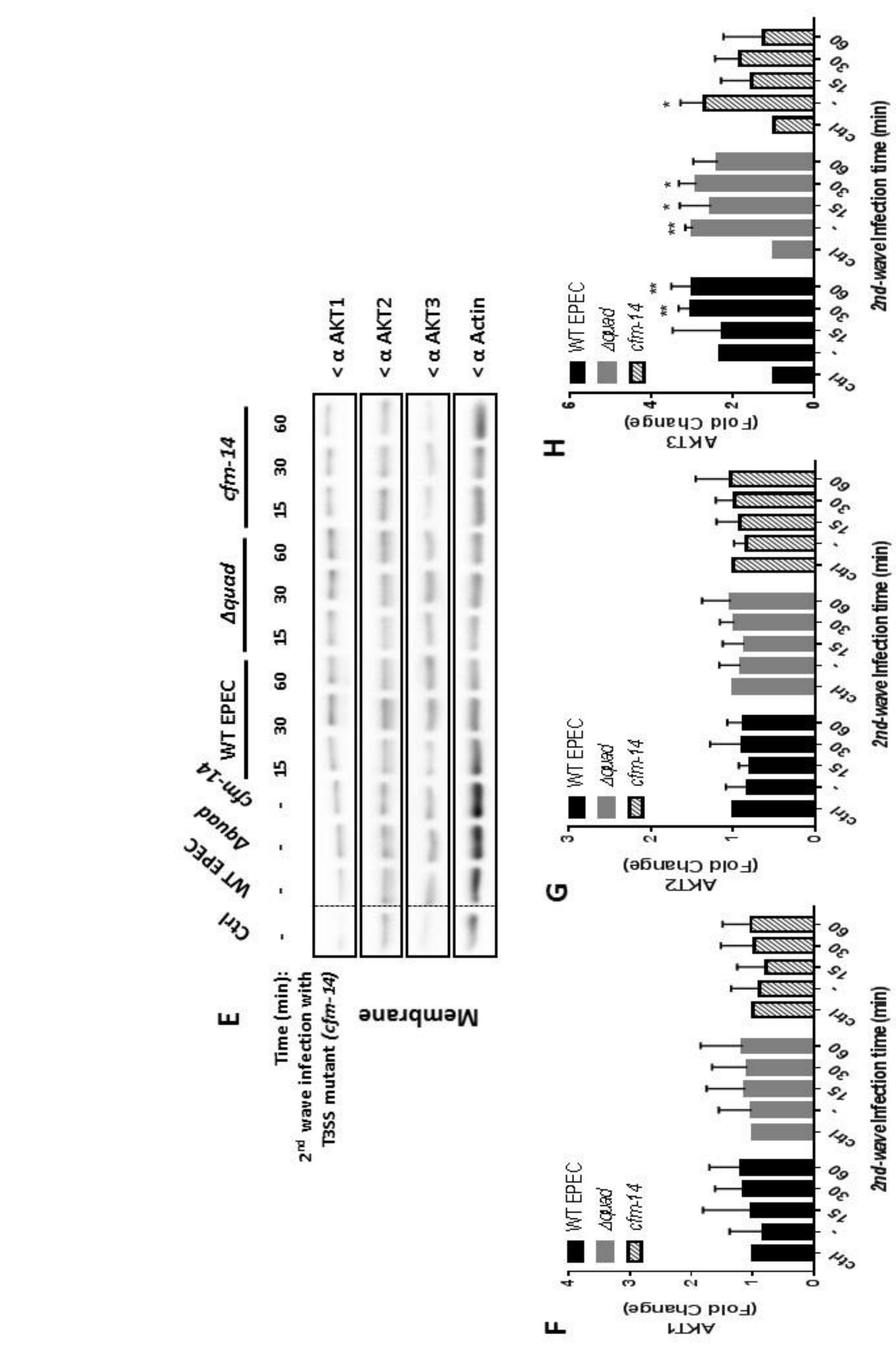

E
Time (min):
2nd wave infection with
T3SS mutant (cfm-14)
Ctrl
WT EPEC
Δquad
cfm-14
WT EPEC
Δquad
cfm-14
15
30
60
Membrane
< α AKT1
< α AKT2
< α AKT3
< α Actin
F
AKT1
(Fold Change)
G
AKT2
(Fold Change)
H
AKT3
(Fold Change)
2nd-wave Infection time (min)

Figure 43 The localisation of AKT (isoforms 1, 2 & 3) is not significantly disrupted by EPEC infection in a T3SS dependent manner.

J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 1 h with the pre-activated WT EPEC, *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) or the T3SS mutant (*cfm-14*) strains. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h (kill extracellular bacteria), followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2nd infection; MOI 100:1) for 15, 30 and 60 min. Cell extracts were isolated after the first-wave infection ('-'; post gentamicin treatment & recovery) or following a second-wave infection with the *cfm-14* strain. (**A & E**) J774A.1 macrophages were fractionated into cytosolic and membrane proteins, before separating by SDS-PAGE (10%) for western blot analysis; probing for anti-AKT1, anti-AKT2, anti-AKT3 and anti-Actin (loading control) antibodies. Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, D, F, G & H**) Densitometry analysis of western blot was performed using ImageLab software, with values representing a fold change in AKT1, AKT2 or AKT3 signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (Ctrl) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (Ctrl; $^{**}P<0.01$ and $^{*}p<0.05$).

5.2.4 The T3SS dependent inhibition of phosphorylated AKT is not linked to notable differences in the localisation of phosphorylated PDK1 (Ser241) or PTEN (Ser380)

As discussed earlier, AKT phosphorylation at Thr308 is regulated by the kinase PDK1 (Alessi *et al.*, 1997b). This phosphorylation is antagonised by the phosphoinositide phosphatase PTEN, converting PI[3,4,5]P3 to PI[4,5]P2 and thus preventing co-localisation of AKT and PDK1 at the membrane (Leslie *et al.*, 2001; Hers *et al.*, 2011). To investigate whether the T3SS dependent inhibition of AKT phosphorylation is linked to T3SS dependent changes in the phosphorylation associated activation of PDK1 (Ser214) or the inhibition of PTEN (Ser380), cell extracts were probed for p-PDK1$_{Ser214}$ and p-PTEN$_{Ser380}$ (Casamayor *et al.*, 1999; Hopkins *et al.*, 2014).

Examining the cytosolic extract revealed very little notable change in the levels of p-PDK1$_{Ser214}$ or p-PTEN$_{Ser380}$ from that of uninfected control after infection (1st wave post gentamicin/recovery) with the WT EPEC, *Δquad* or T3SS mutant (*cfm-14*; Figure 44A-C). Furthermore, this level of p-PDK1$_{Ser214}$ or p-PTEN$_{Ser380}$ was largely maintained after a second-wave infection with the *cfm-14* strain (2nd wave; Figure 44A-C). However, quantification analysis did reveal a small but significant decrease in levels of cytosolic p-PDK1$_{Ser214}$ after this second wave infection for cells pre-infected with the WT EPEC (15 & 30 min post 2nd infection) and *Δquad* (15 min post 2nd infection) strain (Figure 44B). In contrast, analysis of the membrane fraction revealed a small, though rarely significant, increase of p-PDK1$_{Ser214}$ and p-PTEN$_{Ser380}$ in cells pre-infected with the WT EPEC, *Δquad* or T3SS mutant (*cfm-14*; 1st wave infections); and following a second infection with the *cfm-14* strain (2nd wave; Figure 44D-F).

This work suggests there are no notable T3SS dependent changes in the level or subcellular localisation of p-PDK1$_{Ser214}$ (active form) or p-PTEN$_{Ser380}$ (inactive form) that may explain the T3SS dependent inhibition of AKT phosphorylation induced by second wave infection (*cfm-14*).

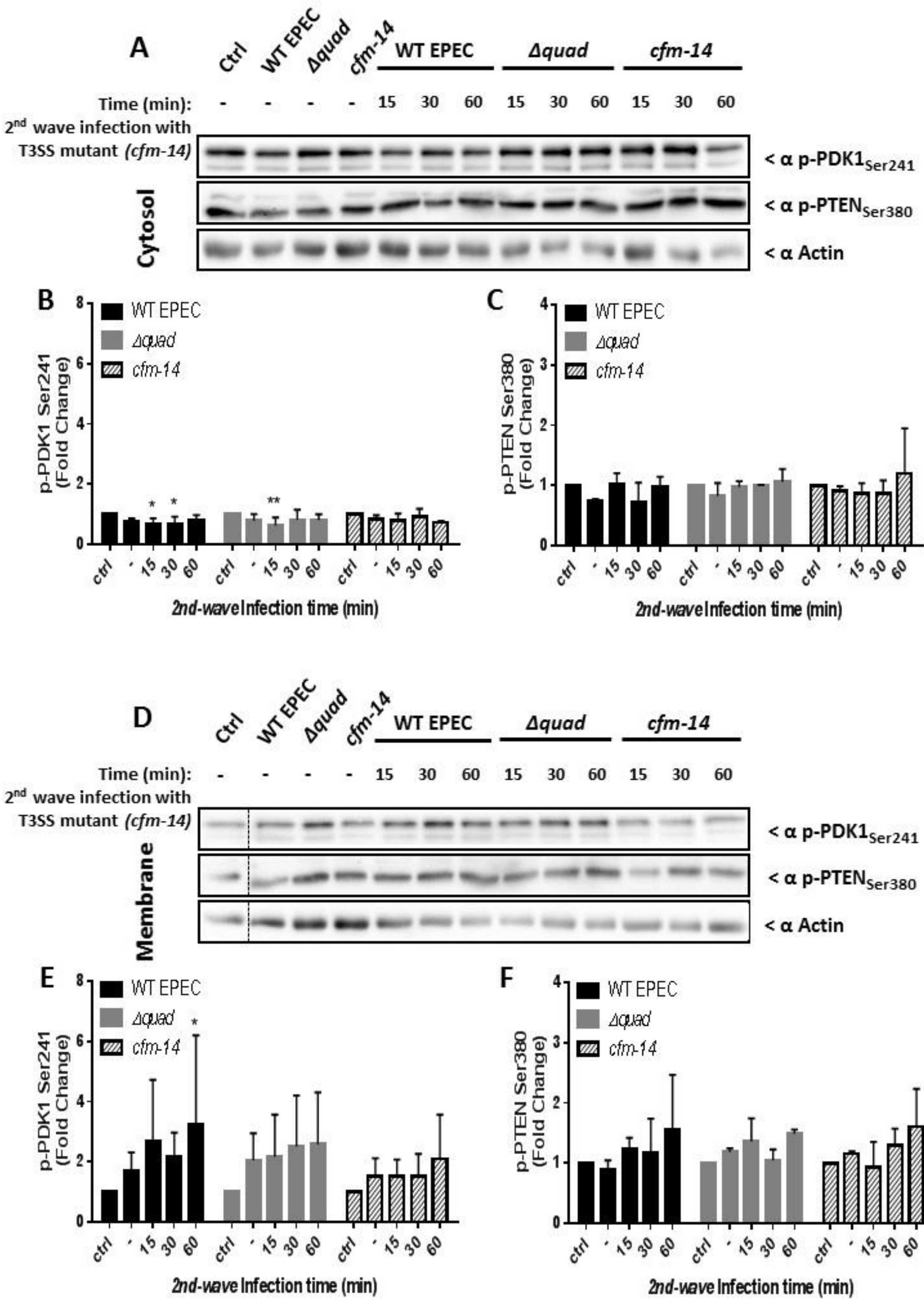

A
Ctrl
WT EPEC
Δquad
cfm-14
WT EPEC
Δquad
cfm-14
Time (min):
15 30 60 15 30 60 15 30 60
2nd wave infection with T3SS mutant (cfm-14)
Cytosol
< α p-PDK1Ser241
< α p-PTENSer380
< α Actin
B
p-PDK1 Ser241 (Fold Change)
WT EPEC
Δquad
cfm-14
2nd-wave Infection time (min)
C
p-PTEN Ser380 (Fold Change)
D
Membrane
E
F

Figure 44 EPEC infection does not impact on the localisation or level of phosphorylated PDK1 (Ser241) or PTEN (Ser380) in a T3SS dependent manner.

J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 1 h with the pre-activated WT EPEC, *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) or the T3SS mutant (*cfm-14*) strains. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h (kill extracellular bacteria), followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2^{nd} infection; MOI 100:1) for 15, 30 and 60 min. Cell extracts were isolated after the first-wave infection ('-'; post gentamicin treatment & recovery) or following a second-wave infection with the *cfm-14* strain. (**A & D**) J774A.1 macrophages were fractionated into cytosolic and membrane proteins, before separating by SDS-PAGE (10%) for western blot analysis; probing for anti- p-$PDK1_{ser241}$, anti-p-$PTEN_{ser380}$ and anti-Actin (loading control) antibodies. Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, E & F**) Densitometry analysis of western blot was performed using ImageLab software, with values representing a fold change in p-$PDK1_{ser241}$ or p-$PTEN_{ser380}$ signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (Ctrl) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (Ctrl; $^{**}P<0.01$ and $^{*}p<0.05$).

5.2.5 EPEC infection decreases the level of phosphorylated cytosolic c-Raf (Ser259) in a T3SS dependent manner

Once fully active, AKT can phosphorylate a number of downstream targets including c-Raf (Ser259) and GSK3β (Ser9) (Manning and Cantley, 2007). To support the capacity of EPEC to prevent AKT phosphorylation and thus its activation in a T3SS dependent manner, cell extracts were probed for p-c-Raf_{Ser259} and p-GSK3β_{Ser9}.

Probing of the cytosolic fractions revealed a notable, though not statistically significant, decrease of p-c-Raf_{Ser259} and p-GSK3β_{Ser9} after the first-wave infection (post gentamicin/recovery) of cells pre-infected with the WT EPEC and *Δquad* strain, but not the T3SS mutant (*cfm-14*; Figure 45A-C). Whilst the second-wave infection with the *cfm-14* strain (2^{nd} wave) was found to induce a modest increase of cytosolic p-c-Raf_{Ser259} and p-GSK3β_{Ser9} in cells pre-infected with the T3SS mutant (*cfm-14*), this was not the case for WT EPEC or *Δquad* mutant (Figure 45A-C). Intriguingly, the second-wave infection of cells pre-infected with the WT EPEC or *Δquad* strain was associated with little change in levels of p-GSK3β_{Ser9}, yet induced a significant time dependent decrease of p-c-Raf_{Ser259} below the level of uninfected control (Figure 45B). By contrast, analysis of the membrane fraction of cells infected with the WT EPEC, *Δquad* or T3SS mutant; as part of a one-wave (post gentamicin/recovery) or two-wave infection strategy, revealed little if any T3SS dependent differences in the p-c-Raf_{Ser259} or p-GSK3β_{Ser9} signals (Figure 45D-F). Quantification analysis demonstrates the increase of membrane p-c-Raf_{Ser259}, but not p-GSK3β_{Ser9}, after first-wave and second-wave infection of cells pre-infected with the WT EPEC, *Δquad* and T3SS mutant (Figure 45E-F).

This work suggests that EPEC infection can prevent, in the cytosol, the increase in phosphorylation of downstream AKT substrates p-c-Raf_{Ser259} and p-GSK3β_{Ser9} in a T3SS dependent manner. Furthermore, the continuing decrease of the p-c-Raf_{Ser259} signal in the cytoplasmic fractions, during second-wave infections of cells pre-infected with the WT EPEC and *Δquad* mutant (not *cfm-14*), may suggest a role for increased protein phosphatase activity. However, with some cell detachment evident during two-wave infections (data not shown), decreased phosphorylation of c-Raf (Ser259) could indicate the loss of host cell viability.

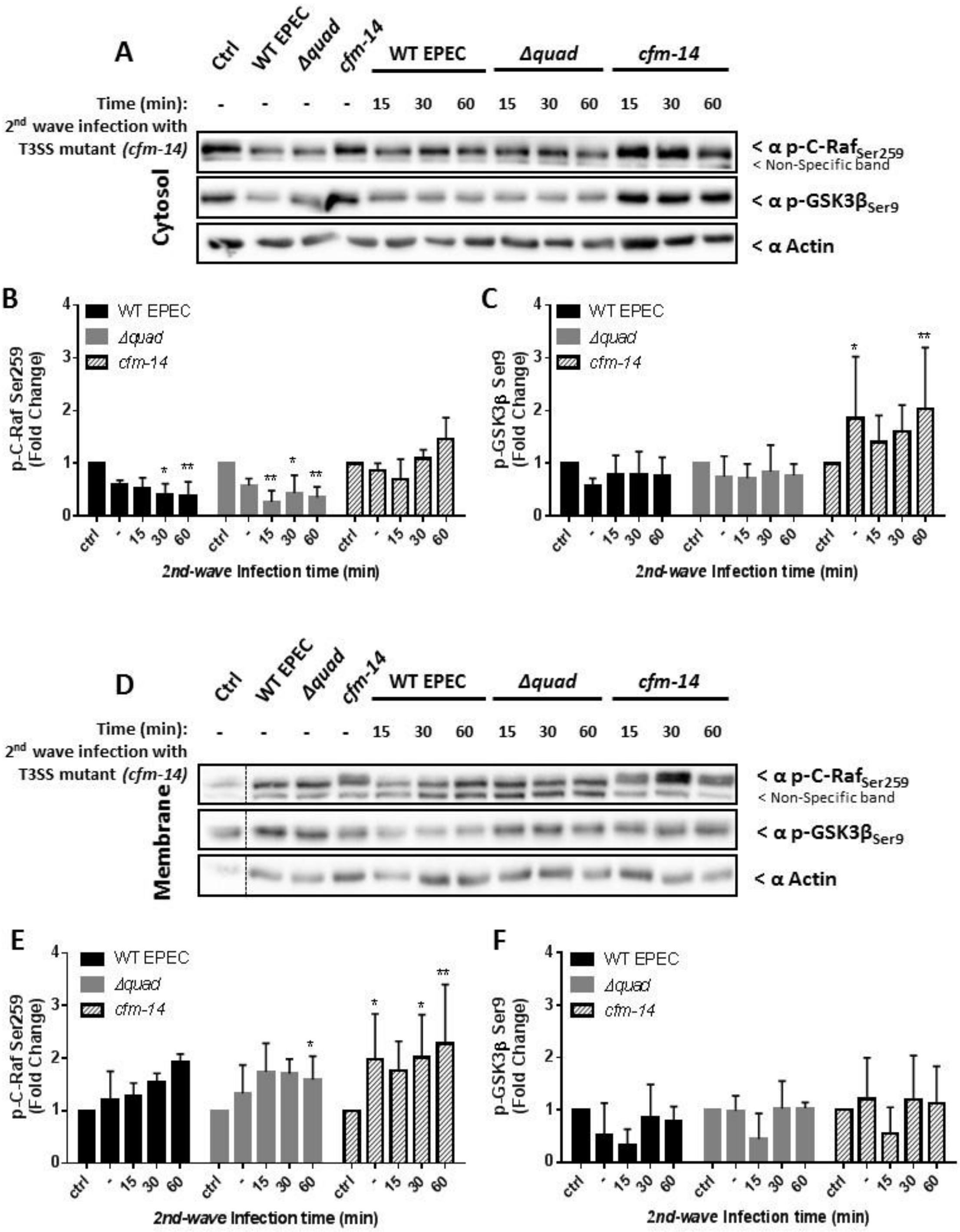

A
Ctrl
WT EPEC
Δquad
cfm-14
WT EPEC
Δquad
cfm-14
Time (min): - - - - 15 30 60 15 30 60 15 30 60
2nd wave infection with
T3SS mutant (cfm-14)
Cytosol
< α p-C-Raf Ser259
< Non-Specific band
< α p-GSK3β Ser9
< α Actin
B
p-C-Raf Ser259 (Fold Change)
WT EPEC
Δquad
cfm-14
ctrl - 15 30 60 ctrl - 15 30 60 ctrl - 15 30 60
2nd-wave Infection time (min)
C
p-GSK3β Ser9 (Fold Change)
WT EPEC
Δquad
cfm-14
2nd-wave Infection time (min)
D
Ctrl
WT EPEC
Δquad
cfm-14
WT EPEC
Δquad
cfm-14
Time (min): - - - - 15 30 60 15 30 60 15 30 60
2nd wave infection with
T3SS mutant (cfm-14)
Membrane
< α p-C-Raf Ser259
< Non-Specific band
< α p-GSK3β Ser9
< α Actin
E
p-C-Raf Ser259 (Fold Change)
WT EPEC
Δquad
cfm-14
2nd-wave Infection time (min)
F
p-GSK3β Ser9 (Fold Change)
WT EPEC
Δquad
cfm-14
2nd-wave Infection time (min)

Figure 45 EPEC pre-infection inhibits the *cfm-14* induced phosphorylation of cytosolic c-Raf (Ser259) and GSK3β (ser9) in a T3SS dependent manner.

J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) for 1 h with the pre-activated WT EPEC, *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) or the T3SS mutant (*cfm-14*) strains. Cells were washed to remove non-adherent bacteria before treating with gentamicin [100µg/ml] for 1 h (kill extracellular bacteria), followed by a second wash step and final incubation in fresh DMEM for 1 h to allow for cell recovery. Post recovery, cells were left uninfected or infected with the *cfm-14* strain (2^{nd} infection; MOI 100:1) for 15, 30 and 60 min. Cell extracts were isolated after the first-wave infection ('-'; post gentamicin treatment & recovery) or following a second-wave infection with the *cfm-14* strain. (**A & D**) J774A.1 macrophages were fractionated into cytosolic and membrane proteins, before separating by SDS-PAGE (10%) for western blot analysis; probing for anti-p-c-Raf_{ser259}, anti-p-$GSK3\beta_{ser9}$ and anti-Actin (loading control) antibodies. Where appropriate, immunoblots were cropped and moved for presentation purposes, indicated by a dashed line. (**B, C, E & F**) Densitometry analysis of western blot was performed using ImageLab software, with values representing a fold change in p-c-Raf_{ser259} or p-$GSK3\beta_{ser9}$ signals, normalised to Actin (loading control) and calculated as a relative change to uninfected (Ctrl) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (Ctrl; **P<0.01 and *p<0.05).

5.2.6 Phagocytosis of T3SS mutant (*cfm-14*) bacteria supports the viability of J774A.1 macrophages in the two-wave infection model

To assess the viability of cells in the two-wave infection model, J774A.1 macrophages were examined for their ability to internalise bacteria following a second-wave infection (T3SS mutant), compared to a standard one-wave infection protocol. Quitard *et al.,* have shown that the capacity of EPEC to inhibit its uptake, through PI3K dependent mechanisms, requires the activity of the LEE effector EspF (Quitard *et al.*, 2006). This T3SS dependent inhibition of phagocytosis was correlated with the decrease of membrane PI[3,4,5]P3, linked to the inhibition of PI3K activity (Celli *et al.*, 2001) and thus upstream of AKT activation (Fayard *et al.*, 2005; Vanhaesebroeck *et al.*, 2012).

Briefly, J774A.1 macrophages were infected with the pre-activated WT EPEC, *Δquad* (lacks LEE encoded Map, EspF, Tir & Intimin) or T3SS mutant (*cfm-14*) at an MOI of 100:1 for 1 h. Cells were then washed and treated for 1 h with either: 1) chloramphenicol for one-wave infections (prevent further effector delivery & enable cell associated bacteria to be fully internalised) then fixed; or 2) gentamicin for two-wave infections (kill extracellular bacteria) before leaving to recover in fresh cell culture media for 1 h. Two-wave infections were then challenged with the GFP expressing T3SS mutant (*cfm-14/pGFP*) at an MOI of 100:1 for 1 h, prior to treating with chloramphenicol (1 h) and fixing cells. The percentage of bacteria internalised by the standard one-wave infection protocol was determined by a differential fluorescence based antibody assay to label extracellular bacteria (Red) and total bacteria (Green). In two-wave infection protocols, second-wave infected bacteria (*cfm-14/pGFP*) were detected by their expression of GFP (Green), with extracellular bacteria identified as those labelled Red (antibody labelling) and Green (GFP).

As reported (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006), infection with the T3SS mutant (*cfm-14*) led to significantly greater number of cell-associated bacteria being internalised than that of the WT EPEC strain (74.9 ± 1.7% & 35 ± 6.2% respectively; $p<0.001$; Figure 46). Furthermore, infection with the *Δquad* mutant, lacking the EspF effector, revealed a similar high percentage of internalised bacteria (62.9 ± 1.9%; $p<0.001$; Figure 46). This latter finding was consistent with previous reports indicating a major defect in the inhibition of PI3K mediated phagocytosis with the *Δquad* mutant (Quitard *et al.*, 2006). Importantly, examining the uptake of GFP expressing T3SS mutant (*cfm-14/pGFP*) in cells pre-infected with the WT EPEC (30.2 ± 1.6), *cfm-14* (74.9 ± 1.7%) or *Δquad* (62.9 ± 1.9%) strain demonstrated that the near identical levels of internalised bacteria as identified by one-wave infections (Figure 46). These findings revealed that cells pre-infected the T3SS (*cfm-14*) or *Δquad* mutants show no defect in internalising second-wave infected (*cfm-14/pGFP*) bacteria, thus supporting host cell viability. The uptake of T3SS mutant (*cfm-14/pGFP*) bacteria by macrophages pre-infected with WT EPEC further supports host cell viability as this relates to internalisation by PI3K independent pathways; not inhibited by EPEC (Celli *et al.*, 2001).

This work supports previous findings demonstrating the inhibition of PI3K mediated phagocytosis in a T3SS dependent manner that requires the effector EspF (Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007; Tahoun *et al.*, 2011). Furthermore, this work suggests that pre-infection with the WT EPEC strain can stably inhibit the uptake of the T3SS mutant (*cfm-14*) for up to 3 h (time between 1^{st} & 2^{nd} wave infections). The ability of cells pre-infected with the *Δquad* and T3SS mutant (*cfm-14*) to internalise *cfm-14/pGFP* bacteria would imply that that these cells are viable and that PI3K is active. Taken together, these findings would suggest the rapid loss of phosphorylated AKT (Set473 & Thr308) in WT EPEC or *Δquad* mutant pre-infected cells is not due to the loss of host cell viability in the two-wave infection model.

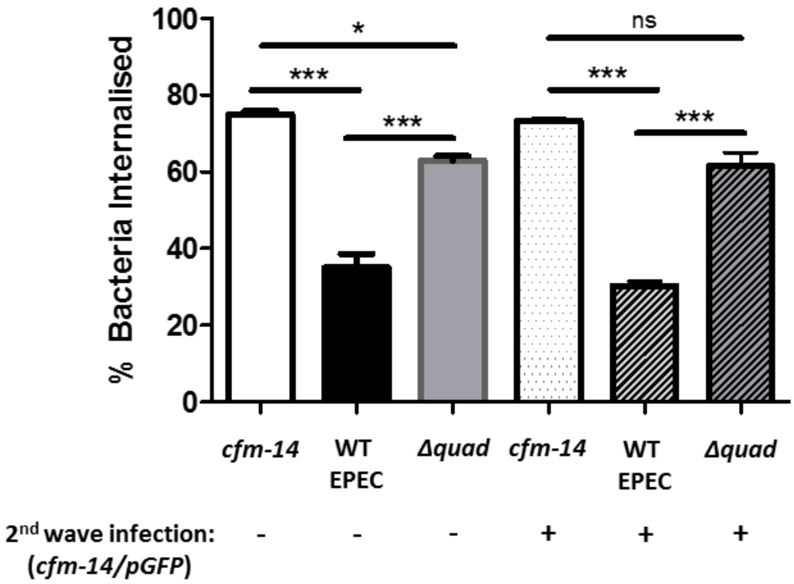


Figure 46 Internalisation of the EPEC T3SS mutant (*cfm-14*) supports the viability of EPEC pre-infected cells.

J774A.1 macrophages were infected for 1 h (MOI 100:1) with the pre-activated WT EPEC, T3SS mutant (*cfm-14*) or *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) strain. After infection, cells were processed for a single or two-wave infection strategy. Single infection; cells were washed and treated with chloramphenicol [25 µg/ml] for 1 h, prior to fixing cells in PFA [2.5%]. Co-infection; cells were washed and infection stopped by 1 h treatment with gentamicin [100 µg/ml], followed by a 1 h recovery period in fresh DMEM and a second infection with the pre-activated *cfm-14/pGFP* strain for 1 h (MOI 100:1). After infection, cells were washed and treated with chloramphenicol [25 µg/ml] for 1h, prior to fixing cells in PFA [2.5%]. Fixed cells were processed for differential immunofluorescence antibody labelling, probing for extracellular bacteria (Red) and total cell associated bacteria (Green) using anti EPEC antibodies. Two-wave infected (*cfm-14/pGFP*) bacteria were distinguished by their expression of GFP (Green), with extracellular bacteria labelled Red (antibody labelling) and distinguished from first-wave infected bacteria by GFP fluorescence (Red/Green co-localisation). Total intracellular bacteria was calculated by subtraction of extracellular bacteria from total bacteria and calculated as a percentage. Values represent the mean ± SD from three independent experiments. Statistical analysis was performed by two-way ANOVA followed by Sidak's post-test $^{*}p<0.05$, $^{***}p<0.001$.

5.2.7 Possible role for protein phosphatases in the rapid T3SS dependent decrease of phosphorylated AKT

Serine/Threonine protein phosphatases play an important role in the dephosphorylation of AKT. As discussed earlier, protein phosphatases PP2A and PHLPP are recognised to selectively dephosphorylate AKT at residues Thr308 and Ser473 respectively (Bayascas and Alessi, 2005), with PP1 also reported to antagonise the phosphorylation of both these residues (Chen *et al.*, 2005; Thayyullathil *et al.*, 2011). Furthermore, PP1 and PP2A are also implicated to dephosphorylate downstream AKT targets p-GSK3β_{Ser9} and p-c-Raf$_{Ser259}$ (Abraham *et al.*, 2000; Jaumot and Hancock, 2001; Hernandez *et al.*, 2010). To investigate the requirement for these protein phosphatases to induce the T3SS dependent decrease of phosphorylated AKT cells were treated with the potent inhibitor of PP1 and PP2A, Calyculin A (Swingle *et al.*, 2007). Thus, J774A.1 macrophages were left untreated or treated with Calyculin A (5 & 100 nM) for 1 h, prior to leaving cells uninfected or infected for 15, 60 and 120 min with the pre-activated T3SS mutant (*cfm-14*) or WT EPEC strain at an MOI of 200:1, with or without inhibitor.

The infection of untreated cells with the T3SS defective mutant (*cfm-14*) demonstrated the strong increase of phosphorylated AKT (Ser473 & Thr308) above the level of uninfected control after 15, 60 and 120 min of infection, with similar results identified in cells pre-treated with 5 nM Calyculin A (Figure 47A & B). Consistent with the literature (Pozuelo-Rubio *et al.*, 2010), treatment of cells with 100 nM Calyculin A led to the dramatic increase of phosphorylated AKT (Ser473 & Thr308) in uninfected cells (Figure 47A & B). Furthermore, subsequent infection with the T3SS mutant (*cfm-14*) for 15, 60 and 120 min sustained this dramatic increase of phosphorylated AKT (Ser473 & Thr308) above background levels (Figure 47A & B). By contrast, infection of untreated cells with the WT EPEC strain led to the expected short transient increase in AKT phosphorylation (Ser473 & Thr308; Figure 47C & D). This increase of phosphorylated AKT was maximal at 15 min, before decreasing rapidly to background levels at 60 and 120 min post infection (Figure 47C & D). Furthermore, this WT EPEC induced transient increase in phosphorylated AKT (Ser473 & Thr308) was also evident in cells pre-treated with 5 nM Calyculin A (Figure 47C & D). Importantly, pre-treatment of cells with 100 nM Calyculin A prevented this rapid T3SS dependent decrease of phosphorylated AKT, demonstrating the sustained increase of p-AKT$_{Ser473}$ and p-AKT$_{Thr308}$

signal after 15, 60 and 120 min with the WT EPEC strain. These results were supported by quantification analysis of p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as a relative change to untreated-uninfected cells (Figure 47B & D). As this work is from a single experiment, these results were not statistically verified. EPEC strain genotype was supported by detection of the host kinase modified form of Tir (T'') in cells infected with the WT EPEC strain (Figure 47C), but not the *cfm-14* mutant (Figure 47A), after 60 min of infection.

This work suggests that the capacity of EPEC to induce a rapid T3SS dependent decrease of phosphorylated AKT can be inhibited by treatment with Calyculin A (100 nM). This would imply that the rapid loss of phosphorylated AKT is caused by the dephosphorylation of AKT at Thr308 and Ser473 by a Calyculin A sensitive protein phosphatase, likely PP1 and/or PP2A.

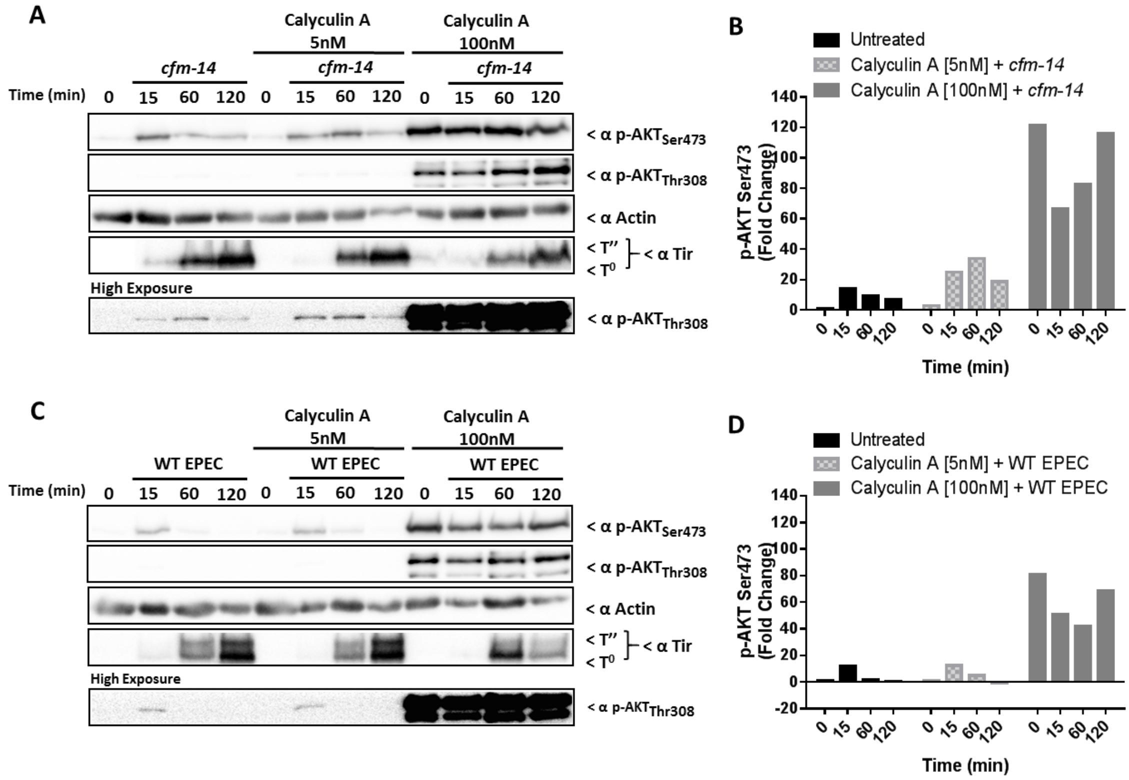


Figure 47 EPEC induced T3SS dependent decrease of phosphorylated AKT is inhibited by phosphatase inhibitor Calyculin A.

J774A.1 macrophages were left untreated or treated with Calyculin A (5 or 100 nM) for 1 h, prior to leaving cells uninfected (0) or infected with pre-activated T3SS mutant (*cfm-14*) or WT EPEC strain (MOI 200:1). Total cell extracts were isolated (1x SDS sample buffer) after 15, 60 and 120 min of infection. (**A & C**) Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{Ser473}$, anti-p-AKT$_{Thr308}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes. (**B & D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{Ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to untreated-uninfected cells.

5.2.8 The T3SS dependent decrease of phosphorylated AKT is insensitive to the treatment of phosphatase inhibitor Okadaic Acid

To examine the relative roles for PP1 and/or PP2A to induce the rapid T3SS dependent decrease of phosphorylated AKT, cells were treated with the phosphatase inhibitor Okadaic Acid, which is selective for PP2A over PP1 at low concentrations (≤100 nM) (Chen *et al.*, 2005; Wiles *et al.*, 2008). Thus, J774A.1 macrophages were left untreated or treated with Okadaic Acid (100 & 400 nM) for 1 h, prior to leaving cells uninfected or infected for 15, 60 and 120 min with the pre-activated T3SS mutant (*cfm-14*) or WT EPEC strain at an MOI of 200:1, with or without inhibitor.

As before, infection of untreated cells with the T3SS mutant (*cfm-14*) led to a strong increase of phosphorylated AKT (Ser473 & Thr308) after 15, 60 and 120 min of infection (Figure 48A & B). Although treatment of cells with Okadaic Acid (100 & 400 nM) did not increase background levels of p-AKT_{Thr308} or p-AKT_{Ser473}, subsequent infection with the T3SS mutant (*cfm-14*) revealed a strong increase of phosphorylated AKT (Ser473 & Thr308), which was most dramatic for cells pre-treated with 400 nM Okadaic Acid (Figure 48A & B). In contrast, infection of untreated cells with the WT EPEC strain led to the expected short transient increase of phosphorylated AKT (Ser473 & Thr308), decreasing to background levels after 60 min post infection (Figure 48C & D). This T3SS dependent transient increase of phosphorylated AKT was also evident in cells pre-treated with Okadaic Acid at 100 and 400 nM (Figure 48C & D). Intriguingly, whilst pre-treatment of cells with 400 nM Okadaic Acid did not prevent the rapid decrease of phosphorylated AKT induced by the WT EPEC strain, p-AKT_{ser473} levels were notably greater than that of untreated WT EPEC infected controls at 60 and 120 min post infection (Figure 48C & D). Again, these results were supported by quantification analysis of p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as a relative change to untreated-uninfected cells. However, as this work is from a single experiment, these results were not statistically verified. EPEC strain genotype was supported by the by the detection of modified form of Tir (T'') in cells infected with the WT EPEC strain (Figure 48A), but not *cfm-14* mutant (Figure 48C); detected after 60 min of infection.

These findings suggest that although 400 nM Okadaic Acid (lesser extent at 100 nM) can reduce the total dephosphorylation of AKT at Thr308 and Ser473, it cannot prevent the rapid

T3SS dependent decrease at 60 and 120 min post infection. This would imply that the T3SS dependent decrease of phosphorylated AKT is dependent on Calyculin A sensitive but Okadaic acid insensitive protein phosphatase, thus arguing against a role for PP1 and/or PP2A.

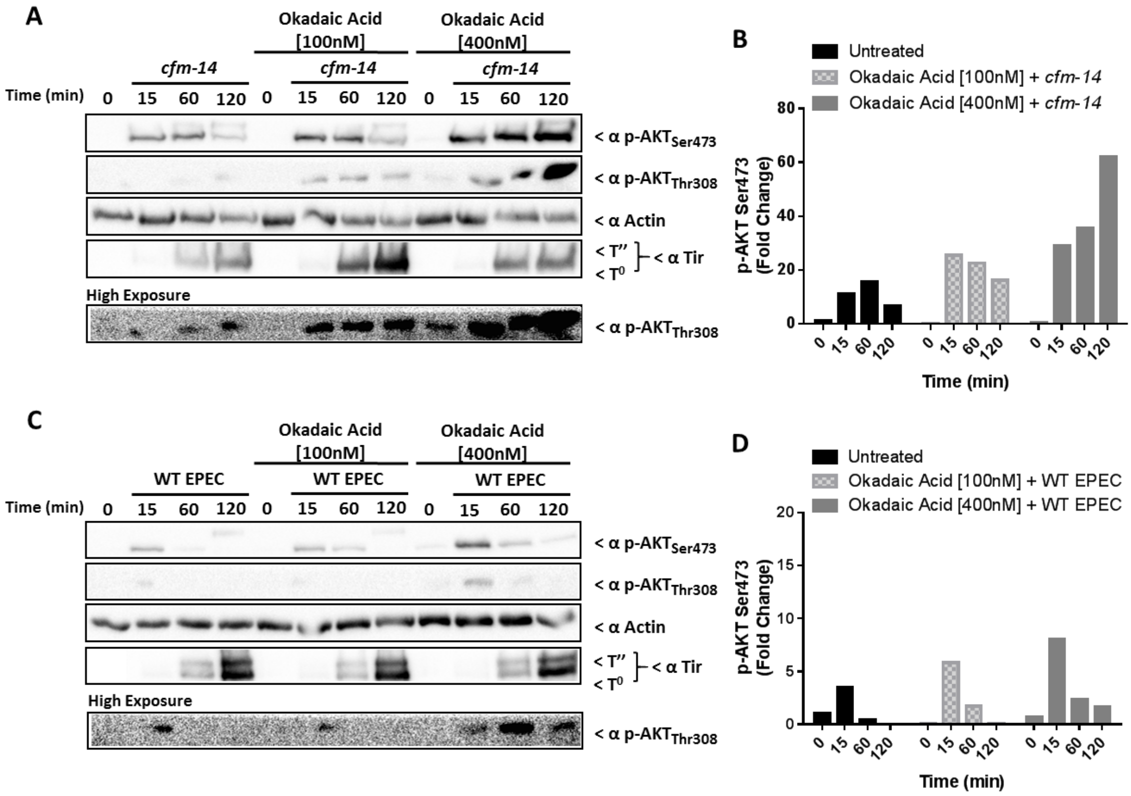


Figure 48 Phosphatase inhibitor Okadaic Acid does not prevent the rapid T3SS dependent decrease of phosphorylated AKT.

J774A.1 macrophages were left untreated or treated with Okadaic Acid (100 & 400 nM) for 1 h, prior to leaving cells uninfected (0) or infected with the pre-activated T3SS mutant (*cfm-14*) or WT EPEC strain (MOI 200:1). Total cell extracts were isolated (1x SDS sample buffer) after 15, 60 and 120 min of infection. (**A & C**) Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{Ser473}$, anti-p-AKT$_{Thr308}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes. (**B & D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{Ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to untreated-uninfected cells.

5.2.9 Calyculin A can restore AKT phosphorylation in cells pre-infected with EPEC

Having demonstrated the ability of EPEC to inhibit AKT phosphorylation in a T3SS dependent manner, studies were undertaken to examine whether cells pre-infected with EPEC could phosphorylate AKT in response to treatment with Calyculin A or following a second-wave infection with the T3SS mutant (*cfm-14*). Thus, J774A.1 macrophages were left uninfected or infected with the pre-activated WT EPEC, *Δquad* or T3SS mutant (*cfm-14*) at an MOI of 200:1 for 1 h. Infections were then stopped and cells treated with gentamicin (kill extracellular bacteria) and Calycilun A (100 nM) for 1 h, before cells were washed and left to recover in fresh cell culture media supplemented with Calyculin A (100 nM) for 1 h. After recovery, all cells were infected with the *cfm-14* strain at an MOI of 100:1 for 1 h. Total cell extracts were isolated and processed for western blot analysis after each stage of the two-wave infection assay: post EPEC infection (1^{st} wave), post gentamicin treatment/recovery, and post infection with the *cfm-14* mutant (2^{nd} wave). In untreated cells, infection with the T3SS mutant (*cfm-14*), but not the WT EPEC or *Δquad* mutant, led to the strong increase of phosphorylated AKT above background levels as expected (Figure 49A). Subsequent treatment of cells with gentamicin and Calyculin A induced the dramatic increase in p-AKT_{ser473} and p-AKT_{Thr308} signals in uninfected cells (Figure 49B). This strong increase of phosphorylated AKT (Ser473 & Thr308) was also evident in cells per-infected with the WT EPEC, *Δquad* and T3SS mutant (*cfm-14*) (Figure 49B). Moreover, the second-wave infection with the *cfm-14* strain, in the presence of Calcyculin A, led to a further increase of phosphorylated AKT in uninfected control, T3SS mutant (*cfm-14*), WT EPEC and *Δquad* mutant pre-infected cells (Figure 49C). This increase of phosphorylated AKT was notably weaker for cells pre-infected with EPEC (*WT EPEC, Δquad* or *cfm-14* strain) than uninfected control cells (Figure 49C). These results are supported by quantification analysis of p-AKT_{ser473} signal normalised to Actin (loading control) and calculated as a relative change to uninfected-untreated cells (Figure 49D). Again, as this work is from a single experiement these findings were not statistically verified. EPEC strain genotype was supported by the absence (*Δquad*) or presence (*cfm-14* & WT EPEC) of Tir, with the detection of the host kinase modified form (T'') supporting the T3SS dependent delivery of Tir with the WT EPEC strain (Figure 49A). Two-wave infection of cells is supported by the strong detection of the unmodified form of Tir (T^0) in all infected cell extracts (Figure 49C). This work suggests the T3SS dependent inhibition of AKT phosphorylation, stimulated by second-wave infection with the T3SS mutant (*cfm-14*), can be blocked/reversed by treatment of cells with phosphatase inhibitor Calyculin A. This finding supports the suggestion that the rapid T3SS dependent decrease of phosphorylated AKT is mediated by a Calyculin A sensitive protein phosphatase, triggering the dephosphorylation of AKT.

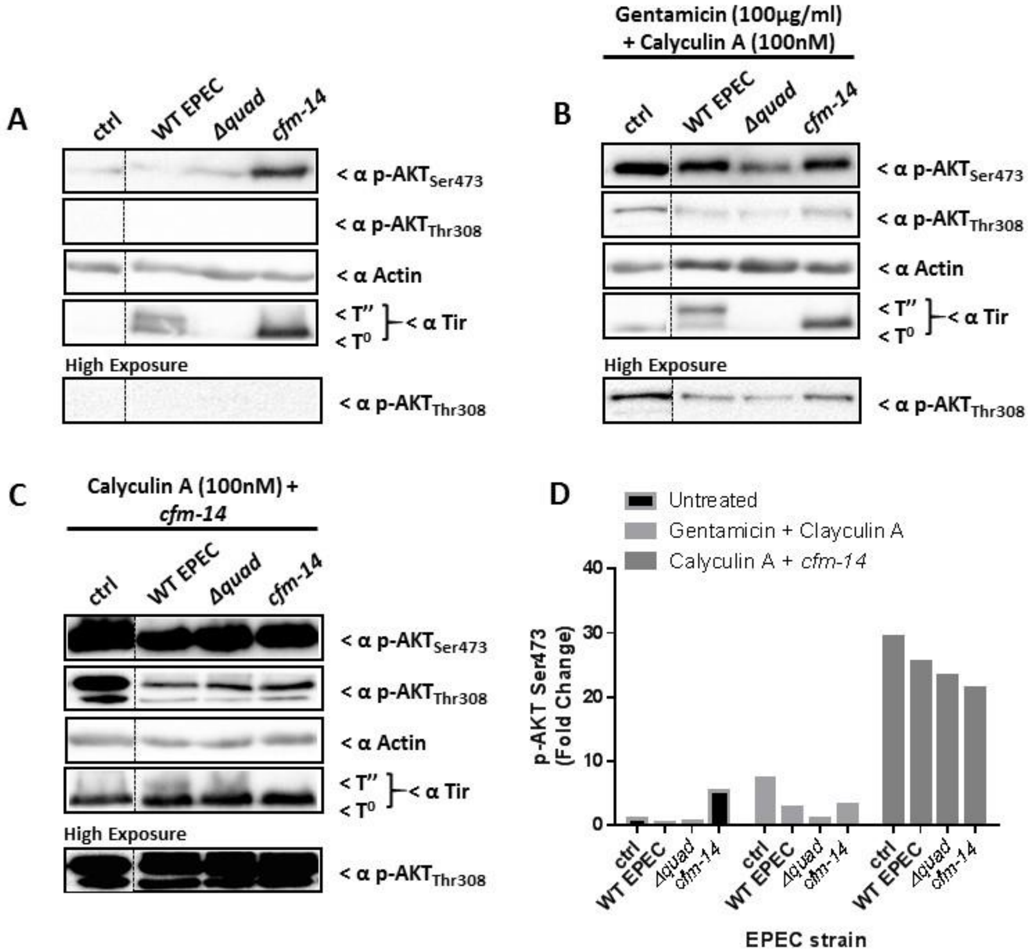


Figure 49 Calyculin A induces AKT phosphorylation in EPEC pre-infected macrophages.

J774A.1 macrophages were left uninfected (Ctrl) or infected (MOI 200:1) with the pre-activated WT EPEC, *Δquad* (lacking LEE genes *map*, *espF*, *tir* & *eae* [Intimin]) or T3SS mutant (*cfm-14*) strains for 1 h. Cells were washed and treated with gentamicin (100 µg/ml; bactericidal levels) and Calyculin A (100 nM) for 1 h, prior to a second infection with the *cfm-14* strain (MOI 100:1) in the presence of Calyculin A for 1 h. Total cell extracts isolated (1x SDS sample buffer) after the first infection (**A**), post gentamicin/Calyculin A treatment (**B**) or following a second infection with *cfm-14* strain (**C**). Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{Ser473}$, anti-p-AKT$_{Thr308}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots represent a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line. (**D**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT$_{ser473}$ signal, normalised to Actin (loading control) and calculated as a relative change to untreated-uninfected cells.

5.3 Discussion

EPEC infection induces a rapid T3SS dependent decrease of phosphorylated AKT, though little is known about how this occurs. To gain insight into this inhibitory mechanism, this study looked to examine the impact of EPEC infection on the phosphorylation of AKT (Ser473 & Thr308), in addition to the localisation of AKT (isoforms 1, 2 & 3) and key signalling associated proteins (p-PDK1$_{Ser241}$, p-PTEN$_{Ser380}$, p-GSK3β_{Ser9} & p-c-Raf$_{Ser259}$). This study suggests the inhibition of AKT phosphorylation by EPEC is not due to notable T3SS dependent changes in localisation of AKT (isoforms 1, 2 & 3), p-PDK1$_{Ser241}$ (active form) and/or p-PTEN$_{Ser380}$ (inactive form). However, this study provides evidence to suggest the rapid T3SS dependent decrease of phosphorylated AKT (Ser473 & Thr308) is linked to the activity of a host activated or bacterial delivered serine/threonine protein phosphatase.

PI3K conversion of PI[4,5]P2 to PI[3,4,5]P3 at the membrane stimulates the recruitment/co-localisation of AKT and PDK1 via their respective PH domains, which is crucial to phosphorylate AKT at Thr308. AKT is then fully activated by a second phosphorylation step at Ser473 before phosphorylating downstream substrates such as GSK3β (Ser9) and c-Raf (Ser259), thereby inhibiting their activity. AKT activation can therefore be broken down into three critical steps: 1) PI[3,4,5]P3 mediated recruitment to the membrane of AKT and PDK1; 2) phosphorylation of AKT at Thr308 and Ser473; and 3) dephosphorylation of AKT, thus terminating AKT signalling.

To investigate these possibilities, studies examined the localisation of proteins associated with key events in the PI3K-AKT signalling pathway. This was initially investigated using a one-wave infection strategy, fractionated into cytosolic and membrane proteins. As expected (Yu *et al.*, 2015), this study confirms the expression of all three AKT isoforms in the macrophage model, with the majority localised to the cytosolic fraction. Furthermore, this work demonstrates for the first time that EPEC infection is associated with the transient localisation of all three AKT isoforms to the membrane, consistent with the literature for the activation of AKT (Astoul *et al.*, 1999). Importantly, this work supports the infection induced increase of AKT phosphorylation, followed by a rapid T3SS dependent decrease to background levels. These findings are consistent with those obtained from total cell extracts, as reported in literature (Goosney *et al.*, 1999; Celli *et al.*, 2001) and this study (see Chapter 3 Figure 13). Although this one-wave infection strategy has identified subtle T3SS dependent differences in the localisation of AKT (isoforms 1, 2 & 3), p-PDK1$_{Ser241}$, p-PTEN$_{Ser380}$ and the phosphorylation of AKT (Ser473), the mechanism behind these differences could not be identified. Due to the unsynchronised infection of the one-wave infection strategy, it was speculated that these T3SS dependent differences could stem from variable populations of cells, at early time points, having sufficient levels of cell associated bacteria to decrease AKT phosphorylation.

This unsynchronised infection associated issue was circumvented by use of a two-wave infection model (Ruchaud-Sparagano *et al.*, 2007). Using this model it was predicted that the pre-infection of cells with EPEC would inhibit, in a T3SS dependent manner, inducers of AKT phosphorylation, which in this study was stimulated by a second-wave infection with the T3SS mutant (*cfm-14*). Indeed, this was shown to be the case with this study revealing the stable inhibition of AKT phosphorylation (Ser473 & Thr308) over a 3 h period (time between 1st & 2nd wave infection) in cells pre-infected with the WT EPEC, but not the T3SS mutant (*cfm-14*). Importantly, this result corroborates findings identified earlier in total cell extracts (see Chapter 3 Figure 19). As expected (Ananthanarayanan *et al.*, 2007), the majority of phosphorylated AKT (Ser473 & Thr308) was detected in the cytosolic fraction, supporting the cycling of AKT away from the plasma membrane once fully activated. Although the level of infection induced phosphorylation of AKT on Ser473 (10-20 fold increase) was notably greater than that of Thr308 (5-10-fold increase) in both fractions (cytosolic & membrane), though not statistically verified, it is important to note that the p-AKT$_{Thr308}$ signal was found to mirror that of p-AKT$_{ser473}$ in cells pre-infected with the WT EPEC or T3SS mutant (*cfm-14*).

In contrast to the one-wave infection strategy, the unorthodox two-wave infection model did not trigger the transient localisation of AKT (isoforms 1, 2 & 3) to the membrane. Although EPEC infection was associated with the increase of AKT3 at the membrane, but not AKT1 or AKT2, this was surprisingly linked to the first-wave infection and sustained in response to a second-wave (T3SS mutant) infection. It is therefore tempting to speculate that the increase of phosphorylated AKT (Ser473 & Thr308) in response to the T3SS mutant (*cfm-14*) in the two-wave infection model is driven by the AKT3 isoform. To confirm this, further investigations could examine the two-wave infection induced phosphorylation of AKT in macrophages deficient of AKT3 and/or by siRNA knockdown of the AKT3 isoform. If confirmed, this model could prove useful to understand how specific AKT isoforms (AKT3) are retained at the membrane following recruitment and why AKT1/2 are not recruited following a second-wave infection.

An interesting observation from the two-wave infection model, for cells pre-infected with the *Δquad* mutant, was the apparent increase in AKT phosphorylation on Thr308 (membrane only), similar to that induced by the T3SS mutant (*cfm-14*). Surprisingly, this increase of membrane p-AKT_{Thr308} signal did not correlate with increased levels of p-AKT_{ser473} at the membrane. This suggests that EPEC may interfere with AKT phosphorylation by two distinct T3SS dependent mechanisms: 1) inducing the loss of AKT phosphorylation, Ser473/Thr308 in the cytosol and Ser473 at the membrane; and 2) inducing the loss of Thr308 phosphorylation of AKT at the membrane. This latter finding implicates a role for one or more proteins lacking from the *Δquad* mutant (Map, Tir, EspF & Intimin) to promote the loss of p-AKT_{Thr308} signal at the membrane. One possibility could be the recruitment of the inositol phosphatase SHIP to the membrane by the EPEC effector Tir (Smith *et al.*, 2010). SHIP is known to dephosphorylate membrane PI[3,4,5]P3 to PI[3,4]P2 and thus antagonise the localisation and activation of AKT (Liu *et al.*, 1999b; Carver *et al.*, 2000). It is therefore tempting to speculate that in the absence of Tir mediated recruitment of SHIP; p-AKT_{Thr308} is retained at the membrane, which may prevent its dephosphorylation.

AKT phosphorylation at Thr308 and Ser473 is reported to increase AKT activity by 100 and 10 fold respectively (Alessi *et al.*, 1996; Alessi *et al.*, 1997b). Phosphorylated AKT regulates the activity of a number of downstream targets, including GSK3β (Ser9) and c-Raf (Ser259), by phosphorylation; thus providing an output for AKT activity. This study illustrates, for the first time, that increased AKT phosphorylation (Ser473 & Thr308), stimulated by 2nd-wave infection in cells pre-infected with the T3SS mutant (*cfm-14*), is linked to the increase of p-$GSK3\beta_{Ser9}$ and p-c-Raf_{Ser259} signals. In contrast, cells pre-infected with the WT EPEC or *Δquad* mutant displayed unaltered levels of cytosolic p-$GSK3\beta_{Ser9}$ in response to the second wave infection, though unexpectedly had significantly reduced levels of p-c-Raf_{Ser259} compared to first-wave infection controls. Therefore, despite the increase of p-AKT_{Thr308} signal in the membrane fraction of *Δquad* mutant pre-infected cells, this did not translate to an increase in AKT activity as would be expected (Vincent *et al.*, 2011). These findings suggested that the decrease in phosphorylated AKT (Ser473 & Thr308) and p-c-Raf_{Ser259} signal may be triggered by dephosphorylation, possibly through the increased activity of serine/threonine protein phosphatases. However, due to notable levels of cell detachment with the WT EPEC and

Δquad mutant in the two-wave infection model (data not shown), it could not be discounted that this decrease in phosphorylation was due to the loss of host cell viability.

It is important to note the well-established detergent based fractionation protocol employed in this study separated host proteins into cytosolic and membrane fractions (Kenny *et al.*, 1997; Kenny, 1999; Warawa and Kenny, 2001). In addition to the plasma membrane, these membrane fractions may contain mitochondria, endoplasmic reticulum (ER) and Golgi membrane components. Therefore, whether localisation of AKT (isoforms 1, 2 & 3), PDK1 and PTEN are indeed localised at the host plasma membrane, and not at organelles, remains to be determined. However, as AKT signalling associated proteins (AKT, PDK1, PTEN and mTORC2) have been identified at the mitochondria and ER, it is possible that AKT activation might also occur at these organelles (Antico Arciuch *et al.*, 2009; Boulbes *et al.*, 2011; Betz and Hall, 2013). It would therefore be of interest to examine the localisation of AKT signalling related proteins by immunofluorescence experiments during EPEC infection. In addition, it should be noted that quantification of host membrane proteins by western blot analysis were normalised against the loading control Actin; consistent with methods reported in the literature (Li *et al.*, 2006; Dasgupta *et al.*, 2015). The suitability of Actin as a membrane marker was supported by the reproducibility of the data following the normalisation of signals. Nevertheless, as Actin is not exclusively a host membrane protein, future work could look to use alternative membrane house keeping protein expressed by macrophages, such as the Na^+/K^+ ATPase (Gaskill *et al.*, 2012).

EPEC inhibition of PI3K mediated phagocytosis, thus upstream of AKT phosphorylation, is linked to the subversive activities of LEE encoded effectors EspB (Iizumi *et al.*, 2007), EspH (Dong *et al.*, 2010) and EspF (Quitard *et al.*, 2006; Martinez-Argudo *et al.*, 2007). Studies by Quitard *et al*., were the first to demonstrate that EPEC strains lacking the EspF effector (e.g. *Δquad* mutant) do not inhibit PI3K mediated phagocytosis (>60% of cell associated bacteria internalised), though they can trigger the rapid loss of phosphorylated AKT similar to WT EPEC (Quitard *et al.*, 2006). Importantly, these results were corroborated in this study revealing the majority cell associated *Δquad* (63%) and *T3SS mutant* (75%) bacteria internalised, in contrast to 35% for the WT EPEC strain. Thus, this work supports the assertion that EPEC inhibition of PI3K activity is not coupled to the T3SS dependent decrease of phosphorylated AKT (Quitard *et al.*, 2006).

Using phagocytosis of EPEC as a marker of PI3K activity and cell viability, cells pre-infected with EPEC strains were examined for the ability to internalise bacteria in a second-wave infection (GFP expressing T3SS mutant, *cfm-14*). This study demonstrates for the first time that cells pre-infected with the WT EPEC, but not *Δquad* or T3SS mutant (*cfm-14*), inhibited the uptake of bacteria by the second wave infection, with <35% of cell-associated bacteria (*cfm-14/pGFP*) shown to be internalised. These findings demonstrated four things: 1) EPEC inhibition of PI3K mediated phagocytosis is stable for up to 3 h (time between 1st & 2nd wave infection); 2) PI3K is active in cells pre-infected with the *Δquad* or T3SS mutant (*cfm-14*); 3) cells pre-infected with the *Δquad* mutant are healthy as capable of internalising second-wave infected (*cfm-14*) bacteria by PI3K dependent and independent mechanisms; and 4) cells pre-infected with EPEC are healthy as internalised second-wave infected (*cfm-14*) bacteria by the PI3K independent pathway (≤40% of bacteria internalised). This work suggests that EPEC can inhibit the PI3K-AKT signalling pathway at multiple points in a T3SS dependent manner and that phosphorylation on Thr308 can be used as a marker of PI3K activity in this infection model (Vincent *et al.*, 2011). Furthermore, this work argues that the decrease in phosphorylated AKT (Ser473 & Thr308) and the downstream substrate p-c-Raf$_{Ser259}$, in cells pre-infected with the WT EPEC and *Δquad* mutant (membrane &/or cytosolic), is not due to the loss of cell viability. Taken together, these findings suggest the decrease in phosphorylated AKT (Ser473 & Thr308) and p-c-Raf$_{Ser259}$ is likely driven by an increase in serine/threonine protein phosphatase activity.

Protein phosphatases, PP1 and PP2A, are estimated to account for more than 90% of the total cellular serine/threonine dephosphorylation events (Faux and Scott, 1996; Sim and Ludowyke, 2002). PP1 is implicated to play a key role in the dephosphorylation of p-GSK3β_{Ser9} (Hernandez *et al.*, 2010), which in addition to PP2A also regulates the phosphorylation p-c-Raf$_{Ser259}$ (Abraham *et al.*, 2000; Jaumot and Hancock, 2001). Furthermore, both PP1 and PP2A, along with the phosphatase PHLPP, are also recognised to dephosphorylate AKT at residues Ser473 and/or Thr308 (Andjelkovic *et al.*, 1996; Chen *et al.*, 2005). The requirement for phosphatases PP1 and/or PP2A to trigger the rapid T3SS dependent decrease of phosphorylated AKT was investigated in this study using the selective phosphatase inhibitors Okadaic acid (PP2A selective at low concentrations; ≤100 nM) and Calyculin A (PP1 and PP2A selective) (Chen *et al.*, 2005; Wiles *et al.*, 2008). This study demonstrates for the first time that pre-treatment of cells with Calyculin A (100 nM), but not

Okadaic acid (100 or 400 nM), can prevent the rapid T3SS dependent decrease of phosphorylated AKT (Ser473 & Thr308) in WT EPEC infected cells. Moreover, this study demonstrates for the first time that the T3SS dependent inhibitory mechanism that rapidly decreases infection induced AKT phosphorylation can be blocked/reversed by treatment with Calyculin A (100 nM) in cells pre-infected with the WT EPEC or *Δquad* mutant. Thus, these findings suggest a key role for a Calyculin A sensitive, but Okadaic Acid insensitive, protein phosphatase to trigger the rapid T3SS dependent decrease of phosphorylated AKT (Ser473 & Thr308).

Inhibition of the T3SS dependent dephosphorylation of AKT in WT EPEC infected cell extracts treated with Calyculin A, but not Okadaic Acid (PP2A selective at ≤100 nM), would suggest a role for the protein phosphatase PP1, but not PP2A (Favre *et al.*, 1997). However, the apparent inability of Okadaic acid at the highest concentration examined (400 nM; PP1 & PP2A selective) to inhibit the T3SS dependent decrease of phosphorylated AKT would argue against this assumption. Whilst it might be tempting to suggest Okadaic Acid did not work in this study, treatment at 400 nM was found to increase the levels of phosphorylated AKT in T3SS mutant (*cfm-14*) infected cells. Though, as these findings using inhibitors Calyculin A and Okadaic Acid were from a single experiment, caution should be taken with these interpretations requiring additional experiments to confirm these results.

Importantly, Calyculin A is also reported to inhibit protein phosphatases PP4, PP5 and PP6 at differing concentrations (Cohen, 1997; Swingle *et al.*, 2007). Whether these phosphatases play a critical role in the T3SS dependent dephosphorylation of AKT cannot be excluded from this study. To better address the requirement for PP1, PP2A and/or other phosphatases, additional studies could examine the impact of other commercially available phosphatase inhibitors, including Tautomycin (PP1 selective), Cantharidin (PP2A selective) and Fostriecin (PP2A & PP4 selective). Alternatively this could be investigated through siRNA knockdown of the catalytic subunits of PP1 (isoforms α1, α2, β, γ1 & γ2), PP2A (isoforms α & β) and/or other protein phosphatases (Wang *et al.*, 2008). How EPEC might trigger the increase in activity of Calyculin A sensitive protein phosphatase(s) is not entirely clear. Moreover, it cannot be discounted that the increase in phosphatase activity is due to a translocated EPEC effector with protein phosphatase function.

It should be noted that in our J774A.1 macrophage cell model, Calyculin A at 100 nM was found to induce the dramatic increase in AKT phosphorylation in uninfected cells. It is therefore concieveable that in our assay this overwhelming inhibitor-induced stimulation of AKT phosphortylation may simply be masking the EPEC induced rapid decline of phosphorylated AKT. This could be due to oversaturation of the p-AKT_{Ser473} signal or, though unlikely, that Calyculin A induced AKT phosphorylation is inaccessible to EPEC-induced dephosphorylation. Although our findings are consistent with the hypothesis for the requirement of a protein phosphatase, additional work is required to corroborate our findings and provide more conclusive evidence towards the role for a Calyculin A sensitive protein phosphatase.

In summary, this study has provided valuable insight into the inhibitory mechanism employed by EPEC to induce the rapid T3SS dependent decrease of phosphorylated AKT. Whilst EPEC is recognised to subvert PI3K activity and PI[3,4,5]P3 availability (Celli *et al.*, 2001; Quitard *et al.*, 2006; Sason *et al.*, 2009), this study suggests that the loss of phosphorylated AKT is not caused by T3SS dependent changes in membrane localisation for AKT (isoform 1, 2 & 3), $PDK1_{ser241}$ and/or p-$PTEN_{ser380}$. Importantly, this study proposes a mechanism whereby EPEC rapidly decreases infection induced AKT phosphorylation by introducing or activating a Calyculin A sensitive protein phosphatase.

Chapter 6 Final Discussion

Studying pathogenic bacteria, such as EPEC, has provided a valuable insight into the mammalian signalling pathways disrupted during infection, including those of inflammation and pro-survival signalling (Krachler *et al.*, 2011; Alto and Orth, 2012; Clements *et al.*, 2012). The capacity of EPEC to subvert these signalling cascades is dependent on the T3SS, critical for the translocation of up to 24 proven effector proteins (Dean and Kenny, 2009; Iguchi *et al.*, 2009; Deng *et al.*, 2012). The ability of EPEC to disrupt the PI3K-AKT signalling pathway has previously been described, though how precisely EPEC subverts AKT activity is unknown. The aim of this project was to investigate the virulent mechanism(s) and bacterial factor(s) necessary for EPEC to trigger the rapid decrease of phosphorylated AKT. This study provides novel information to suggest the capacity of EPEC to inhibit AKT phosphorylation is stable, involving multiple mechanisms and linked to the increased activity of protein phosphatase(s); pathogen delivered or host activated. Although the effectors responsible for the inhibition of AKT phosphorylation were not identified, this study discounts a key role for up to 21 of 24 published EPEC effector proteins. Thus, this work implicates a role for unexamined effectors (LifA, NleJ & EspC), 6 experimentally supported effectors (identified by bioinformatics; Dean *et al.,* unpublished), and/or EPEC effectors not yet identified. Importantly, this study suggests the decline of phosphorylated AKT by unknown effector(s) is/are likely dependent on the LEE encoded chaperone, CesT.

Previous studies examining the ability of EPEC to trigger the rapid decline of phosphorylated AKT have been limited, with the majority of work undertaken in a macrophage cell model (Celli *et al.*, 2001; Quitard *et al.*, 2006). In these studies, the capacity of EPEC to both stimulate and decrease AKT phosphorylation in a PI3K dependent manner was reported, illustrating a characteristic T3SS-dependent transient increase in AKT phosphorylation over a 90 min infection period (Celli *et al.*, 2001; Quitard *et al.*, 2006). These observations were made by non-quantitative western blot analysis for the phosphorylation of AKT at Ser473, but not Thr308; the latter representing the first step in AKT activation by PDK1 (Alessi *et al.*, 1996; Alessi *et al.*, 1997b). Moreover, these interpretations were made from infected total cell extracts and provided little insight into the mechanism(s) behind this inhibitory process.

This study has employed a number of different strategies to investigate the mechanism(s) responsible for the T3SS-dependent decrease in phosphorylated AKT. Using a quantitative approach, this study examined for the first time the impact of EPEC infection on AKT phosphorylation at Ser473 and Thr308; and reported on the activity of AKT by assessing its ability to phosphorylate downstream targets c-Raf and GSK3β. Through the development of a two-wave infected fractionated macrophage cell model this work has uncovered previously unrecognised T3SS-dependent changes in the membrane localisation and phosphorylation of AKT. Furthermore, by examining the impact of EPEC infection using a single and two-wave infection strategy this study has revealed that the T3SS-dependent inhibition of AKT phosphorylation is stable and not readily reversible for up to 3 h post-infection, implicating an inhibitory mechanism involving a host activated or bacterial delivered serine/threonine protein phosphatase. Thus, this work has significantly extended previous observations, providing greater insight into the disruptive capacity of EPEC on AKT activity.

The work presented in this study suggests the capacity of EPEC to inhibit phosphorylation-associated activation of AKT is not linked to T3SS-dependent changes in the membrane localisation of AKT (isoforms 1, 2 & 3) or key associated signalling proteins (PDK1 & PTEN). Instead, this study suggests the rapid dephosphorylation of AKT is linked to the activity of serine/threonine protein phosphatase(s); either bacterial delivered or host activated. This was supported using two-wave infection models, revealing a rapid T3SS-dependent decrease of phosphorylated AKT (Ser473 & Thr308) and cytosolic c-Raf (Ser259) in EPEC infected cells. These findings were corroborated by phosphatase inhibitor data, revealing the inhibition of EPEC-induced T3SS-dependent decrease of phosphorylated AKT after treatment with Calyculin A. However, since this latter result is from a single experiment, additional work is required to confirm these observations. Moreover, since protein phosphatase activity was not measured directly during EPEC infection, future experiments could look to examine this using commercially available phosphatase assay kits. In addition, it would be interesting to examine the impact of EPEC infection on cells transfected with constitutively active forms of AKT such as m/p-AKT and ΔPH-AKT. m/p-AKT possesses a myristoylation signal that enables PI[3,4,5]P3 independent membrane translocation, resulting in AKT hyper activation (Andjelkovic *et al.*, 1997), whilst ΔPH-AKT lacks the PH domain resulting in an altered conformation that exposes the Thr308 residue thus enabling cytosolic ΔPH-AKT to be phosphorylated by PDK1 (Alessi *et al.*, 1997a). The prediction would be that EPEC infection should lead to the decreased phosphorylation of these constitutively active and phosphorylated forms of AKT. Such studies could lend support to the findings of a protein phosphatase-mediated T3SS-dependent decrease of phosphorylated AKT, which is not due to a defect in membrane recruitment of AKT.

Protein phosphatases PP1 and PP2A regulate the activity of almost two thirds of the >500 kinases encoded in the human genome, accounting for 90% of total eukaryotic dephosphorylation events. The regulation of these protein phosphatases is highly complex. PP1 and PP2A are composed of a catalytic subunit bound to one or more regulatory subunits which regulate their subcellular localisation, catalytic activity and substrate specificity (Cohen, 2002; Sim and Ludowyke, 2002; Shi, 2009). Furthermore, these catalytic and regulatory subunits are also regulated by post-translational modifications such as phosphorylation and methylation (Sim and Ludowyke, 2002). Thus, how EPEC might precisely regulate/increase the activity of host phosphatases to dephosphorylate AKT is not entirely clear.

It is becoming increasingly recognised that pathogenic bacteria target the PI3K/AKT signalling pathway as an important step to establishing an infection, with most bacteria associated with AKT activation and only a few linked to AKT dephosphorylation. For example, secretion of the pore-forming toxin HlyA by uropathogenic *E. coli* (UPEC) is reported to inhibit AKT phosphorylation at residues Ser473 and Thr308 (Wiles *et al.*, 2008). Moreover, other pore forming toxins including aerolysin and α-toxin have also been shown to inhibit AKT phosphorylation (Wiles *et al.*, 2008). Although the mechanism behind this inhibition remains to be defined, Wiles *et al.*, have identified a critical role for phosphatases PP1 and PP2A (Wiles *et al.*, 2008). This seemingly aberrant activation of protein phosphatases was linked to a toxin-induced increase of osmotic stress in target cells. Indeed, osmotic stress has been shown to decrease AKT phosphorylation through the activation of protein phosphatases (Parrott and Templeton, 1999; Plescher *et al.*, 2015). Although EPEC does not encode a homologous pore-forming toxin, EPEC can induce pore formation by the secretion of effectors EspB and EspD. It was therefore speculated that EPEC might trigger AKT dephosphorylation by a similar mechanism. However, infection of J774A.1 macrophages with the EPEC strain TOEA7*Δcore::km*, which has a functional T3SS and translocon for effector delivery, did not trigger the decline of phosphorylated AKT. Furthermore, treatment of cells with dextran (Mr ~100,000 & ~20,000), which is known to block EspB/EspD membrane pores thus limiting osmotic stress (Ide *et al.*, 2001; Wiles *et al.*, 2008), did not prevent the rapid T3SS-dependent decrease of p-AKT$_{ser473}$ signal (data not shown). Taken together, this would argue against a role for EspB/EspD pore formation and osmotic stress in the rapid decline of phosphorylated AKT.

PP1 and PP2A are implicated in the regulation of phosphorylated AKT (Ser473 & Thr308), GSK3β (Ser9) and c-Raf (Ser259). It is therefore significant that inhibition of these phosphatases by Calyculin A prevented the rapid decline of phosphorylated AKT induced by EPEC infection. However, the inability to reproduce these results using the inhibitor Okadaic Acid (PP1 & PP2A selective at concentrations ≥ 100 nM) argued against a role for both these phosphatases in the T3SS-dependent dephosphorylation of AKT (Chen *et al.*, 2005; Wiles *et al.*, 2008). Importantly, Okadaic Acid treatment did lead to a small increase in EPEC induced AKT phosphorylation in J774A.1 macrophages, supporting inhibitor function. Taken together, this implied a role for a Calyculin A-sensitive, but Okadaic Acid-insensitive, protein phosphatase. However, as both inhibitors are reported to target the same phosphatases (PP4, PP5, PP6 & PP7) with similar IC50's (Swingle *et al.*, 2007), but for PP1 and PP2A, this result seems difficult to comprehend. Since Okadaic Acid is significantly (100 fold) less permeable than that of the potent phosphatase inhibitor Calyculin A (Favre *et al.*, 1997; Herzig and Neumann, 2000), it is tempting to suggest that PP1 and/or PP2A were not fully inhibited by Okadaic Acid (at 100 or 400 nM) in this model. This would suggest the need to measure PP1 and PP2A activity after treatment with Okadaic acid and Calyculin A. Although the data presented are consistent with the role for a protein phosphatase, that is likely sensitive to Calyculin A inhibition, it cannot be discounted that this inhibitor at high concentrations is simply masking the EPEC-induced decline of phosphorylated AKT. Since Calyculin A is able to stimulate AKT phosphorylation at high concentrations (100 nM), additional work is required to confirm whether this inhibition of a T3SS dependent decrease of phosphorylated AKT is indeed due to the inhibition of protein phosphatases and not caused by oversaturation of the p-AKTSer473 signal. Specific targeting of the catalytic subunits of protein phosphatases by siRNA could thus provide an alternative and more direct method of examining the role of Calyculin A sensitive phosphatase such as PP1, PP2A and/or other protein phosphatases in EPEC-induced dephosphorylation of AKT.

The ability of EPEC to induce the extensive dephosphorylation of host phosphotyrosine proteins has previously been identified (Kenny and Finlay, 1997; Goosney *et al.*, 1999; Celli *et al.*, 2001; Quitard *et al.*, 2006). Whether this is linked with the rapid dephosphorylation of AKT has never been examined, thus it would be interesting to investigate the impact of serine/threonine and tyrosine phosphatase inhibitors on the T3SS-dependent dephosphorylation of host proteins such as AKT. One possibility could be that EPEC secretes and/or activates a dual specificity phosphatase that targets both tyrosine and serine/threonine phosphorylated proteins. Examining the phosphorylation status of alternative targets downstream of AKT (e.g. Bad, Bax & FOXO transcription factors) and/or all serine/threonine phosphorylated proteins during EPEC infection could provide insight into the specificity of the increased phosphatase activity.

As previously discussed, bacterial manipulation of the host phophoproteome is not uncommon, linked to the direct or indirect activity of effector proteins (Rogers *et al.*, 2011; Grishin *et al.*, 2015; Scholz *et al.*, 2015). The results of this study suggest that EPEC induced rapid dephosphorylation of AKT is either due to an effector with protein phosphatase function or caused by the indirect increase of host phosphatase activity. The *Yersinia* effector YopH is one example of an effector with phosphatase function, inducing the tyrosine dephosphorylation of several host proteins (Andersson *et al.*, 1996). Indeed, YopH has been shown to induce the dephosphorylation of AKT, which is dependent of its tyrosine phosphatase activity (Sauvonnet *et al.*, 2002). Whilst previous studies have suggested that EPEC does not secrete an effector with Tyrosine phosphatase function, it is important to note that these studies only examined secreted EPEC effectors (Goosney *et al.*, 1999). Furthermore, these studies did not examine for effectors with serine/threonine phosphatase activity (Goosney *et al.*, 1999). To resolve the question of a direct or indirect effector-mediated increase in phosphatase activity, this study sought to identify the virulent factors necessary to trigger the rapid T3SS-dependent decrease of phosphorylated AKT.

To investigate the requirement for the EPEC effector repertoire, this study examined available EPEC strains lacking subsets of LEE and/or Nle effector proteins. The work presented in this study demonstrates for the first time that 21 EPEC effectors do not play a critical role in the rapid T3SS dependent dephosphorylation of AKT. This collective elimination of 21 effectors was achieved by examining the role for 7 LEE and 14 of the most studied Nle effectors in isolation, in addition to the recently generated complex EPEC effector mutant TOEA7*Δcore::km* (unable to deliver 20 of 24 known EPEC effectors). However, whether there exists cooperation between LEE and Nle-encoded effectors to induce the rapid decline of phosphorylated AKT cannot be discounted. Whilst this is unlikely, this would suggest the need to examine an EPEC strain lacking the 14 Nle effectors and all known LEE effectors, but for the translocation critical effector EspB.

T3SS effector chaperones CesF and CesT play a critical role in translocation of most EPEC effector proteins; implicated to promote effector stabilisation, maintain effector conformation suitable for translocation and targeting of the effector to the T3SS apparatus (Ghosh, 2004). An intriguing result of this study was the identification of a CesT chaperone dependent decrease of infection induced AKT phosphorylation in J774A.1 macrophages. This study revealed that infection with CesT deficient strains (*ΔcesT*, *Δcore::km*, *gorf3Δcore::km* & TOEA7*Δ*core::km) displayed a T3SS mutant-like defect in ability to induce a rapid decrease of phosphorylated AKT. Crucially, re-introduction of the CesT chaperone - encoded on a plasmid - into a tested CesT deficient (*Δcore::*km) mutant restored the capacity of this strain to induce the rapid dephosphorylation of AKT. Thus, this work implicated the role for a CesT dependent effector to induce AKT dephosphorylation.

The CesT chaperone is recognised to play a critical role in the efficient translocation of 5 LEE (EspH, Map, EspZ, EspF & Tir) and 8 Nle (EspJ, NleG, NleH1, NleH2, NleB1, NleB2, NleC & NleA) effector proteins (Thomas *et al.*, 2005; Mills *et al.*, 2008; Mills *et al.*, 2013; Ramu *et al.*, 2013). Having already discounted the role for 7 LEE and 14 Nle effectors, this suggested the requirement for an unknown CesT dependent effector; thus implicating a role for unexamined effectors (EspC, LifA & NleJ), 6 experimentally supported effectors (Dean *et al*., unpublished) and/or effectors not yet identified. Whether effectors EspC, LifA and NleJ require the CesT chaperone for effector translocation is unknown (Thomas *et al.*, 2007; Ramu *et al.*, 2013).

To date, no function has been reported for the recently identified effector NleJ (Deng *et al.*, 2012). In contrast, the serine protease auto-transporter of EPEC (SPATE), EspC is reported to induce host cell death through degradation of the focal adhesion kinase (FAK) and by activation of the mitochondrial apoptotic pathway (Vidal and Navarro-Garcia, 2008; Navarro-Garcia *et al.*, 2014; Serapio-Palacios and Navarro-Garcia, 2016). Interestingly, LifA (lymphocyte inhibitory factor A) is reported to promote EPEC cell attachment, host immunosuppression and disruption of epithelial barrier function (Klapproth *et al.*, 2000; Babbin *et al.*, 2009; Klapproth, 2010). Indeed, this large multifunctional toxin, of approximately 360 kDa, is reported to encode three enzymatic activities: glycosyltransferase, protease and aminotransferase. Whether, LifA also possess protein phosphatase activity is an intriguing possibility. To confirm the requirement for one or more of these effectors in the dephosphorylation of AKT would require the deletion of these effector genes; ideally generating an EPEC strain lacking all LEE and all known Nle effectors.

EPEC induced host cell toxicity in J774A.1 macrophages was a prominent problem during this study; evident in WT EPEC infected cells, which was more prominent for EPEC strains lacking the EspZ effector and/or CesT chaperone (critical for EspZ translocation into target cells) (Shames *et al.*, 2010; Shames *et al.*, 2011; Roxas *et al.*, 2012). Indeed, EPEC strains lacking the CesT chaperone were associated with significant cell detachment, increased cytotoxicity and the dramatic loss of phosphorylated AKT by the end of infection (90/120 min post infection). Whilst it might be tempting to suggest the T3SS-dependent loss of phosphorylated AKT during EPEC infection was due to increased host cell toxicity and loss of cell viability in this study, several results would argue against this possibility. These include: 1) the capacity to restore AKT phosphorylation, stimulated by second-wave infection, in EPEC pre-infected cells following treatment with the phosphatase inhibitor Calyculin A; 2) the ability of macrophages to internalise the *Δquad* mutant by PI3K mediated phagocytosis; and 3) the ability of macrophages to internalise WT EPEC by PI3K independent mechanisms (<40% internalised) (Celli *et al.*, 2001; Quitard *et al.*, 2006). These latter findings were supported by one-wave and two-wave infection strategies.

Crucially, this toxicity associated loss of phosphorylated AKT was absent in macrophages infected with the TOEA7*Δcore::km* mutant (lacks CesT chaperone), inducing a T3SS mutant-like p-AKT_{ser473} profile. This strain, lacking all published Nle effectors (except EspC, LifA & NleJ) and 6 genes of the central 'core' LEE region (*map*, *espH*, *tir*, *cesT*, *cesF* & *eae* [Intimin]) is thus theoretically unable to express or deliver up to 20 of 24 published EPEC effectors. This strain not only supports a critical role for CesT, but also discounts a key role for the EspB effector in the T3SS-dependent decrease of phosphorylated AKT. Furthermore, this strain suggests that the toxicity-associated decline in phosphorylated AKT is linked to the activity of one or more of the 14 Nle effectors lacking from the TOEA7*Δcore::km* mutant. Surprisingly, significant infection induced cell detachment was evident after infection with the TOEA7*Δcore::km* mutant on HeLa cells, thus highlighting important differences with the macrophage cell model.

The HeLa cell model was developed to circumvent the identified issues of EPEC induced cell detachment/toxicity and avoid limitations of poor transfection efficiency in J774A.1

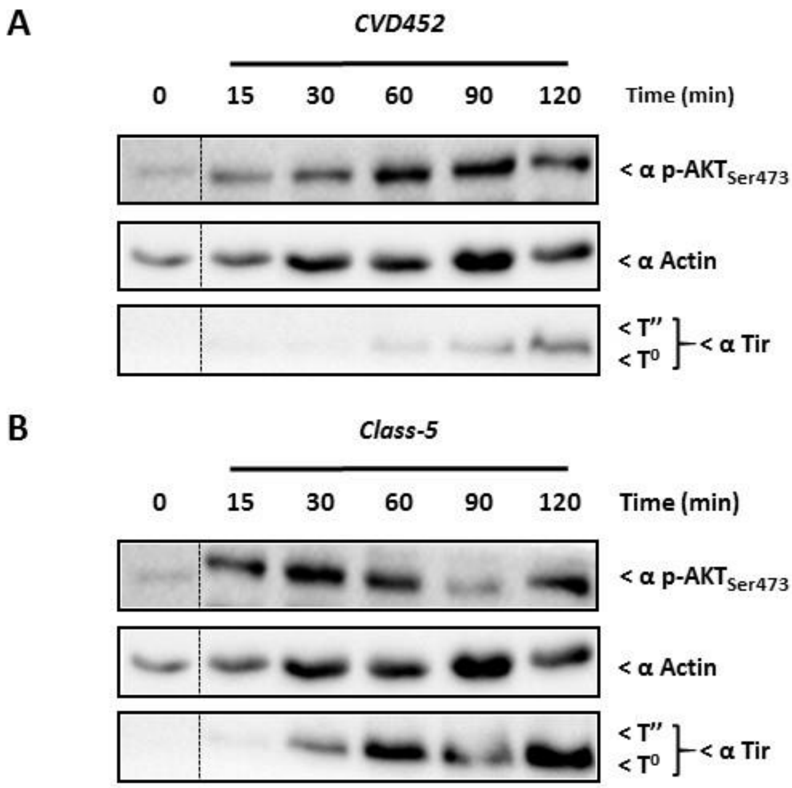
macrophages that could limit future investigations. This model could then be used to aid identification and characterisation of the EPEC effector(s) required to inhibit AKT phosphorylation. Although the HeLa cell model is reportedly more resistant to EPEC induced cell detachment (Kenny *et al*., unpublished), this was found not to be the case using our infection protocol; revealing notable levels of EPEC induced cell detachment, which increased significantly for strains lacking the EspZ effector and/or CesT chaperone. Due to the absence of infection induced AKT phosphorylation in this HeLa cell model, this study examined the ability of EPEC to decrease/inhibit EGF stimulated AKT phosphorylation. Importantly, infections with EPEC strains failed to reproduce key findings identified in J774A.1 macrophages of a CesT dependent decrease of phosphorylated AKT. This discrepancy between models was linked to the ability of EPEC to induce the degradation of the EGFR, reported to be mediated by EspF (induce EGFR degradation) and EspZ effectors (prevents EGFR degradation) (Roxas *et al.*, 2014). Importantly, modification of the EGFR was also evident following infection with the TOEA7*Δcore::km* mutant (unable to deliver EspF or EspZ effectors), implicating a role for other effectors that alter the EGFR. Whether this altered EGFR is indeed functional was not examined in this study. These findings tentatively suggest that degradation/modification of the EGFR is responsible for the inhibition of EGF induced AKT phosphorylation with TOEA7*Δcore::km* mutant infected cells. However, as this is from a single experiment, further work is required to confirm these observations before drawing firm conclusions.

The results of the HeLa cell model therefore suggest three possibilities: 1) EPEC inhibition of EGF induced AKT phosphorylation is due to cell death/toxicity; 2) inhibition of EGF triggered AKT phosphorylation is due to EPEC induced cleavage of the EGFR; or 3) involves a CesT independent mechanism. This latter possibility seems unlikely as this would suggest there are two different mechanisms (CesT dependent & CesT independent) which EPEC inhibits AKT phosphorylation in HeLa cells and J774A.1 macrophages. To resolve these questions would require further development and optimisation of the HeLa cell model. For example, studies could transfect the EspZ effector into HeLa cells to prevent EPEC induced toxicity and host cell detachment. In addition, studies could look to use a receptor independent mechanism for AKT phosphorylation such as transfection of constitutively active forms of AKT (m/p-AKT &/or ΔPH-AKT), as discussed earlier. Alternatively, agonists of AKT phosphorylation other than EGF could be used to examine the ability of EPEC to inhibit AKT phosphorylation, such as TNFα.

Although the HeLa cell model in its current form does not seem to be the best model to identify the EPEC effectors responsible for inhibiting AKT phosphorylation, this model could prove useful for future transfection studies to examine and characterise candidate EPEC effectors in isolation. Future work should therefore continue to develop the macrophage model, to overcome the identified limitations of increased cell detachment and reported poor transfection efficiency, to interrogate the EPEC effectors and mechanism required to inhibit infection induced AKT phosphorylation.

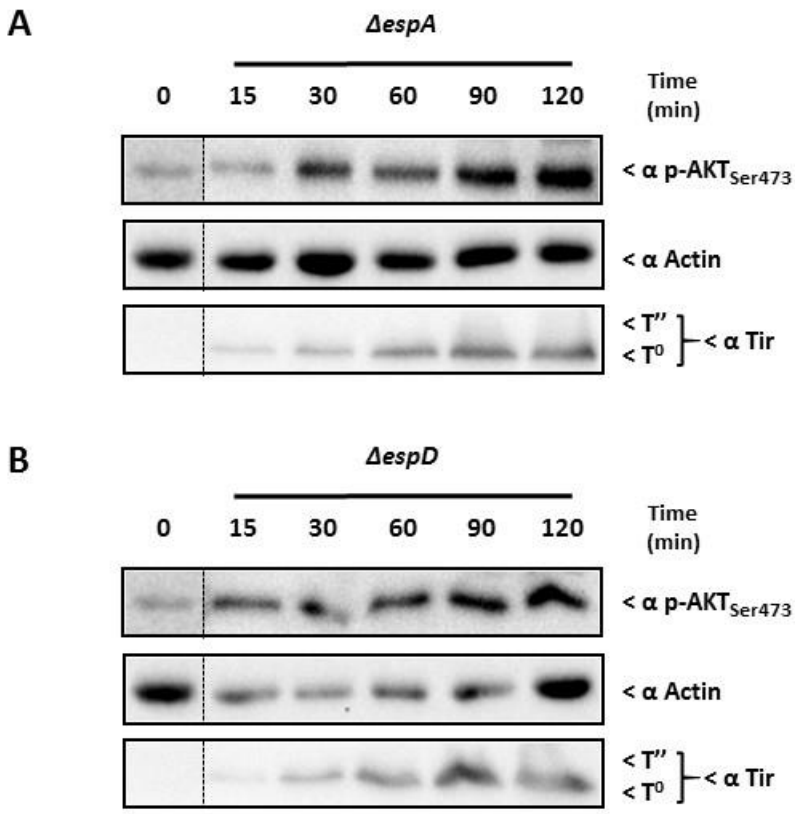
To conclude, this study has provided novel insights into the role of the EPEC effector repertoire in the T3SS-dependent decrease of phosphorylated AKT. Furthermore, by interrogating the mechanisms regulating AKT activation in the macrophage cell model, this study proposes a critical role for a host activated or bacterial delivered protein phosphatase to induce the rapid dephosphorylation of AKT. These experiments have set the groundwork to enable future studies to identify the virulence factors responsible and uncover its mechanism for inhibiting AKT phosphorylation, thus providing insight into the role of AKT and protein phosphatases in EPEC induced disease.

Appendix



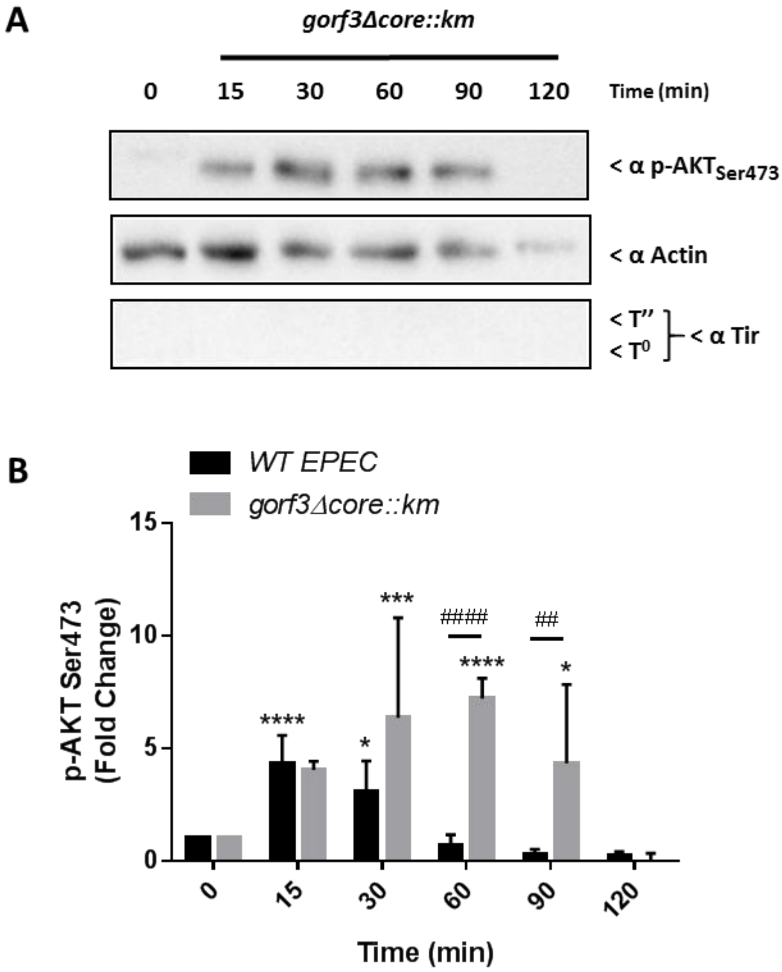
Appendix Figure 1 EPEC induced decrease of phosphorylated AKT requires a functional T3SS.

(**A & B**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated T3SS mutant *CVD452* (*ΔsepB*) or *Class-5* strains. Total cell extracts were was isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots presented are of a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.



Appendix Figure 2 The T3SS dependent decrease of phosphorylated requires translocon proteins EspA and EspD.

(**A & B**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with pre-activated *ΔespA* (lacks EspA) or *ΔespD* (lacks EspD) strains. Total cell extracts were was isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT$_{ser473}$, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Western blots presented are of a single experiment, with immunoblots (where appropriate) cropped and moved for presentation purposes, indicated by a dashed line.


A
gorf3Δcore::km
0
15
30
60
90
120
Time (min)
< α p-AKT Ser473
< α Actin
< T''
< T0
< α Tir
B
WT EPEC
gorf3Δcore::km
p-AKT Ser473 (Fold Change)
15
10
5
0
****
*
***
####
****
##
*
Time (min)

Appendix Figure 3 The T3SS dependent decrease of phosphorylated AKT is dependent on one or more genes encoded on the central 'core' LEE region.

(**A**) J774A.1 macrophages were left uninfected (0) or infected (MOI 200:1) with the pre-activated *gorf3Δcore::km* (lacking the Nle effector EspG2/Orf3, LEE effector EspG & proteins of the central core LEE region [EspH, Map Tir, CesF, CesT & Intimin] strain. Total protein cell extracts were isolated (1x SDS sample buffer) at 15, 30, 60, 90 and 120 min post infection. Protein samples were separated by SDS-PAGE (10%) for western blot analysis, probing for anti-p-AKT_{ser473}, anti-Tir and anti-Actin (loading control) antibodies. Host kinase mediated modification of Tir, dependent on its T3SS delivery into host cells, is indicated by an apparent increase in molecular mass from its unmodified (T^0) to its fully modified form (T'') (Kenny *et al.*, 1997). Where appropriate, immunoblots were cropped and moved for presentation purposes. (**B**) Densitometry analysis of western blots was performed using ImageLab software, with values representing a fold change in p-AKT_{ser473} signal, normalised to Actin (loading control) and calculated as relative change to uninfected control (0) cells. Quantification data are from a minimum of three independent experiments, with values shown being the mean ± SD. Values for p-AKT_{ser473} levels from WT EPEC infected cell extracts are taken from Figure 13. Statistical analysis was performed by two-way ANOVA followed by Dunnett's post-test for comparison against uninfected control cells (****p<0.0001, ***p<0.001. **p<0.01 and *p<0.05) or by Sidak's post-test for comparison of values at each corresponding time point (####p<0.0001, ###p<0.001, ##p<0.01 and #p<0.05).

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