

Regulation of host tyrosine kinases by the
Escherichia coli* and *Salmonella
ADP-ribosyltransferases EspJ, SeoC and SboC

Ph.D. Thesis

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Candidate's declaration

I, Dominic Pollard, declare that this thesis constitutes my own work and that any external contributions are appropriately acknowledged or referenced from hereon in.

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Abstract

The human pathogens enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) and *Salmonella enterica* are responsible for a significant proportion of the global diarrhoeal disease burden. EPEC, EHEC and the mouse-restricted pathogen *C. rodentium* (CR) make up the attaching and effacing (A/E) pathogens. CR is used to model EPEC/EHEC pathogenesis *in vivo*, where it colonises the apical surface of mouse intestinal mucosae, destroying microvilli and inducing colonic crypt hyperplasia (CCH) and diarrhoeal disease symptoms. The type three secretion system (T3SS) is instrumental to the infectivity of the A/E pathogens, as well as *Salmonella*, through the injection of sets of effector proteins into the host cell cytosol for the manipulation of host cell signalling.

Prior to this study the A/E pathogens T3SS effector EspJ was identified as a novel enzyme that can disrupt FcγR- and C3R-mediated phagocytosis, dependent upon a functional ADP-ribosyltransferase (ART) domain. *In vitro* EspJ was shown to inhibit Src kinase through simultaneous amidation and ADP-ribosylation of a conserved kinase domain residue, E310. This study shows that the inhibition of phagocytosis is conserved by the EspJ homologues *Salmonella enterica* subsp. *salamae* and *arizonae* SeoC, *S. bongori* SboC and *Y. enterocolitica* YspJ. SeoC is secreted by the *Salmonella* pathogenicity island (SPI)-1 T3SS but does not impact *Salmonella* entry into host cells. An *in vitro* proteomics assay showed that EspJ_{EHEC} could ADP-ribosylate a multitude of host non-receptor tyrosine kinases (NRTK), including Src and its inhibitor Csk which were also ADP-ribosylated by SeoC and SboC, but not YspJ. Finally, a proteomics comparison of IECs purified from mice infected with CR expressing functional or non-functional EspJ, suggested that EspJ modulates host inflammation and the immune system *in vivo*.

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Abbreviations

GI	Gastro intestinal
ACN	Acetonitrile
2YnAd	Alkyne-tagged adenosine
A/E	Attaching and effacing
ADPr	ADP-ribosylation
AIEC	Adherent invasive <i>E. coli</i>
AJ	Adherens junction
ART	ADP-ribosyltransferase
ARTC	Clostridial toxin like ART
ARTD	Diphtheria toxin like ART
ATP	Adenosine triphosphate
AUC	Analytical ultracentrifugation
AzRB	Azide arginine biotin capture reagent
AzRTB	Azide arginine TAMRA biotin capture reagent
bART	Bacterial ADP-ribosyltransferase
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
Cbp	Csk binding protein
CCH	Colonic crypt hyperplasia
CENHP	Centromere protein H
CGD	Chronic granulomatous disease
CIP	Calf intestinal phosphatase
CR	Citrobacter rodentium
CR3	Complement receptor 3
Csk	C-terminal Src kinase
CT	Cholera toxin
DAEC	Diffusely adherent <i>E. coli</i>
DAG	Diacyl glycerol
DAMP	Damage associate molecular patter
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Media
DMP	Dimethyl pimelimidate
DSS	Dextran sulphate sodium
DT	Diphtheria toxin
DUOX	Dual oxidase
EAEC	Enteropathogenic <i>E. coli</i>
ECM	Extracellular matrix
eEF2	Eukaryotic elongation factor 2
EGF	Epidermal growth factor
EHEC	Enterohaemorrhagic <i>E. coli</i>

EIEC	Enteroinvasive <i>E. coli</i>
eNAD	2-ethynyl-adenosine-NAD
EPEC	Enteropathogenic <i>E. coli</i>
Esc	<i>E. coli</i> secretion apparatus component
Esp	<i>E. coli</i> secreted protein
ETEC	Enterotoxigenic <i>E. coli</i>
FA	Formic acid
FACS	Fluorescence activated cell sorting
Fak	focal adhesion kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FRET	Fluorescence energy transfer
GAP	Guanine activating factor
GDI	guanosine dissociation inhibitor
GEF	Guanine exchange factor
GO	Gene ontology
GPCR	G-protein coupled receptor
GST	Glutathione-S-transferase
GuHCl	Guanidinium hydrochloride
HC	haemorrhagic colitis
HUS	Haemolytic uremic syndrome
IEC	intestinal epithelial cell
IFN	Interferon
IFT20	intraflagellar transport protein 20
IgG	Immunoglobulin G
IKK	IκK kinase
IL1R	Interleukin-1 receptor
ILK	Integrin linked kinase
IMAC	Immobilised metal ion affinity chromatography
iNOS	Inducible NOS
IP3	Inositol-(1,4,5)-triphosphate
IPA	Ingenuity pathway analysis
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRAK	IL1 receptor associated kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine activation motif
ITIM	Immunoreceptor tyrosine inhibitory motif
IκB	Inhibitor of NFκB
Jak	Janus kinase
JAM	Junction adhesion molecule
KEA	Kinase enrichment analysis
LCA	Leukocyte common antigen
LDH	Lactate dehydrogenase
LEE	Locus of enterocyte effacement

LPS	lipopolysaccharide
LT	Heat labile toxin
MAPK	Mitogen activated kinase
MAR	Mono-ADP-ribosylation
MBP	Maltose binding protein
MKK	MAPK kinase
MR	Mannose receptor
MTOC	Microtubule organising centre
NAD ⁺	Nicotinamide adenosine dinucleotide
NEMO	NFκB essential modifier
NHE	Na ⁺ /H ⁺ exchanger
Nle	<i>Non-LEE encoded</i>
NLR	NOD-like receptor
NOD	Nucleotide oligomerisation domain-containing
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NRTK	Non-receptor tyrosine kinase
NTS	Non-typhoidal <i>Salmonella</i>
OD	Optical density
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PAR	Poly-ADP-ribosylation
PARG	PAR-glycohydrolase
PARP	Poly-ADP-ribosyltransferase
PBS	Phosphate buffered saline
PBST	PBS-Tween20
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Peptidoglycan
PH	Plekstrin homology
PI3K	phosphatidylinositol-3-kinase
PI3P	phosphatidylinositol-3-phosphate
PIP2	Phosphatidylinositol-(4,5)-biphosphate
PIP3	Phosphatidylinositol-(3,4,5)-biphosphate
PMA	phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PTM	Post translational modification
PTP	Protein tyrosine phosphatase
Ptprc	Protein tyrosine phosphatase receptor type C
PVDF	Polyvinyl fluoride
RIC8A	Synmembrin-8
RLR	RIG-I like receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RTK	Receptor tyrosine kinase
<i>S. arizonae</i>	<i>S. enterica</i> subspecies <i>arizonae</i>
<i>S. salamae</i>	<i>S. enterica</i> subspecies <i>salamae</i>
SCV	<i>Salmonella</i> -containing vacuole
SDM	Site directed mutagenesis
Sec	General secretory
SEC-MALLS	Size exclusion chromatography multi angle laser light scattering
SFK	Src family kinase
SH	Src homology
SIRT	Sirtuin
Stx	Shiga-like toxin
SNX	Sorting nexin
SPI	<i>Salmonella</i> pathogenicity island
Stage	Stop and go extraction
STEAEC	Shiga toxin producing enteroaggregative <i>E. coli</i>
STEC	Shiga toxin producing <i>E. coli</i>
T1SS	Type one secretion system
T2SS	Type two secretion system
T3SS	Type three secretion system
T4SS	Type four secretion system
T5SS	Type five secretion system
T6SS	Type six secretion system
TAB	TAK1 binding protein
TAE	Tris-acetate-EDTA
Tat	Twin arginine translocation
TCA	Tricarboxylic acid
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
Tir	Translocated intimin receptor
TJ	Tight junction
TLR	Toll-like receptor
TMT	Tandem mass tag
TNF	Tumor necrosis factor
TNFR1	TNF factor receptor 1
UPEC	Uropathogenic <i>E. coli</i>
WAVE	WASP-family verprolin-homologous protein
WRC	WAVE regulatory complex
X2K	Expression 2 kinase
ZO	Zonula occludens
ΔART	EHEC EspJ R79A catalytic mutant strain

Chapter 1: Introduction

Chapter 1: Introduction

1 Bacterial pathogenesis

The bacteria-host relationship may take one of many forms. Bacteria and host can exist in a harmonious state beneficial to both parties, in which case bacteria are referred to as commensals, and together constitute the host's natural microbiome. Alternatively, bacteria might infect a host resulting in disease onset. These so-called pathogenic bacteria can be classified as frank/primary pathogens which are able to cause disease within individuals of normal health. Alternatively, opportunistic pathogens are not usually harmful, but may take advantage of immunocompromised individuals or those with an altered microbiota which can result from changes in diet, and stress. Opportunistic pathogens may ordinarily be part of a healthy microbiota or be acquired from the external environment.

1.1 Diarrhoeagenic bacteria *Escherichia coli* and *Salmonella*

Diarrhoea remains a serious public health issue and is the second leading cause for morbidity and mortality in children under five, accounting for 1 in 9 child deaths worldwide (Liu et al., 2012; Walker et al., 2012). The frequency and severity of diarrhoeal diseases are increased in mid- to low-income countries where poor environmental hygiene, water sanitation and malnutrition are most prevalent. The principal component for treatment of diarrhoeal diseases is oral rehydration therapy to replace essential fluids and electrolytes (Riddle et al., 2016). Anti-diarrhoeal agents may be administered which inhibit intestinal peristalsis, promote intestinal reabsorption and may possess anti-secretory properties. While most cases are mild and self-limiting, more severe infections especially in the young and immunocompromised individuals may be treated with anti-microbial agents. Nevertheless, the use of antibiotics is often not recommended in non-complicated cases as it can increase adverse effects (Goldwater and Bettelheim, 2012; Sirinavin and Garner, 1999). The causative agents for such diarrhoeal

diseases may be bacterial pathogens including *Salmonella* spp., *Yersinia* spp. and *Escherichia coli* which colonise the gastro-intestinal (GI) tract.

E. coli and *Salmonella* are both members of the *Enterobacteriaceae* family and share over 100 million years of common ancestry (Ochman and Wilson, 1987). The *Enterobacteriaceae* are a group of Gram-negative rod-shaped bacteria that are non-sporulating and facultative anaerobes. Members can be distinguished by clinical tests for motility, lactose fermentation and strain specific surface antigens. Both *E. coli* and *Salmonella* are motile bacteria capable of colonising the GI tract, but *E. coli* and not *Salmonella* is a lactose fermenter. Most *E. coli* strains are non-pathogenic and comprise a significant proportion of the GI tract flora. However, a subset of pathogenic *E. coli* strains can cause a range of intestinal and extra-intestinal conditions including urinary tract infections, sepsis/meningitis and diarrhoeal disease. While *Salmonella* may occupy the GI tract harmlessly, it is one of the most commonly isolated food-borne human pathogens worldwide (Scallan et al., 2011). The pathogenicity of these bacteria is attributed to the acquisition of mobile genetic elements encoding adhesins, toxins and secretion systems. Such diversity is exemplified by the comparison of laboratory strain *E. coli* K12 strain MG1655 with pathogenic Shiga-toxin producing *E. coli* (STEC) strain EDL933 and Uropathogenic *E. coli* (UPEC), which showed that only 39.2% of the proteome is shared between the three bacteria (Welch et al., 2002).

1.1.1 Diarrhoeagenic *Escherichia coli* epidemiology

A subset of *E. coli* pathotypes have been traditionally implicated in endemic and epidemic cases of diarrhoeal disease. These are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) (Kaper et al., 2004; Nataro and Kaper, 1998). More recently two new pathotypes have been identified; adherent invasive *E. coli* (AIEC) and Shiga-toxin producing enteroaggregative *E. coli* (STEAEC). In

developing countries where poor sanitation is the major predecessor of diarrhoeal diseases, EPEC, EAEC and ETEC are the most successful pathotypes. Contrastingly, in developed countries where infections are primarily associated with outbreaks of contaminated food sources, EIEC, STEC, and the STEC member enterohaemorrhagic *E. coli* (EHEC) are most frequently contracted (Clements et al., 2012).

I. Invasive diarrhoeagenic E. coli (AIEC and EIEC)

The AIEC pathotype is an invasive strain of *E. coli* most commonly associated with Crohn's Disease. They can invade and replicate within macrophages without stimulating cell death signalling, while inducing release of pro-inflammatory TNF- α , contributing to Crohn's Disease inflammatory symptoms (Darfeuille-Michaud, 2002). However, AIEC is considered a pathobiont as they can exist harmoniously within the gut mucosae and usually only cause harm when the intestinal barrier is genetically or environmentally compromised (Martinez-Medina and Garcia-Gil, 2014).

EIEC is mostly associated with food and travel related outbreaks (Newitt et al., 2016) and is genetically similar to the invasive pathogen *Shigella*. EIEC falls into the category of type III secretion system (T3SS)-expressing *E. coli* along with the extracellular pathogens EPEC and EHEC, translocating tens of effector proteins into host cells enabling the invasive cycle. These effectors partially overlap with the repertoire of both the EPEC/EHEC and *Shigella* T3SS though much of the EIEC pathogenesis appears to be the result of the *Shigella* proteins IpaA, IpaB, IpaC and IpdD (Killackey et al., 2016).

II. Extracellular diarrhoeagenic E. coli

In the developing world ETEC is the most commonly isolated diarrhoeagenic *E. coli*, responsible for around 20% of diarrhoea caused by enteropathogenic bacteria in children under five years old (Qadri et al., 2005). The ETEC heat-labile enterotoxin (LT) and/or the heat-stable

enterotoxin (ST) exacerbate diarrhoeal symptoms through their perturbation of ion homeostasis.

DAEC are characterised by a diffuse pattern of adherence to the entire surface of Hep-2 cells *in vitro* and have been isolated from numerous cases of diarrhoeal disease, most commonly from children under 5 years old and particularly those below 12 months (Nataro and Kaper, 1998; Scaletsky et al., 2002). However, some studies fail to show a difference in DAEC prevalence between diarrhoea patients and control patients (Gunzburg et al., 1993; Scaletsky et al., 2002).

EAEC and STEAEC are typified by their ‘stacked-brick’ adherence to epithelial cells, such as Hep-2 (Nataro et al., 1992; Weintraub, 2007) which is regulated by the aggregative adhesive fimbriae (AAF) and the secreted protein Dispersin (Sheikh et al., 2002). EAEC is the second leading cause of traveller’s diarrhoea worldwide (Scavia et al., 2008). Some EAEC strains have recently been identified with the Shiga toxin (Stx), and these strains (STEAEC) have been reported in outbreaks to cause haemolytic uremic syndrome (HUS), a disease symptom more typical of the Stx producing EHEC strains (Bielaszewska et al., 2011; Paddock et al., 2013).

III. *Enteropathogenic E. coli (EPEC) and Enterohaemorrhagic E. coli (EHEC)*

Together with the mouse-restricted pathogen *Citrobacter rodentium* (CR), EPEC and EHEC make up the so-called attaching and effacing (A/E) pathogens, characterised by the formation of filamentous actin rich pedestals beneath the attached bacteria along with the destruction of the surrounding enterocyte brush border microvilli, in a T3SS-dependent manner (Frankel et al., 1998). The resulting reduction in the absorptive surface disrupts ion transport between the GI lumen and enterocytes contributing to diarrhoeal symptoms. Classically EHEC are believed to have evolved from EPEC via acquisition of the Shiga toxin (Stx) (Karmali et al., 1983), however, there are also Stx-independent differences in epidemiology (Nataro and Kaper, 1998;

Spears et al., 2006). EPEC transmission is via the faecal-oral route and usually acquired from contaminated water sources. Reservoirs are mostly human but have also been observed in dogs and cattle. EPEC infections are most common in young infants in the developing world, causing watery diarrhoea, vomiting and fevers, and have associated mortality rates between 10% and 40% (Deborah Chen and Frankel, 2005). Contrastingly, EHEC infections are more prevalent in the developed world infecting both the young and the elderly. Ruminants make up the main reservoirs of EHEC, and outbreak sources include contaminated bovine food products, fruits and vegetables (Berger et al., 2010; Ferens and Hovde, 2011). Diarrhoeal disease symptoms may develop into haemorrhagic colitis (HC) and HUS, characterised by bloody diarrhoea and acute renal failure. Stx is an AB₅ toxin that following binding to the glycolipid globotriaosylceramide (Gb3) receptor and internalisation into intestinal epithelial cells (IEC), cleaves 28S ribosomal RNA inhibiting protein synthesis (Johannes and Römer, 2010). Stx is responsible for HC and HUS manifestation via the disruption of protein synthesis in enterocytes and endothelial cells resulting in their death (Nataro and Kaper, 1998).

IV. Citrobacter rodentium and the mouse model of A/E pathogen infection

EPEC and EHEC are not able to colonise or form A/E lesions on the intestinal mucosae of mice. However, CR is a natural pathogen of mice and shares the colonisation mechanisms displayed by EPEC and EHEC in humans. Therefore, the CR mouse model of infection is considered the ‘gold standard’ infection model for EPEC and EHEC pathogenesis, and has also been used to investigate numerous human intestinal disorders including Crohn’s disease, ulcerative colitis and colon tumorigenesis (Chandrakesan et al., 2014; Collins et al., 2014; Higgins et al., 1999). CR is transmitted via the faecal-oral route, resulting in colitis and colonic crypt hyperplasia (CCH), which is characterised by increased epithelial regeneration and repair mechanisms resulting in a thickened colonic mucosa. At the peak of infection CR represents 1-3% of the intestinal microbiota and 10⁹ colony forming units per gram in the colon (Collins

et al., 2014). During infection CR intimately attaches to the host IECs, initially colonising the caecal patch, a lymphoid structure of the caecum, and eventually occupying the distal colon and rectum by day four post infection. The bacterial load peaks at day eight before CR is cleared, via the shedding of infected epithelial cells from first the caecum and then the colon. CR is fully cleared, two to three weeks post infection (Collins et al., 2014).

CR encodes a fully functional T3SS along with an effector protein repertoire that shares all 22 of the core effectors in EPEC strain E2348/69 (Petty et al., 2010). Crucially this means that CR is an invaluable tool to investigate the *in vivo* roles of T3SS effectors. Accordingly, the impacts of individual effectors on colonisation, CCH and immune responses has been characterised (Kelly et al., 2006; Mundy et al., 2005; Pallett et al., 2017; Pearson et al., 2017), though the subtle role of an effector is often masked by the contributions of potentially 50 others.

1.1.2 *Salmonella* spp. epidemiology

Salmonella is reported to cause at least 1.2 million illnesses per annum, resulting in some 450 fatalities in the United States alone, and an estimated 1.3 billion cases worldwide (Scallan et al., 2011). The *Salmonella* genus is divided into two species, *S. bongori* and *S. enterica*. *S. bongori* is primarily associated with reptiles, and while capable of infecting humans, is only responsible for a minor proportion of reported illnesses (Fookes et al., 2011). There are six *S. enterica* subspecies; *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* which are further divided into over 2600 serovars based upon serotypes defined by their O (lipopolysaccharide, LPS) and H (flagellar) antigens (Grimont and Weill, 2008; Guibourdenche et al., 2010) (Scallan et al., 2011). Together these intracellular bacteria can cause a range of symptoms including fever (typhoidal), enterocolitis/diarrhoea, bacteraemia or asymptomatic carriage. The *enterica* subspecies can be divided into two groups; the causative agent of typhoid fever – *Salmonella enterica* subspecies *enterica* serovar Typhi (*S. Typhi*), and

non-Typhoidal *Salmonella* (NTS) which cause a self-limiting diarrhoeal disease called Salmonellosis. While NTS has a wide range of wild and domestic animal reservoirs including ruminants, birds and reptiles (Mermin et al., 2004; Rodriguez et al., 2006) and is capable of causing disease in these hosts and humans, *S. Typhi* is human-restricted.

I. Non-Typhoidal Salmonella (NTS)

Most reported Salmonellosis cases result from infections with *S. enterica* subsp. *enterica*. For example, a recent report from the Netherlands found that only 0.15% of human Salmonellosis was caused by non-*enterica* subspecies (Mughini-Gras et al., 2016). Thus, research into the infection strategies of the remaining *S. enterica* subspecies and *S. bongori*, is limited to date. The non-*enterica* subspecies can cause sporadic disease in mammals, most commonly within children and immuno-compromised individuals (Hall and Rowe, 1992; Lakew et al., 2013; Lee et al., 2016; Mahajan et al., 2003; Nair et al., 2014) and several fatalities have been reported as a result of these rarer subspecies (Abbott et al., 2012). In particular, incidences resulting from these rarer subspecies have increasingly been observed in relation to the growing popularity of reptiles as pets (Mermin et al., 2004). *S. enterica* subspecies *arizonae* is the most common non-*enterica* subspecies isolated from humans causing a wide range of infections including gastroenteritis, bacteraemia, septic arthritis and osteomyelitis. Subspecies *salamae* was responsible for 200 cases of Salmonellosis and bacteraemia between 2004 and 2012 in England and Wales (Nair et al., 2014). Interestingly subspecies *houtenae* is most commonly associated with brain infections such as meningitis (Lamas et al., 2018). Taken together, clinical evidence suggests that as a proportion of cases, those caused by non-*enterica* subspecies are more likely to cause extra-intestinal disease, particularly in individuals with underlying conditions. This perhaps reflects lower pathogenic capabilities than their counterparts in the *enterica* subspecies. Due to the rarity of reported human infections from these subspecies, little is known about their *in vivo* infection process.

NTS caused by serovars from the *enterica* subspecies, such as *S. Typhimurium*, induces a self-limiting localised gastroenteritis. It is estimated that 1% of cases lead to bacteraemia, though this figure is increased in immunocompromised individuals especially in sub-Saharan Africa (Feasey et al., 2012; Graham, 2010). *S. enterica* localise to the apical intestinal epithelium where they translocate across the epithelial barrier by similar methods to *S. Typhi*, including M cell transcytosis at the Peyer's patches (Hallstrom and McCormick, 2011; Jensen et al., 1998). Bovine, swine and rabbit infections produce an enterocolitis more similar to human infections than the mouse models do. This is most severe in the caudal ileum, cecum and proximal colon (Santos et al., 2001). Here the bacteria induce inflammatory responses, including immune cell infiltration, crypt abscesses, epithelial cell death, oedema and fluid secretion. A hallmark of this infection is neutrophil recruitment within 1-3 h of infection, and diarrhoeal symptoms begin within 8-72 h post colonisation (Tsolis et al., 1999; Wray and Sojka, 1978). Within humans, symptoms occur within a similar time frame mainly affecting the large bowel and occasionally the jejunum, duodenum and stomach.

1.2 Gram-negative bacterial secretion systems

The delivery of proteins from the bacterial cytoplasm to the surrounding environment or into other bacterial/mammalian cells is essential for bacterial survival and pathogenesis (Green and Mecsas, 2016; Tseng et al., 2009). A protein must cross two membranes in Gram-negative bacteria to be secreted, and if translocated into target cells, the host plasma membrane too. To overcome this barrier Gram-negative bacteria have evolved up to six divergent secretion systems (named type 1 - 6) (Figure 1.1). The type one secretion system (T1SS) spans both the inner and outer membranes while in the case of the type three, four and six secretion systems (T3SS, T4SS and T6SS) bacterial inner and outer membranes and the target cell membrane are crossed by one macromolecular complex. Conversely, types two and five (T2SS and T5SS) rely upon the general secretory (Sec) and twin arginine translocation (Tat) pathways to

transport proteins from the cytoplasm to the periplasm, before facilitating protein movement across the outer membrane themselves. Together these systems perform a wide range of functions and share homology to a diversity of structures from prokaryotes, eukaryotes and viruses.

In ETEC the T2SS is responsible for the transport of the heat labile toxin (LT) (Tauschek et al., 2002), whereas EHEC utilises the T2SS for transport of the proteinase/mucinase StcE essential for colon colonisation through mucin degradation (Lathem et al., 2002), and the EPEC T2SS exports SslE which contributes to biofilm formation (Baldi et al., 2012). Substrates of the T5SS pathway are also reliant upon the Sec apparatus for transport to the periplasm, but once here facilitate their own export by utilisation of their beta barrel structure. EPEC strain E2348/69 delivers the cytotoxic serine protease EspC via this mechanism into host cells, disrupting focal adhesion kinase (Fak) and spectrin leading to cell detachment (Navarro-Garcia et al., 2014). Similarly, the homologous EHEC EspP causes perturbations in multiple pathways including the complement cascade and biofilm formation (Weiss and Brockmeyer, 2013). The Sec-Tat-independent T1SS, which closely resembles ATP-binding cassette (ABC) transporters (Symmons et al., 2009; Thomas et al., 2014), transports substrates often involved in bacterial virulence; for example the UPEC HlyA haemolysin protein which leads to host cell rupture allowing bacteria to cross mucosal barriers (Welch et al., 1981). The ancestry of the T4SS can be traced to bacterial DNA conjugation systems, and are able to transport monomeric proteins, or protein-protein and protein-DNA complexes to prokaryotic or eukaryotic cells (Cascales and Christie, 2003). Due to their range of substrates, T4SS can function in DNA conjugative transfer, uptake or release as well as effector protein translocation and are involved in the pathogenesis of bacteria such as *Legionella pneumophila* and *Helicobacter pylori* (Backert and Meyer, 2006). The most recently discovered secretion system is the T6SS which translocates proteins directly from the bacterial cytoplasm most commonly to competing bacteria or into

eukaryotic cells. Here, effector proteins are crucial for bacterial competition and virulence in eukaryotic cells. The T6SS shares homology to T4 bacteriophage tails and may have arisen from inverted phage tails, reversing the direction of protein transport (Pukatzki et al., 2007). While EPEC doesn't encode a T6SS, it is utilised by EAEC (Felisberto-Rodrigues et al., 2011), EHEC and CR (Gueguen and Cascales, 2013; Petty et al., 2010) and there is evidence for its use in intra-bacterial competition for CR (Gueguen and Cascales, 2013)

1.2.1 The type three secretion system

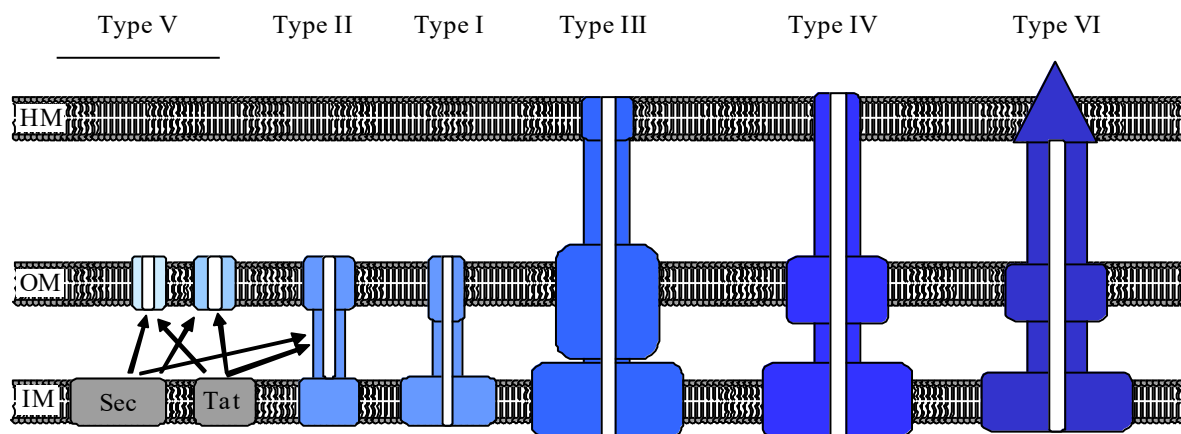


Figure 1.1: Schematic of bacterial secretion systems.

Types I to VI secretion systems are displayed in relation to bacterial inner (IM) and outer membranes (OM), as well as the target host membrane (HM). Image adapted from (Tseng et al., 2009).

In 1991 it was observed that *Yersinia* Yop proteins were translocated in a Sec-independent manner (Michiels et al., 1990), and it was less than ten years later that this macromolecular machine termed the type three secretion system (T3SS) was visualised in *Salmonella* by negative stain electron microscopy (Kubori et al., 1998). Subsequent research has led to an intricate understanding of regulation, assembly and energising of this 3.5 MDa injectisome, and the effects of the protein effectors translocated by it in a range of plant and mammalian

pathogens. The following sections outline the T3SS using *E. coli* nomenclature, highlighting certain aspects that differentiate the A/E pathogen and *Salmonella* machines.

The needle-like structure of the T3SS (Figure 1.2) that protrudes from the bacterial surface is built upon the multi-ring basal body that bears a close resemblance to the bacterial flagellin. This basal body consists of three membrane-embedded multimeric rings connected by a periplasmic inner rod connecting the channels created in the bacterial inner and outer membranes, and the export apparatus that sits on the cytoplasmic side of the inner membrane. This export apparatus, comprised of proteins *E. coli* secretion apparatus component (Esc)R/S/T/U/V, nucleates T3SS assembly recruiting ring components which are exported via the Sec pathway (Diepold et al., 2011). EscD and EscJ form inner membrane-spanning and membrane-tethered rings respectively and EscD interacts with the outer membrane EscC ring (Creasey et al., 2003; Ogino et al., 2006). These rings are adjoined by the inner rod channel formed of multiple EscI units, which forms the base from which the multimeric EscF needle assembles. Ring insertion is followed by the formation of the EscO C-ring/sorting complex which in flagellar motors is proposed to provide torque. The EscO C-ring has also been proposed in *Salmonella* to play a role in regulating the order of early/mid/late- effector translocation, and form a platform upon which the energising ATPase complex forms (Biemans-Oldehinkel et al., 2011; Pallen et al., 2005). This ATPase which is composed of the ATPase EscN and its positive and negative regulators EscO and EscL (Biemans-Oldehinkel et al., 2011; Romo-Castillo et al., 2014), has remarkable structural similarity to the flagellar and F1 ATPase, and powers the translocation of effector proteins.

The EPEC EscF needle is 23 nm long and 8-9 nm wide making it the shortest observed in comparison to *Salmonella enterica* (25-80 nm), *Yersinia pestis* (41 nm), *Yersinia enterocolitica* (58 nm) and *Shigella flexneri* (45-50 nm). Contrastingly, needle length is tightly conserved within species. Many models have been proposed for needle length regulation which

is known to rely upon T3SS specificity switches or molecular rulers. In EPEC needle length is regulated by the interaction of EscP with the assembling EscF subunits and the cytosolic EscU, the latter causing a switch in substrate specificity from needle to filament and translocon components (Creasey et al., 2003; Feria et al., 2012; Sal-Man et al., 2012).

The EspA filament structure is unique to A/E pathogens and elongates the T3SS protrusion. Its length varies but can reach up to 600 nm (Crepin et al., 2005; Knutton et al., 1998). It is capped by the EspB/EspD translocon which forms a 3-5 nm pore in the host membrane (Ide et al., 2001) completing the continuous channel from the bacterial to mammalian cytosols. This triggers a second substrate specificity switch from mid- to late-effector proteins facilitated by the SepD-SepL-CesL complex (O'Connell et al., 2004). It has recently been shown that the ruler protein EscP also plays a role in this second switch, via its interaction with SepL (Shaulov et al., 2017). As the SepL-EscP interaction is calcium dependent, this could explain the previously described role of calcium in the second switch (Deng et al., 2005; Ide et al., 2003; Kenny et al., 1997a; Shaulov et al., 2017).

Chaperones are also crucial throughout the T3SS assembly, and for translocation of early-/mid-/late-effector proteins. Typically small acidic proteins, these chaperones are key to guiding structural components and effectors to the export complex, partitioning subunits to prevent their premature homo- or hetero-oligomeration in the bacterial cytosol, and regulating the hierarchy of late-effector translocation. Eight T3SS chaperones have been studied and grouped into Class 1A with one substrate (CesF and CesL), Class 1B with several substrates (CesT), Class II specific to translocon subunits (CesD, CesD2, CesAB, and CesA2), and Class III for needle subunits (CesA2/EscG) (Gaytán et al., 2016).

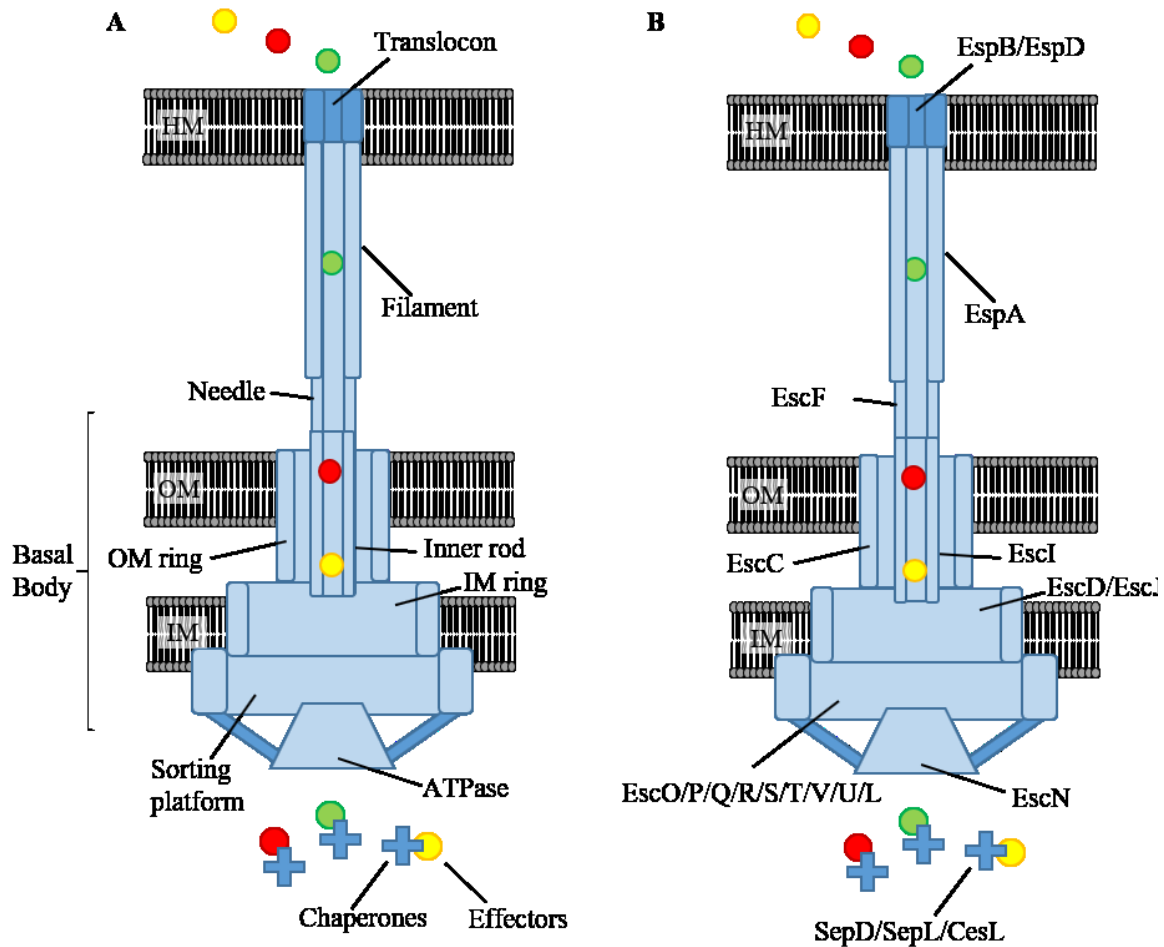


Figure 1.2: Schematic of the T3SS architecture

(A) The T3SS basal body consists of several membrane-spanning ring-like structures crossing the bacterial inner membrane (IM) and outer membrane (OM), and powers the translocation of bacterial effector proteins delivered by chaperones to the sorting platform. The T3SS needle protrudes from the basal body and is tipped by the translocon pore-forming complex that punctures host membranes (HM). This creates a continuous channel from the bacterial to host cytosol for direct delivery of bacterial effector proteins. (B) A/E pathogen structural subunits of the T3SS.

1.3 T3SS-directed pathogenesis

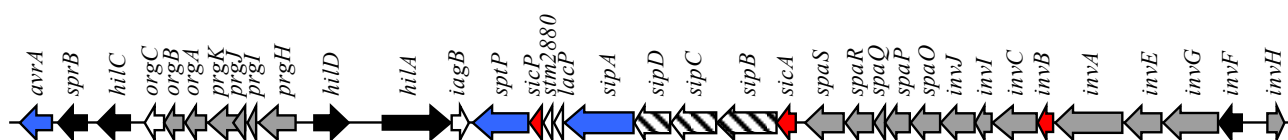
1.3.1 Pathogenicity islands

The T3SS of *Salmonella* and *E. coli* translocate an arsenal of effector proteins into the cytosol of host cells. These macromolecular machines along with several effectors are encoded within mobile genetic elements named pathogenicity islands, which due to their characteristic low GC content are presumed to have been acquired by horizontal gene transfer.

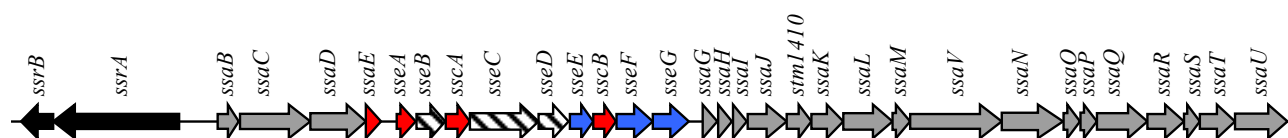
EPEC, EHEC and CR express a single functional T3SS, encoded within the highly conserved locus of enterocyte effacement (LEE) pathogenicity island (Elliott et al., 2002; Jarvis et al., 1995; McDaniel et al., 1995). This 35.6 kb island in EPEC encodes the essential virulence factors for the effacement of intestinal microvilli and the intimate attachment of bacteria, and results in the actin rich pedestals on cultured epithelial cells typical of A/E pathogens. The LEE harbours 5 operons (LEE1-5) encoding 41 open reading frames (ORFs) including structural T3SS components, chaperones and several translocated effectors (Figure 1.3).

While over twenty *Salmonella* pathogenicity islands (SPIs) have been described, *Salmonella* strains are dependent upon the presence of two for virulence; the SPI-1 (Galán et al., 1989; Mills et al., 1995) and SPI-2 (Coburn et al., 2005; Ochman et al., 1996; Shea et al., 1996) (Figure 1.3). Like the LEE, both islands encode the structural components, chaperones and some effectors of an operational T3SS but components and functions differ in many ways (Dieye et al., 2009). The 40.2 kb SPI-1 encodes 41 genes required for the invasion of phagocytic cells, non-phagocytic cells and M-cells. After invasion, certain SPI-1 effector proteins contribute to the early biogenesis of a specialised intracellular compartment, the *Salmonella* containing vacuole (SCV) which serves to avoid autophagosomal degradation of *Salmonella*. Later acidification of the vacuolar lumen activates expression of the *Salmonella* SPI-2 T3SS. The 39.8 kb SPI-2 encodes 44 genes for the regulation of the latter SCV maturation stages to prevent lysosomal fusion and degradation of the compartment. Furthermore, they utilise the host cell microtubule network to control the movement of the SCV. Initially the SCV is directed towards the perinuclear region near the microtubule organising centre (MTOC) at the Golgi apparatus, a spatial localisation shown to maximise bacterial replication. At later stages of infection the SCV moves towards the cell periphery where *Salmonella* are presumably released into the intestinal lumen allowing the spreading of infection (Haraga et al., 2008; LaRock et al., 2015).

B - SPI-1



C - SPI-2



■ Machinery ■ Effector ■ Translocon ■ Regulator ■ Chaperone ■ Intimin ■ Putative orf

Schematic representation of the (A) EPEC locus of enterocyte effacement (LEE) and (B/C) *Salmonella* pathogenicity island (SPI)-1 and SPI-2. T3SS structural components/machinery are shown in grey, translocon subunits in diagonal black lines,

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these tools allow intricate control of the host cell and while a great deal is now understood, there remains a vast amount of unknown information about these effectors, especially within an *in vivo* setting.

1.3.2 Induction of diarrhoeal symptoms

The IECs form a crucial barrier to the external environment of the intestinal lumen; denying toxins, antigens and microbes access to the lamina propria, and performing selective absorption of nutrients, electrolytes and water. This single layer of cells is organised into crypts and villi, constantly renewed by the action of stem cells, and covers a surface area of about 400 m² making it the largest mucosal surface in the body (Peterson and Artis, 2014). *Salmonella* and the A/E pathogens utilise both T3SS-dependent and T3SS-independent mechanisms to disrupt protein synthesis and secretion, water and ion transport and the cell-cell junction integrity of the intestinal epithelium, each contributing to the diarrhoeal disease symptoms typical of these enteric pathogens (Figure 1.4).

One of the hallmarks of EPEC and EHEC infections is the destruction of IEC microvilli, resulting in a greatly reduced absorptive surface area which contributes to diarrhoea onset. This effacement was historically attributed to the impact of the bacterial outer membrane protein intimin and effectors Tir, Map, and EspF which when deleted in concert, removed the ability of EPEC to cause destruction of microvilli (Dean et al., 2006). Simultaneously, the T3SS translocon component EspB interrupts the actin-myosin interaction crucial for maintaining microvilli structure (Iizumi et al., 2007).

IEC cell to cell connectivity formed through tight junctions (TJs), adherens junctions (AJs) and desmosomes create a physical barrier between the intestinal lumen and the basal lamina. AJs and desmosomes maintain strong adhesion between cells, while TJs enable selective permeability to solutes (Suzuki, 2013). TJs are composed of the transmembrane proteins

occludin, claudins, junctional adhesion molecules (JAMs) and tricellulin, and cytosolic scaffolding proteins including zonula occludens (ZO) proteins. *Salmonella* and EPEC/EHEC use T3SS-effectors to alter TJ composition and permeability (Boyle et al., 2006; Simonovic et al., 2000), resulting in decreased transepithelial resistance, ZO-1 expression and occludin phosphorylation, all features of functional tight junctions. During EPEC and EHEC infections while Map, EspF, EspM and EspG disrupt tight junction composition (Dean and Kenny, 2004; Glotfelty et al., 2014; Simovitch et al., 2010), NleA inhibits the regeneration of TJs by interrupting protein trafficking. Map and EspF together disrupt the Crumbs polarity complex that is essential to IEC polarisation and TJ assembly (Tapia et al., 2017). NleA inhibits COPII vesicle fusion and protein trafficking by binding Sec24, a critical component of the COPII coat (Kim et al., 2007; Thanabalasuriar et al., 2010). Similarly, *Salmonella* effectors SipA, SopB, SopE and SopE2 have been linked to TJ and barrier function disruption (Boyle et al., 2006), while AvrA was described as a TJ stabiliser (Liao et al., 2008). Again, the direct mechanisms of these perturbations are unclear but TJ disruption may be reliant upon the ability of SopB, SopE and SopE2 to activate Rho-GTPases (discussed later).

Disruption of normal ion absorption processes is also crucial to the onset of diarrhoea caused by enteric pathogens, and the resulting fluid accumulation has been attributed to EPEC/EHEC intimin and effectors Tir, Map, EspF and EspG/EspG2, and *Salmonella* SipA, SopA, SopB, SopD and SopE2. EspF and EspG work in concert to relocate aquaporin water channels from the cell membrane to the cytosol, contributing to increased fluid accumulation in the lumen (Guttman et al., 2007). The sodium/glucose co-transporter SGLT1 is an important contributor to IEC water absorption that is inactivated by the cooperative efforts of intimin, Tir, Map and EspF. Though the exact mechanism of inactivation is unclear, it seems the reduction in activity is also an effect of the relocation of SGLT1 and the destruction of microvilli (Dean et al., 2006). EspF also disrupts the activity of the key Na^+/H^+ exchanger (NHE)-3 (Hodges et al., 2008)

whereas in EPEC Cl^- ion absorption is perturbed by the actions of EspG and EspG2, which cause the relocation of the Cl^-/OH^- exchanger DRA from the apical surface to intracellular vesicles, through an unknown mechanism (Gill et al., 2007). Chloride ion secretion is inhibited by *Salmonella* SopB, an multi-functional effector also important for *Salmonella* invasiveness. SopB indirectly reverses the inhibitory effect of EGF on chloride secretion, via SopB inhibition of phosphatidylinositol-3-kinase (PI3K) (Bertelsen et al., 2004). Though the combined effects of *Salmonella* SipA, SopB, SopD, SopE2 and SipB appear to modulate fluid accumulation during *Salmonella* infections, a single *sipB* mutation is equally attenuating as the combined *sipA/SopABDE2* mutant, suggesting the effect of SipB is likely due to its role in the translocation of effectors, and not a direct effect (Zhang et al., 2002). Aside from the aforementioned effects of SopB on chloride secretion, there is little direct evidence that *Salmonella* directly modulates water and ion transporters and it has been proposed that effects on fluid retention could be a result of the tissue damage caused by the rapid recruitment of neutrophils during the early stages of *Salmonella* infections (Nunes, 2008).

Despite the efforts and advancements towards complete understanding of the disease mechanisms of diarrhoea induced by *Salmonella* and A/E pathogens, much is still unclear and it is increasingly apparent that it is a multifactorial process controlled not only by direct effects on fluid and ion transporters and cell-cell junctions, but also the indirect effects of the host immune response and physical damage caused by the bacterial infection (Viswanathan et al., 2008).

1.3.3 Cytoskeleton modulation

Salmonella and the A/E pathogens translocate effectors for the manipulation of the host cell actin cytoskeleton, but with remarkably different consequences. While *Salmonella* relies upon the perturbation of actin signalling to invade epithelial cells, EPEC and EHEC destroy the microvilli structures *in vivo* and create actin rich pedestals on cultured cells. In combination

with this the host cytoskeleton is key to many aspects of the host immune response (Mostowy and Shenoy, 2015).

I. A/E pathogen Tir-induced actin rich pedestal formation

Pedestal formation is dependent on the presence of the EPEC/EHEC outer membrane protein intimin, together with its T3SS-secreted receptor, Tir (Donnenberg et al., 1993a, 1993b; Kenny et al., 1997b). The Tir-intimin interaction has been reported to have affinities of 10 nM and 1 nM in EPEC and EHEC respectively, facilitating a very tight attachment of these bacteria to host cells (Ross and Miller, 2007). These two proteins are also essential for colonisation *in vitro* and *in vivo*, and A/E lesion formation (Deng et al., 2003).

Tir is the first effector to be translocated into the host cell (Rosenshine et al., 1996; Thomas et al., 2007; Wang et al., 2008). After translocation it inserts into the apical plasma membrane with a hairpin loop topology, leaving the N- and C- termini cytosolic. EPEC and EHEC Tir share around 60% sequence identity and each elucidate the recruitment of host actin nucleators and adaptors through different mechanisms which converge upon the actin nucleation promoting factor N-WASP (Lommel et al., 2004). The N-termini of EPEC and EHEC Tir are interchangeable and are proposed to localise Tir to the cytoskeleton via interactions with several focal adhesion proteins (Goosney et al., 2000; Kenny, 2001). The divergent C-terminus orchestrates the cytoplasmic signalling that regulates actin polymerisation (Lommel et al., 2004). This divergence is demonstrated by the fact that Tir_{EPEC} is able to restore EHEC- Δtir pedestal formation, but Tir_{EHEC} cannot complement EPEC- Δtir (Kenny, 2001). The following signalling events describe pedestal formation characterised in EPEC O127:H6 or EHEC O157:H7.

Tir_{EPEC} signals through critical C-terminal tyrosines and phosphorylation of these residues is essential for actin polymerisation (Kenny et al., 1997b) (Figure 1.5). These phosphorylation

events are enhanced by the clustering of Tir upon intimin binding and the phosphorylation of Tir_{EPEC}-Y474 (EPEC E2348/69) is regarded as the most crucial for pedestal formation *in vitro* (Kenny et al., 1997b). Numerous tyrosine kinases have been attributed the role of Tir phosphorylation, including members of the Src and Abl family kinases and Tir dependent pedestal formation can be ablated by broad range pharmacological inhibitors of these families (Hayward et al., 2009; Phillips et al., 2004; Swimm et al., 2004a, 2004b). Conversely, as more specific inhibitors or individual tyrosine kinase knockdown cell lines still form pedestals upon EPEC infection, it appears that multiple redundant kinases are utilised for Tir phosphorylation (Swimm et al., 2004a). Recruitment of host adapter proteins Nck1 and Nck2 is enabled by these phosphorylation events, which bind through their Src homology (SH)-2 domains to a 12 residue, Y474-containing motif of Tir (Campellone et al., 2002; Gruenheid et al., 2001). Subsequently, via the three Nck SH3 domains, N-WASP is recruited which in turn sequesters the Arp2/3 actin nucleating complex (Buday et al., 2002; Gruenheid et al., 2001; Miki and Takenawa, 2003; Stradal et al., 2004).

Contrastingly, tyrosine phosphorylation is not present in actin pedestals facilitated by EHEC, Tir_{EHEC} lacks the Y474 critical to Tir_{EPEC} and pedestal formation is not reliant upon Nck (DeVinney et al., 2001; Kenny, 1999). Instead Tir_{EHEC} binds the IMD domains of host adapters IRTKS and IRSp53, which also possess SH3 domains enabling the transduction of actin regulation. The non-LEE encoded effector EspF_U/TccP (24% identity with EspF), not found in EPEC, is recruited by IRSp53 and IRTKS and in turn binds to and activates N-WASP through its C-terminal proline rich region, and its N-WASP activating helical domain (Campellone et al., 2004; Cheng et al., 2008; Garmendia et al., 2004).

There are also exceptions to these canonical EPEC and EHEC pedestal formation pathways. It has been shown that EPEC can form pedestals in a Nck- independent manner, relying upon Y474 and Y454 phosphorylation, though this is a far less efficient process (Campellone and

Leong, 2005). Furthermore, there are data to suggest that EHEC can form pedestals in an N-WASP independent manner (Vingadassalom et al., 2010). It is also true the pathways described above are not always specific to EPEC or EHEC; *espFu/tccp* has been identified in O119 (typical EPEC) and O55 (atypical EPEC) strains (Garmendia and Frankel, 2005), and the homologous *tccp2* found in 95% of non-O157 EHEC strains, was also conserved in all tested lineage 2 EPEC strains but not lineage 1 strains (Ogura et al., 2007; Whale et al., 2007). Conversely, over 90% of non-O157 EHEC strains that harboured *tccp2*, also expressed a Tir capable of signalling via Nck (Ogura et al., 2007).

Despite extensive research, the ultimate role of A/E pathogen induced pedestals remains to be clarified. While one could postulate that Tir-driven pedestal formation may have arisen as an anti-phagocytosis mechanism, evidence suggests that functional Tir is directly responsible for the inhibition of EPEC uptake by macrophages (Celli et al., 2001). Alternatively, this intimate attachment may render EPEC/EHEC/CR more resistant to diarrhoeal flow mediated detachment. Other T3SS effector proteins have links to the regulation of pedestal formation, including EspH and Map which are involved in the regulation of Rho GTPases, the high jacking of which is fundamental to the strategies employed by A/E pathogens and *Salmonella*.

II. Modulation of Rho GTPases

Rho GTPases are part of the Ras-related small GTPase family, and are key to the regulation of actin and microtubule dynamics, vesicular trafficking, cell polarity, cytokinesis and phagocytosis (Caron and Hall, 1998; Heasman and Ridley, 2008). These Rho GTPase molecular switches cycle between active GTP bound and inactive GDP bound forms and usually regulate proteins through direct interactions. Guanine nucleotide exchange factors GEFs replace GDP with GTP, activating Rho GTPases while GAPs enhance the hydrolysis of GTP to GDP, promoting Rho GTPase inhibition. Finally, guanine nucleotide dissociation inhibitors (GDIs) lock Rho GTPases in their inactive GDP bound state. Cdc42, Rac1 and RhoA

are the best characterised members, regulating filopodia formation, lamellipodia/ruffle and stress fibre formation respectively (Hall, 1998).

The A/E pathogens employ multiple mechanisms to modulate these Rho GTPases (Figure 1.4), and central to this control are Map, EspM and EspT; members of a family of bacterial Rho GTPase GEFs that share a common WxxxE motif (Alto et al., 2006; Bulgin et al., 2010). This motif is also found in *Shigella* IpgB1/IpgB2 and *Salmonella* SifA/SifB, though no GEF or actin modulating activities have been demonstrated for SifA/SifB to date. EspM and Map are GEFs for RhoA and Cdc42 respectively, inducing stress fibre (EspM) and filopodia formation (Map) (Arbeloa et al., 2008; Huang et al., 2009). The EPEC/EHEC effector EspW activates Rac1 leading to actin ruffling and ‘flower-like’ structures, via an unknown mechanism (Sandu et al., 2017). In contrast to other A/E pathogen T3SS GEFs EspH has been shown to inactivate DH-PH (Dbl-homology/Plekstrin-homology) RhoGEFs (Dong et al., 2010). This aids the transition from early filopodia formation to Tir-dependent pedestal formation (Wong et al., 2012), and inhibits the actin polymerisation necessary for phagocytosis (discussed later). Due to their lack of homology with mammalian DH-PH GEFs, EspH does not inhibit bacterial GEFs. This enables A/E pathogens to simultaneously inhibit host GEFs and replace them with bacterial GEFs. Finally, the effector EspV found in 16% of EPEC and EHEC strains appears to modulate actin cytoskeletal dynamics, via an unknown mechanism, resulting in nuclear condensation, cell rounding and dendrite-like projections (Arbeloa et al., 2011).

Many bacteria also take control of Rho GTPases to facilitate invasion of host cells. Activation of Rho GTPases leads to recruitment and activation of actin nucleating proteins from the WASP and WASP-family verprolin-homologous protein (WAVE) families, which in turn activate the Arp2/3 complex required for actin nucleation and reorganisation. Interestingly the EPEC GEF EspT, which is found in less than 2% of EPEC strains and no EHEC strains (Arbeloa et al., 2009), activates Cdc42 and Rac1 enabling the invasion of epithelial cells by

these rare strains of a typically extracellular bacterium (Bulgin et al., 2009). *Salmonella* SopE and SopE2 are GEFs which activate Cdc42, Rac1 and RhoG facilitating invasive behaviour (Hardt et al., 1998). SopE and SopE2 are supported by SopB which modulates the cytoskeleton by indirectly activating Cdc42 and RhoG but not Rac1, via an unknown mechanism (Patel and Galán, 2006). Furthermore the inositol phosphatase activity of SopB can produce membranous phosphatidylinositol-3-phosphate (PI3P), which recruits GEFs Arp1 and ARNO, in turn stimulating WAVE regulatory complex (WRC) (Humphreys et al., 2012). The combined importance of SopE/SopE2/SopB is exemplified by the lack of invasiveness in a *sopE*, *sopE2*, *sopB* triple mutant, although their presence is not universally conserved within the *Salmonella* genus (Desai et al., 2013; Miold et al., 2001). Post *Salmonella* invasion of host cells, the activity of these bacterial GEFs is reversed by the SPI-1 GAP SptP which inhibits Rho GTPases enabling host cell recovery (Galán and Fu, 1999; Hardt et al., 1998).

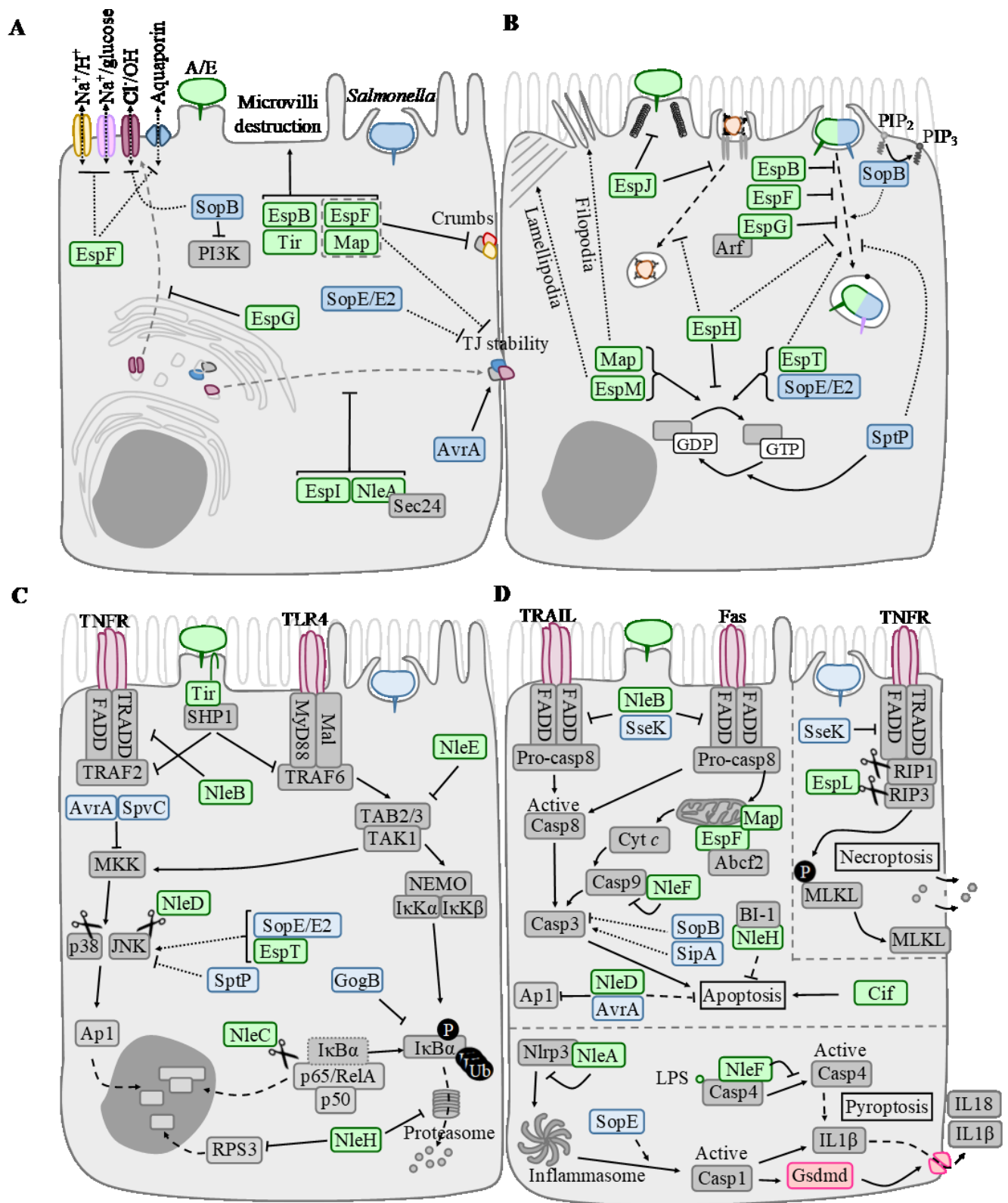


Figure 1.4: Manipulation of host cell signalling by A/E pathogen and *Salmonella* T3SS effector proteins

Disruption of (A) ion transportation, microvilli and cell adhesions, (B) cytoskeletal dynamics and bacterial internalisation, (C) inflammatory and (D) cell death signalling pathways by *Salmonella* (blue) and A/E pathogen (green) proteins. Scissors = cleavage, bar-headed lines = inhibition, arrow headed solid lines = activation, arrow headed dashed lines = protein/complex movement, arrow /bar-headed dotted lines = indirect activation/inhibition, P = phosphorylation, Ub = ubiquitination.

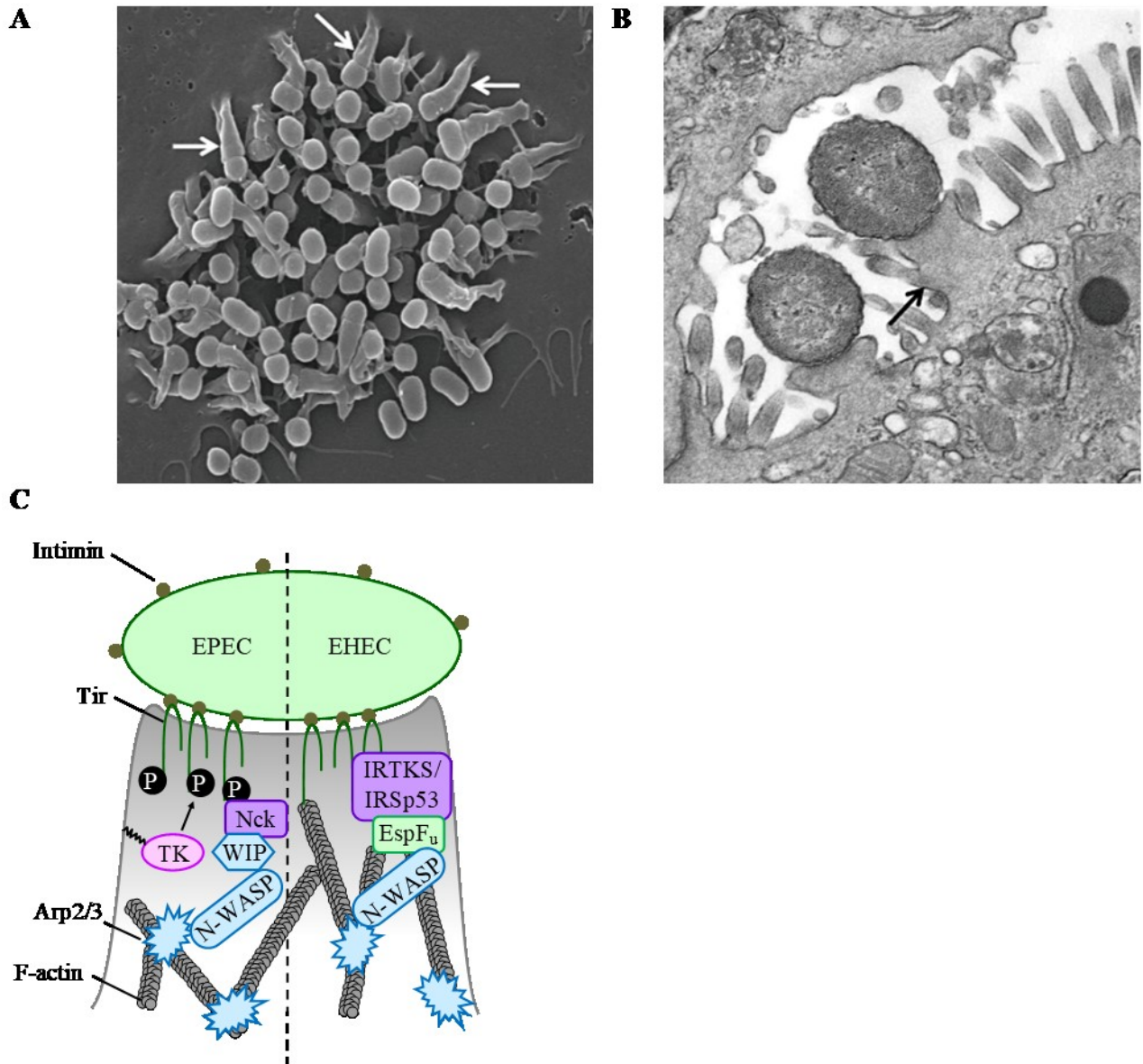


Figure 1.5: EPEC and EHEC pedestal formation

(A) Scanning electron micrograph of showing EPEC-induced pedestals on cultured epithelial cells. (B) Transmission electron micrograph showing the intimate attachment of EPEC and microvilli effacement on intestinal cells. (C) Schematic showing the major players involved in coordinating EPEC and EHEC pedestal formation. For EPEC and EHEC the T3SS effector Tir inserts into the host membrane and via its extracellular domain, interacts with the bacterial outer membrane protein intimin stimulating receptor clustering. For EPEC, host tyrosine kinases phosphorylate Tir intracellular tyrosines which recruits adapter protein Nck through its SH2 domains. Nck in turn recruits WIP. For EHEC there is no tyrosine phosphorylation of Tir. Instead host adapters IRTKS and IRSp53 bind to Tir and recruit the EHEC T3SS effector EspFU. N-WASP is bound and activated by WIP (EPEC) or EspFU (EHEC) which stimulates the actin nucleating Arp2/3 complex resulting in actin reorganisation and pedestal formation. A and B from Wong *et al* 2011.

1.4 The host response to infection

The host immune system has developed pathogen recognition receptors (PRRs) to enable the detection of microbial pathogens through conserved pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs) resulting from infection or wounding. The detection of such PAMPs and DAMPs by PRRs leads to a rapid inflammatory response that is accompanied by innate immunity mechanisms such as pathogen internalisation and destruction by phagocytosis. Inflammatory signalling downstream of PRRs induces the expression and release of pro-inflammatory molecules such as interleukin (IL)1 β , IL8 and TNF- α , which amplify inflammation as well as recruiting and activating innate immune cells such as dendritic cells (DC), neutrophils and macrophages. These cells provide roles such as pathogen internalisation and digestion as well as the production of microbicidal mucus, peptides and reactive oxygen species (ROS).

Following microbe internalisation, the presentation of pathogen antigens to lymphocytes by dendritic cells, and the release of further pro-inflammatory molecules activates cells of the adaptive immune system. Intertwined with these responses, host cells possess numerous programmed cell death pathways which as well as being crucial to development, and the removal of faulty cells, offers a mechanism of protection against infection by the sacrifice of infected cells. Not only does this prevent further bacterial replication, but often results in the release of ‘danger signals’ at the same time as exposing pathogens to a now activated immune system.

IEC function extends beyond forming an impenetrable barrier, and ion/nutrient homeostasis. They are also pivotal to maintaining intestinal immune system homeostasis; they must maintain non-inflammatory conditions in the presence of a multitude of commensal bacteria yet mount an immune response to infection by pathogenic bacteria. This is complicated by the numerous PAMPs shared by commensal and pathogenic bacteria, plus the presence of a healthy repertoire

of extracellular and intracellular PRRs in IECs (reviewed in (Peterson and Artis, 2014)). This is in part managed by the polarised distribution of Toll-like receptor (TLR) PRRs towards the basolateral side of IECs, allowing a strong response to basolateral microbes and dampening the response to luminal bacteria. Furthermore, the recognition of dangerous microbes relies on the detection of virulence factors such as secretion systems and toxins that enter the IEC.

Extracellular Gram-negative bacteria are most likely detected by TLR-2, TLR4 and TLR5 through recognition of peptidoglycan (PG), LPS or flagella, respectively. However, once internalised, bacteria be sensed by RIG-I like receptors (RLR), nucleotide-binding oligomerisation domain-containing (NOD) receptors or NOD-like receptors (NLRs) through a range of PAMPs including nucleic acid, peptidoglycan, LPS, flagella and T3SS components. The mutation of TLRs and NODs increases susceptibility to bacterial infection (Poltorak et al., 1998).

1.4.1 Inflammatory and ROS signalling

TLR-2/-4/-5 engagement of PAMPs stimulates receptor dimerisation and NF κ B and MAPK proinflammatory signalling pathways are transduced. TLR dimerization is followed by recruitment of adapter proteins MyD88, Mal, TRIF or TRAM, the combination of which depends on the specific TLR. As an example, TLR4 stimulates two signalling pathways after recruitment of either MyD88-Mal or TRIF-TRAM complexes. MyD88-Mal binding is followed by recruitment and phosphorylation of IL1 receptor-associated kinase (IRAK)-4 and IRAK1/2 which stimulates activity of the TRAF6 E3 ubiquitin ligase. TRAF6 ubiquitinates itself as well as TAK1 kinase and the I κ B kinase (IKK) subunit NEMO (NF κ B essential modifier). TAK1 recruitment of the TAK1-binding protein (TAB)-2 and TAB3 activates TAK1 which stimulates MAPK and NF κ B pathways.

Under non-inflammatory conditions proteins of the NFκB family (p65, p50, p52, c-Rel and RelB) are held in the cytoplasm by the inhibitor of NFκB (IκB). Following activation by TAK1, IKK phosphorylates IκB promoting its ubiquitination and degradation. This releases NFκB subunits which form homo- or hetero-dimers and translocate to the nucleus where they activate the expression of pro-inflammatory cytokines. Alternatively, TAK1 phosphorylates MAPK kinases (MKK) which subsequently phosphorylate MAPKs p38 and JNK leading to activation of AP-1 transcription factors that regulate expression of genes involved in apoptosis, cell proliferation and inflammation. In the TRIF-TRAM dependent pathway TRAF6 may be recruited stimulating the NFκB and MAPK pathways as previously described. Alternatively, the E3 ligase TRAF3 is recruited and activates the IKK-related kinases TBK1 and IKKε. These kinases subsequently phosphorylate interferon regulatory factor (IRF)-3 a transcription factor that stimulates expression of type I interferon genes.

Proinflammatory cascades downstream of multiple receptors aside from TLRs converge upon TAK1 activation including events downstream of the tumour necrosis factor receptor 1 (TNFR1), interleukin-1 receptor (IL1R) and the intracellular NOD1/2 PRRs. Upon sensing of intracellular peptidoglycan, NOD1/2 interact with RIPK2 which in turn stimulate TAK1 activity (Park et al., 2007). IL1β binding to the IL1R stimulates a MyD88-dependent signalling pathway, whereas the TNFα-TNFR1 interaction causes recruitment of death domain containing adapters TRADD and RIPK1, the ubiquitination of which recruits and activates IKKγ and TAK1. The induction of inflammatory signalling by PAMPs and DAMPs is intrinsically intertwined with multiple cell death pathways. For example, after TNFR1 stimulation in certain circumstances TRADD may recruit the adapter protein FADD instead of RIPK1 inducing a cell death cascade (Micheau and Tschopp, 2003).

I. T3SS-mediated modulation of inflammation and immune responses

The T3SS effectors of *Salmonella* and the A/E pathogens have been shown both to stimulate and dampen each numerous of the innate responses at various points of infection (Figure 1.4). While *Salmonella* SPI-1 induces a large inflammatory burst and neutrophil recruitment during the invasive stages of infection, at later time points this is limited largely by several SPI-2 substrate proteins. Comparatively, EPEC and EHEC cause a relatively low level of inflammation during infection and most of its inflammatory modulators are immunosuppressants.

As well as their contribution to invasion as previously described, the *Salmonella* SPI-1 GEFs SopE, SopE2 and EPEC EspT activate ERK/JNK/p38 MAPK pathways resulting in AP-1 and NFκB activation (Bulgin et al., 2009; Hobbie et al., 1997; Raymond et al., 2011). Later during *Salmonella* infection inflammation is reversed by GAP activities of the more stable SPI-1 GAP SptP which acts in concert with SpvC, AvrA, and GogB to dampen the immune response (Lin et al., 2003; Murli et al., 2001). GogB, blocks the ubiquitylation and degradation of IκB, preventing NFκB activation (Pilar et al., 2012), a function presumed to be shared by the EPEC/EHEC effector EspK (Vlisidou et al., 2006). The SPI-1 effector AvrA has acetyltransferase activity towards MAPKKs resulting in the inhibition the NFκB and JNK/AP-1 signalling (Collier-Hyams et al., 2002; Jones et al., 2008; Ye et al., 2007), simultaneously stabilising intestinal tight junctions and attenuating bacterial invasion (Lin et al., 2016). Finally, the SPI-1 and SPI-2 effector SpvC dephosphorylates MAPKs via its phosphothreonine lyase activity (Haneda et al., 2012; Mazurkiewicz et al., 2008).

In a similar manner EPEC and EHEC T3SS effectors NleE, NleB, NleC and Tir are immunosuppressors that modulate the NFκB pathway at multiple stages (reviewed in (Raymond et al., 2013)). NleE is a methyltransferase that stalls signalling by modifying the TAB2/TAB3, preventing their association with TRAF6 (Nadler et al., 2010; Zhang et al.,

2011). Aside from orchestrating pedestal formation Tir inhibits TRAF6 ubiquitination through its immunoreceptor tyrosine inhibitory motif (ITIM) which recruits the tyrosine phosphatases SHP-1 and SHP-2 and promotes an inhibitory SHP-1-TRAF6 association (Yan et al., 2012, 2013). NleC and NleD are metalloproteases that prevent inflammatory transcription factor activation by cleaving the p65 subunit of NF κ B, or the MAPKs p38 and JNK respectively. (Baruch et al., 2011; Pearson et al., 2011; Yen et al., 2010). The kinases NleH1 and NleH2 disrupt the ubiquitylation and degradation of I κ B inhibiting the NF κ B response to TNF- α (Royan et al., 2010), and NleH1 blocks the translocation of NF κ B transcriptional complexes via its interaction with ribosomal protein S3 (RPS3) (Gao et al., 2009). The crosstalk between inflammation and cell death is exemplified by the glycosyltransferase NleB which inhibits both processes by introducing a novel post translational modification (PTM) onto death domain containing proteins, ablating signalling pathways stimulated by TNF or Fas-ligand (Newton et al., 2010; Pearson et al., 2013).

1.4.2 Pro-inflammatory and anti-inflammatory cell death

Apoptosis, necroptosis and pyroptosis represent the major cell death pathways employed in response to bacterial infections (Rudel et al., 2010). Simultaneously, the regular exfoliation and regeneration of intestinal epithelial cells complements these protective strategies. Apoptosis can be stimulated by either extrinsic, transmembrane death receptor mediated pathways (TNF-R1, FasL and Apo2/3) or intrinsic, mitochondria mediated pathways culminating in the release of pro-apoptotic cytochrome *c*. Apoptosis is a non-inflammatory cell death pathway dependent on non-inflammatory caspases-8/-2/-3/-6/-7. It is characterised by early cell shrinkage and chromatin condensation followed by late membrane blebbing and the formation of bacteria-containing apoptotic bodies which are engulfed by phagocytic cells. Contrastingly, pyroptosis and necroptosis are inflammatory forms of cell death that result in membrane rupture and release of pro-inflammatory cytokines into the extracellular

environment. Pyroptosis may be stimulated by detection of bacterial flagellin or T3SS components by NAIP-NLRC4 and LPS detection by NLRP3 or caspase-4 (human) (Miao et al., 2006, 2010). Their detection stimulates formation of large macromolecular complexes called inflammasomes which process and activate inflammatory caspases such as caspase-1, which then activate IL1 β and IL18 by proteolysis of pro-IL1 β and pro-IL18. Activation of the pore forming protein Gasdermin D by caspase-1 leads to membrane rupturing and release of pro-inflammatory cytokines (He et al., 2015). Necroptosis proceeds via the activities of RIPK1 and RIPK3 culminating in the activation MLKL which subsequently affects ion transportation causing swelling and membrane rupture (Hildebrand et al., 2014). Finally, necrosis is a caspase-independent response that involves inflammation, nuclear swelling, membrane rupture, and release of cytosolic components.

I. T3SS mediated regulation of cell death and adhesion

EPEC infections display the early hallmarks of apoptosis but don't stimulate late features such as membrane blebbing and nuclear fragmentation, suggesting that they employ anti-apoptotic strategies (Crane et al., 2001). Indeed, EPEC and EHEC possess pro-apoptotic effectors; EspF, EspH, Map and Cif, and anti-apoptotic effectors; NleH1/NleH2, NleB, NleF, NleD and EspZ. In addition, recent evidence has demonstrated effectors that also prevent inflammatory cell death (Pearson et al., 2017). While *Salmonella* induces apoptosis during the infection of epithelial cells in a caspase-3 and -8 dependent manner (Kim et al., 1998), the infection of macrophages stimulates a rapid caspase-1 dependent pyroptosis (Brennan and Cookson, 2000; Hersh et al., 1999). In line with this, *Salmonella* possesses both pro- and anti-cell death effectors regulating multiple aspects of a cell's fate (Figure 1.4).

II. Inflammatory cell death – pyroptosis and necroptosis

NAIPs can recognise T3SS rod proteins EPEC/EHEC EscI, and *Salmonella* SPI-1 PrgJ but not SPI-2 SsaI (Miao et al., 2010). By switching to SPI-2 expression after initial invasion

Salmonella turns off this NLRC4 induced pyroptotic response. Simultaneously, *Salmonella* has been demonstrated to down-regulate the expression of flagellin after invasion, to further avoid detection by the NAIP-NLRC4 axis at later stages of infection.

During the inflammatory stage of *Salmonella* infection, SPI-1 translocon protein SipB activates caspase-1 via a direct interaction while the secreted effector SopE stimulates caspase-1 indirectly through the activation of Rho-GTPases Cdc42 and Rac (Hersh et al., 1999; Müller et al., 2009). Both SipA and SipC have been demonstrated to regulate the trafficking of the host membrane protein PERP, a protein with many roles including caspase activation (Hallstrom and McCormick, 2016; Hallstrom et al., 2015). Despite this multitude of effectors promoting inflammatory cell death, *Salmonella* also inhibits necroptotic pathways through the actions of SseK1 and SseK3 which inhibit death domain proteins by glycosylation in a similar manner to EPEC/EHEC NleB (Günster et al., 2017; Pearson et al., 2013).

EPEC/EHEC EspL, NleA and NleF inhibit caspase-dependent inflammatory pathways. NleA inhibits caspase-1 dependent IL1 β activation via its interaction NLRP3 (Yen et al., 2015). The pyroptotic signalling transduced by human caspase-4 (mouse caspase-11) detection of bacterial LPS is inhibited via the direct interaction of NleF with caspase-4, dampening early IL18 release and neutrophil recruitment in the CR infection of mice (Pallett et al., 2017). In addition, EspL has recently been shown to inhibit necroptotic pathways through its cysteine protease cleavage of key RHIM domain containing proteins RIPK1, RIPK3, TRIF and DAI/ZBP1 (Pearson et al., 2017).

III. Non-inflammatory cell death – apoptosis

EPEC/EHEC effectors EspH, EspF, Map and Cif stimulate the intrinsic apoptotic pathways. The N-terminus of EspF targets it to the mitochondrial membrane where it is imported into the mitochondrial matrix by host machinery and destabilises the membrane potential leading to

cytochrome *c* release and caspase activation (Crane et al., 2001; Nagai et al., 2005; Nougayrède and Donnenberg, 2004). In addition, EspF interacts with the ABC transporter Abcf2, thought to have a role in caspase inhibition. This interaction induces Abcf2 degradation, with presumably resulting in increased caspase activation (Nougayrède et al., 2007). The T3SS GEF, Map possesses an N-terminal mitochondrial localisation sequence (Kenny and Jepson, 2000; Ma et al., 2006). Here it perturbs the mitochondrial membrane potential, inducing cytochrome *c* release, inducing the intrinsic apoptosis pathway (Kenny and Jepson, 2000; Ma et al., 2006; Papatheodorou et al., 2006). Finally, the effector Cif stabilises the cell cycle at G1/S and G2/M causing a delayed form of apoptosis. It does this through deamidation of NEDD8, preventing the neddylation of cullin-RING ubiquitin ligases which are required for cell cycle progression (Marchès et al., 2003; Morikawa et al., 2010; Samba-Louaka et al., 2008, 2009; Taieb et al., 2006; Toro et al., 2013).

To maintain full regulation of apoptosis, *Salmonella*, EPEC and EHEC also translocate effectors that block both the intrinsic (AvrA, NleD, NleB, NleF) and extrinsic (NleH1/2, EspZ, SopB) apoptotic pathways. The anti-inflammatory activities of *Salmonella* AvrA and EPEC/EHEC NleD also inhibit the API-1 induced transcription of pro-apoptotic genes (Baruch et al., 2011; Wu et al., 2012). Similarly the aforementioned activities of EPEC NleB and *Salmonella* SseK1/SseK3 inhibit death receptor signalling necessary for apoptosis (Gao et al., 2013; Günster et al., 2017; Li et al., 2013; Pearson et al., 2013; El qaidi et al., 2017). In addition to its roles in modulating caspase-1 and caspase-4 mediated inflammation NleF specifically inhibits FasL-stimulated extrinsic apoptosis by preventing downstream cleavage of caspase-3, caspase-8 and RIPK1 (Pollock et al., 2017). Within epithelial cells SipA activates caspase-3 and SipA's subsequent cleavage by activated caspase-3 is thought to contribute to neutrophil recruitment (Lee et al., 2000). Additionally, in macrophages SipA activation of caspase-3 stimulates macrophage apoptosis (McIntosh et al., 2017). EPEC/EHEC NleH1 and NleH2

inhibit intrinsic apoptosis via their interaction with Bax Inhibitor-1 (BI-1) which ordinarily prevents mitochondrial permeabilization and cytochrome *c* release mediated by Bax. By interacting with BI-1, the kinase NleH promotes inhibition of Bax in a kinase independent manner (Hemrajani et al., 2010). The absence of the EPEC/EHEC EspZ causes an increase in the cleavage and activation of caspases-3, -7 and -9 and downstream apoptosis, while the presence of EspZ protects epithelial cells against staurosporine stimulated intrinsic apoptosis, but not TNF stimulated extrinsic apoptosis (Roxas et al., 2012). Interestingly, EspZ has been shown to regulate T3SS effector translocation and it has been hypothesised that its absence allows uncontrolled injection of pro-apoptotic effectors (Berger et al., 2012). In addition, EspZ interacts with CD98 at the plasma membrane promoting cellular adhesion and survival through Fak, pro-survival kinase Akt, and integrin signalling pathways (Shames et al., 2010). EspO is another effector that stabilises focal adhesions, but through its interaction with integrin linked kinase (ILK) (Kim et al., 2009). EspO has proposed apoptotic regulatory roles via a direct interaction with the anti-apoptotic protein Hax-1 (PhD thesis (Constantinou, 2013)). Absence of the *Salmonella* pro-inflammatory effector SopB causes increased epithelial caspase-3 cleavage and intrinsic apoptosis. In a similar vein SopB stabilises the activation of the pro-survival kinases Akt and protein kinase B (Knodler et al., 2005).

The multitude of cell death regulatory effectors employed by EPEC/EHEC and *Salmonella*, and their large crossover with the modulation of inflammatory pathways demonstrates not only how finely balanced the bacterial suite of effectors are, but the difficulties bacteria face when perturbing such a complex network of host signalling pathways.

1.4.3 Phagocytosis

Professional phagocytes (macrophages, neutrophils and dendritic cells) are crucial to the innate immune response by engulfing and destroying bacteria before generating peptides for MHC antigen presentation. In *cis*-phagocytosis, the recognition of bacterial features stimulates

bacterial internalisation (Peiser et al., 2000). Contrastingly, *trans*-phagocytosis relies upon the opsonisation of bacteria with immunoglobulin-G (IgG) or the complement component iC3b, which are recognised by the phagocyte Fc γ and CR3 receptors respectively (Caron and Hall, 1998).

In IgG-dependent phagocytosis, the Fc portion of IgG antibodies are recognised by Fc γ -receptors (Fc γ R) (Anderson, 1990) mediating receptor clustering. Fc γ R cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) domains are then phosphorylated by the Src family (SFK) tyrosine kinases (Ghazizadeh et al., 1994; Hamada et al., 1993) stimulating recruitment of a range of proteins including Syk kinase, Nck and CrkII through their SH2 domains. Downstream pathways central to the dynamics of phagocytosis include the regulation of actin reorganisation, delivery of endomembrane to the site of internalisation, and modulation of membrane lipid composition. RhoGTPase GEFs Vav and ELMO/DOCK180 are activated by Syk and CrkII, respectively. The resulting GTP-bound Rac and Cdc42 activate N-WASP/WAVE through direct interaction, stimulating the Arp2/3 signalling pathway. Syk phosphorylation of the linker for activation of T-cells (Lat) allows recruitment and phosphorylation of Gab2/Grb2 along with Nck, phosphatidylinositol-(4,5)-diphosphate (PIP₂) and the Src family kinase Hck can activate N-WASP and downstream Arp2/3-driven actin polymerisation. A dynamic balance of PIP₂, phosphatidylinositol-(3,4,5)-biphosphate (PIP₃) and diacyl glycerol (DAG) in the membrane is required for successful particle internalisation. Syk and SFKs phosphorylate PI3K which processes PIP₂ to PIP₃ and stimulates endomembrane delivery to the site of internalisation through Arf GEFs. PIP₃ also recruits the Tec family kinases enabling their activation of phospholipase C (PLC) γ which converts PIP₂ to inositol-1,4,5-trisphosphate (IP₃) and DAG respectively. Myosin X is recruited by PIP₃ and IP₃ stimulates the mobilisation of calcium from the endoplasmic reticulum, both of which are crucial to late stages of phagocytosis (Figure 1.6).

While Fc γ R-mediated internalisation is perhaps the best characterised mechanism for phagocytosis, many other phagocytic receptors exist including C-type lectins, integrins and scavenger receptors, and these can work in synergy or with numerous co-receptors to increase the efficiency of engulfment. C-type lectins are PRRs that recognise carbohydrate sugars such as β -1,3-glucans of fungal pathogens. Scavenger receptors recognise a variety of ligands including bacterial LPS, but it seems that they may act as co-receptors not able to orchestrate phagocytosis alone (Freeman and Grinstein, 2014). Integrins are heterodimeric receptors with diverse functions, comprised of combinations of numerous α - and β - subunits. The best characterised in terms of phagocytosis is $\alpha_M\beta_2$, otherwise known as Mac-1 or CR3, which can coordinate phagocytosis of both opsonised and non-opsonised particles (Le Cabec et al., 2002; Hynes, 2002). Fc γ R and CR3 work in concert to facilitate internalisation. Signalling downstream of the Fc γ R activates Rap GEFs which stimulate the unfolding of integrins, while integrin engagement has been shown to increase the mobility of Fc γ R and other receptors (Freeman and Grinstein, 2014). CR3 binding of C3bi-opsonised particles stimulates recruitment of Talin which in turn sequesters key cytoskeletal proteins paxillin, vinculin and α -actinin. Simultaneously ligand engagement stimulates SFK and Syk activation which can phosphorylate and regulate the activities of talin-recruited proteins as well as crucial GEFs such as Vav1 (Lowell, 2011). Though exact mechanisms are unclear, the absence of Syk greatly impairs CR3-mediated phagocytosis (Shi et al., 2006).

The oxidative burst is an intrinsic part of the defence against pathogens, through the generation of large quantities of ROS and reactive nitrogen species (RNS), and the activation of proteases that degrade phagocytosed microbes. Nitric oxide synthases (NOS) generate microbicidal nitric oxide (NO) from arginine. The inducible NOS (iNOS) is regulated at the transcriptional level, with its expression stimulated by proinflammatory cytokines through MAPK, NF κ B and Jak-Stat signalling cascades in response to a microbial stimulus (Stuehr and Marletta, 1985;

Wink et al., 2011). ROS are produced by the NADPH oxidases (NOX) 1-5 and dual oxidase (DUOX) 1 and 2. However, during phagocytosis NOX2 is the predominant NOX at force (Singel and Segal, 2016). Its activation depends upon the translocation of cytoplasmic subunits p40-*phox*, p47-*phox* and p67-*phox* plus Rac GTPase to the membrane bound gp91-*phox* p22-*phox* heterodimer (Figure 1.7). The functional complex converts molecular oxygen to a superoxide anion, the downstream conversion of which leads to generation of potent antimicrobial molecules such as hydrogen peroxide and hypohalous acid. Loss of function NOX2 variants may result in immune disorders such as chronic granulomatous disease (CGD) which involve severe bacterial and fungal infections (O'Neill et al., 2015). NOX functions can be activated downstream of pathogen recognition, for example through the LPS-TLR4 signalling axis (Lee et al., 2012), or via stimulation of opsonin receptors such as FcγRs and complement receptors, and their expression may be primed by a variety of cytokines such as TNF-α and IFN-γ (Almeida et al., 2005; Blaser et al., 2016). While the production of ROS and NOS is critical to the host innate immune response, they also function to regulate an increasing number of processes including antigen presentation, the adaptive immune response, dampening of inflammation and wound healing during infection (Singel and Segal, 2016).

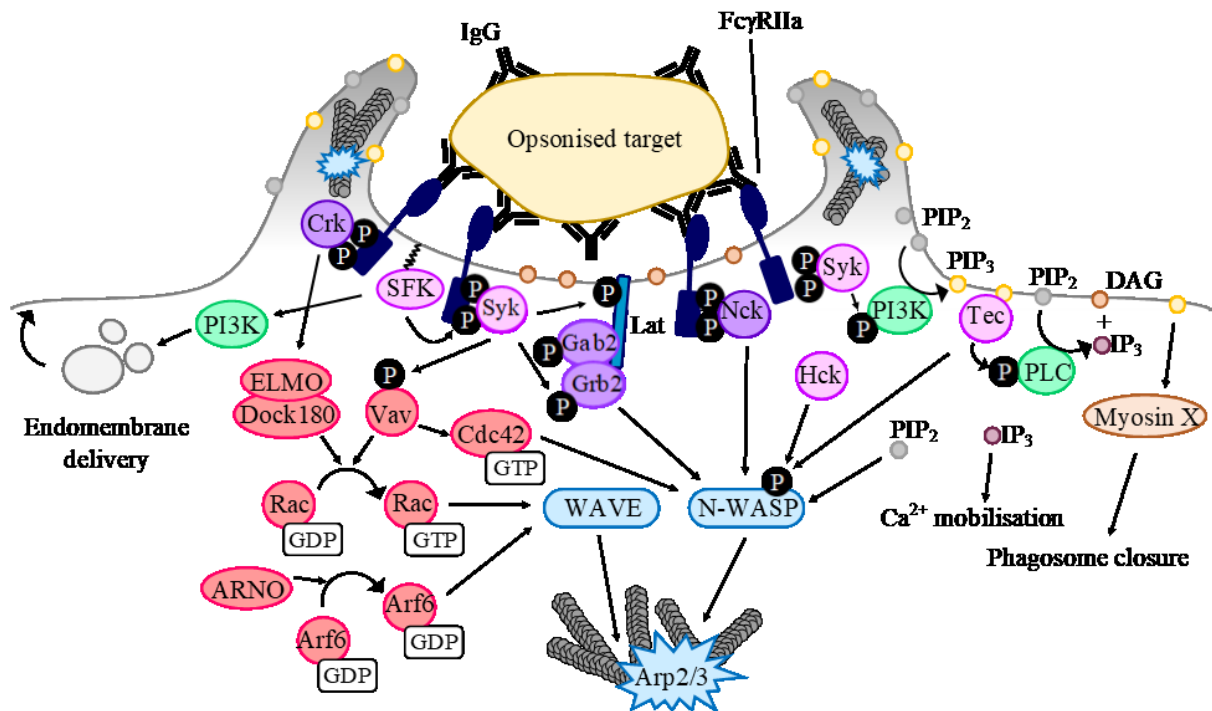


Figure 1.6: FcγRIIa mediated phagocytosis signalling events

A schematic for the signalling pathways that regulate FcγRIIa mediated phagocytosis. IgG-opsonised particles are bound by the extracellular domains of FcγRIIa which stimulates receptor crosslinking and phosphorylation of FcγRIIa intracellular ITAM domains by Src family kinases (SFK). This induces recruitment of Syk kinase and the adapter proteins Nck or CrkII (Crk) via their SH2 domains. Syk recruits multiple proteins regulating several pathways. Phosphorylation of the transmembrane protein Lat enables recruitment and phosphorylation of Gab2/Grb2 which in turn recruit and activate N-WASP. N-WASP is also activated by Nck, active Cdc42, phosphatidylinositol-(4,5)-biphosphate (PIP₂), and the SFK Hck. The GEFs Vav and ELMO/DOCK180 are activated through Syk and CrkII respectively, and in turn stimulate Rac, which activates WAVE. Alternatively, activation of Arf6 by the GEF ARNO also indirectly activates WAVE. N-WASP and WAVE complexes regulate the Arp2/3 actin nucleating complex. Membrane composition is dynamically regulated by PI3K and PLCγ which catalyse the conversion of PIP₂ to phosphatidylinositol-(3,4,5)-biphosphate (PIP₃) or inositol 1,4,5-trisphosphate (IP₃) and diacyl glycerol (DAG) respectively. Different membrane ratios of PIP₂, PIP₃ and DAG are crucial to steps in phagocytosis. Furthermore, IP₃ induces calcium mobilisation from the endoplasmic reticulum, and PIP₃ activates myosin X which are both crucial to late stages of phagocytosis.

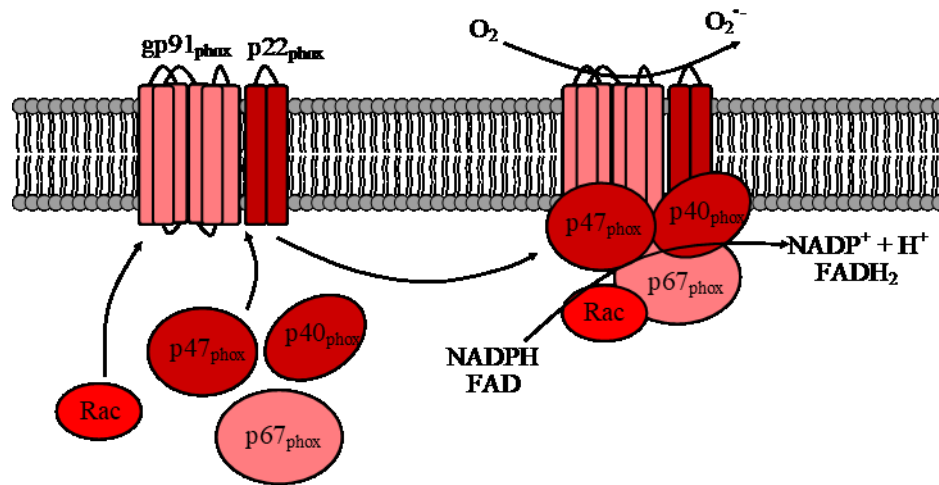


Figure 1.7: The NOX2 NADPH oxidase complex

Schematic showing the activation of the NOX2 NADPH oxidase complex. Transmembrane subunits gp91-*phox* and p22-*phox* in isolation and inactive in resting phagosomes (left). Recruitment of cytoplasmic p47-*phox*, p40-*phox*, p67-*phox* and Rac1/Rac2 leads to complex activation (right). The active complex is able to trigger electron transfer from NADPH to FAD to enable the reduction of molecular oxygen to superoxide.

I. T3SS mediated regulation of phagocytosis

How *Salmonella* uses T3SS-secreted GEFs to drive its entry into epithelial cells has been explained in earlier sections. Within the SCV, the SPI-2 genes are crucial to the exclusion of NOX from the phagosomal membrane (Vazquez-Torres et al., 2000). Contrastingly, the extracellular pathogens EPEC and EHEC have multiple T3SS effectors that prevent bacterial internalisation (Figure 1.4). The T3SS translocon component EspB also localises to phagocytic cups during infection (Iizumi et al., 2007) where it binds myosin proteins involved in the mechanics of phagocytosis, with anti-phagocytic and microvillus effacement results. This is enabled by the disruption of the actin-myosin interaction, by binding the actin-binding site of myosin-1c with 20-fold higher affinity than actin (Iizumi et al., 2007). As well as causing focal adhesion disruption, cell rounding and detachment, EspH inhibits *cis*- and *trans*-phagocytosis

during infection. Its inhibition of DH-PH GEFs ablates the Rho GTPase activation necessary for actin polymerisation and phagocytosis (Caron and Hall, 1998; Dong et al., 2010). As well as promoting cell death and detachment, EspF can inhibit *cis*- but not *trans*-phagocytosis (Marchès et al., 2008; Quitard et al., 2006). Through interactions with actin, profilin and sorting nexin-9 (SNX-9) (Alto et al., 2007; Peralta-Ramírez et al., 2008), EspF interrupts PI3K dependent internalisation, though details are not fully understood. EspG has been shown to inhibit the phagocytosis of EPEC and EHEC via indirect inhibition of the WRC; the EspG-Arf6 interaction prevents Arf6 activation of the Arf1 GEF ARNO, and the EspG-Arf1 interaction prevents Arf1 activation of WRC both contributing to the inhibition of WRC-dependent phagocytosis (Humphreys et al., 2016). In contrast, EspJ ablates FcγR- and C3R-mediated *trans*-phagocytosis, utilising its ART activity to inhibit the tyrosine kinase Src, the details of which will be expanded upon in later sections (Marchès et al., 2008; Young et al., 2014).

1.5 Tyrosine kinases

Phosphorylation, the transfer of a phosphate group from adenosine triphosphate (ATP) to a target protein, is a reversible PTM reported to affect at least 75% of the human proteome (Sharma et al., 2014) with the number of human phosphor-sites estimated in the hundreds of thousands (Boersema et al., 2010; Vlastaridis et al., 2017). Accordingly, phosphorylation participates in the most cell signalling pathways. In eukaryotes this PTM is estimated to occur almost entirely on serine, threonine and tyrosine residues at an estimated ratio of 90:10:0.5 (S:T:Y), but can also occupy other residues including histidine (Hunter and Sefton, 1980). The small proportion of tyrosine phosphorylation is surprising when considering that 90 human tyrosine-specific protein kinases have been identified. Proposed explanations include the tight regulation of tyrosine kinases which are only activated under specific conditions, the short half-life of pTyr residues due to the action of phosphotyrosine phosphatases (PTPs), and the fact that the role of pTyr is usually regulatory to protein function and not an essential mediator

of protein structure (Hunter, 2009). The mis-regulation of tyrosine kinases has been associated with a multitude of diseases, and these kinases have long been attractive therapeutic targets (Cohen, 2002; Zhang et al., 2009).

Tyrosine phosphorylation is involved in a range of signalling processes including growth factor and cytokine responses, cell adhesion via integrins, metabolic processes, cell cycle control through the regulation of cyclin dependent kinases, and transcriptional regulation by the modulation of Stat transcription factors. Tyrosine kinases can be sub-classified as receptor or non-receptor tyrosine kinases (RTKs and NRTKs). RTKs consist of an extracellular N-terminal domain responsible for binding activating ligands such as growth factors and cytokines or other transmembrane proteins. This is followed by a transmembrane domain and cytoplasmic C-terminal kinase containing domain which transmits the extracellular signal. Ligand binding initiates RTK clustering and activation of the C-terminal kinase domain by autophosphorylation. These tyrosine phosphorylation sites allow the docking of proteins with SH2 domains which include adapter proteins, other kinases, E3 ubiquitin ligases, or PTPs. NRTKs are soluble proteins which may be membrane anchored via PTMs such as myristoylation or palmitoylation. They contain a highly conserved catalytic domain (also known as SH1) accompanied by regulatory domains which vary depending on the sub class of NRTK (Figure 1.8) (Hunter, 2009). In the case of the SFKs these are SH3 and SH2 domains which perform auto-regulatory roles as well as providing substrate specificity through binding proline-rich regions and pTyr residues respectively.

A similar SH3-SH2-SH1 core is conserved within Abl, Csk and Tec NRTK families (Figure 1.8) (Hubbard and Till, 2000). On top of this, Abl/Arg kinases have F-actin binding and DNA-binding domains, and are involved in regulating cell cycle, morphology and motility. Tec, Btk, Itk, Bmx and Rlk make up the second largest NRTK family after the SFKs. These kinases harbour a pleckstrin homology (PH) domains which bind phosphatidylinositol lipids for

membrane tethering, where they serve to stimulate PLC γ and calcium mobilisation, regulating actin dynamics and cell polarisation downstream of B- and T-cell receptors (Schwartzberg et al., 2005).

In Syk/Zap-70 kinases a second SH2 domain replaces the SH3 domain enabling their recruitment to phosphorylated ITAM of activated T cell-, B cell- and Fc γ -receptors. Fak and Janus-family kinases (Jak)s lack both SH3 and SH2 folds. Fak instead encodes an N-terminal integrin binding domain and a C-terminal focal adhesion binding domain and is integral to signalling downstream of integrins and growth factor receptors responsible for the regulation of cell adhesion and motility (Mitra et al., 2005). Jaks have four N-terminal Jak homology domains for cytokine-receptor targeting and a second, inactive kinase domain which serves a regulatory purpose. Downstream of numerous cytokine and growth factor receptors, Jaks phosphorylate and activate the signal transducers and activators of transcription (Stat) which stimulate the expression of genes for the regulation of cell proliferation, differentiation, migration and apoptosis (O'Shea et al., 2015). Interestingly, the Abl, Tec, Syk, Fak and Jak kinases can all be regulated by SFKs which partially explains the diversity of roles attributed to these NRTKs.

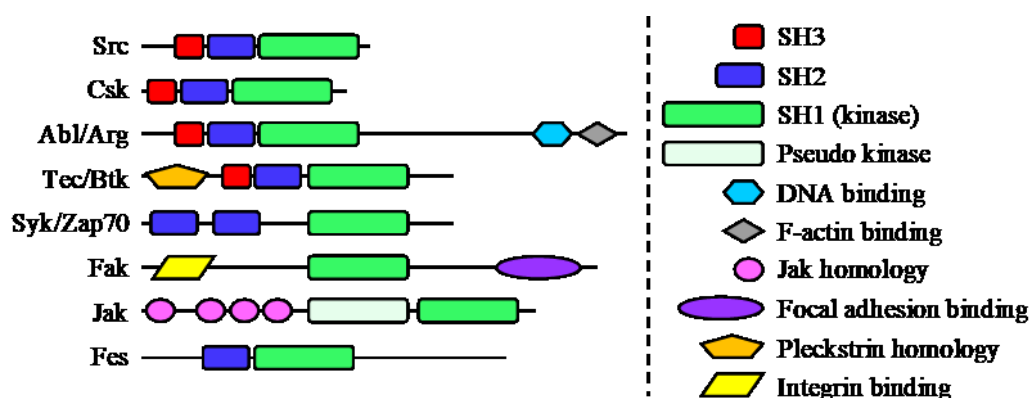


Figure 1.8: Domain organisation of NRTK families

Schematic representation of the domain organisation of representative NRTK family members. Diagrams represent amino to carboxy terminus from left to right. Figure adapted from Hubbard and Till, 2000.

1.5.1 Src family kinases and Csk

Studies of Src kinase led to the discovery of tyrosine phosphorylation (Hunter and Sefton, 1980). First identified in the oncogenic retrovirus Rous sarcoma virus (Brugge and Erikson, 1977), v-Src was later proven to be present as a mammalian gene, c-Src. The SFKs is the largest sub-family of non-receptor tyrosine kinases with nine members; the ubiquitously expressed Src, Fyn, Yes and Yrk, and those found primarily in haematopoietic cells Hck, Lck, Lyn, Fgr and Blk. A cell may express numerous SFKs at differential levels, and in numerous subcellular localisations.

In addition to the SH3-SH2-SH1 core, SFKs possess an N-terminal membrane targeting myristoylation/palmitoylation site. C terminal to the kinase domain is a short carboxy terminal domain containing an inhibitory phosphorylation site Y527 (Boggon and Eck, 2004). Phosphorylation of Y527 by the C-terminal Src kinase (Csk) causes SFKs to adopt an inactive closed conformation in which Y527 interacts with the SH2 domain (Figure 1.9). In fact, under basal cellular conditions it has been reported that 90-95% of c-Src is Y527 phosphorylated (Cooper et al., 1986). A Y527F mutation, prevents phosphorylation by Csk resulting in constitutively active Src. This inactive conformation can be disrupted upon SH2 or SH3 binding their interaction partners, or through phosphatase-mediated dephosphorylation of Y527. Maximal activation is then facilitated by unfolding of the SFK and autophosphorylation of Y416 within the activation loop of the kinase domain (Figure 1.9) (Boggon and Eck, 2004).

While Csk too possesses kinase, SH2 and SH3 domains, it lacks a C-terminal regulatory tyrosine and the N-terminal membrane anchoring PTM sites. In contrast to the negative regulatory roles of SH2 and SH3 in SFKs, these domains seem to have an activating role in Csk through intramolecular interactions with the small lobe of the kinase domain. In fact deletion of Csk SH2/SH3 domains decreases kinase activity by two thirds (Jamros et al., 2010). Csk is spatially regulated by the so-called Csk binding protein (Cbp) which through its

interaction with the Csk SH2 domain simultaneously recruits Csk to the cell membrane, increasing its affinity for Src, and Csk catalytic activity (Figure 1.9) (Saitou et al., 2014; Wong et al., 2005). This interaction is enabled by the phosphorylation of Cbp, an event thought to be facilitated by SFKs, creating a negative feedback loop of SFK activity (Kawabuchi et al., 2000; Yasuda et al., 2002). Furthermore, it has been demonstrated that Csk can also be activated via an interaction with G $\beta\gamma$ (Lowry et al., 2002) and by PKA phosphorylation of Csk (Vang et al.,

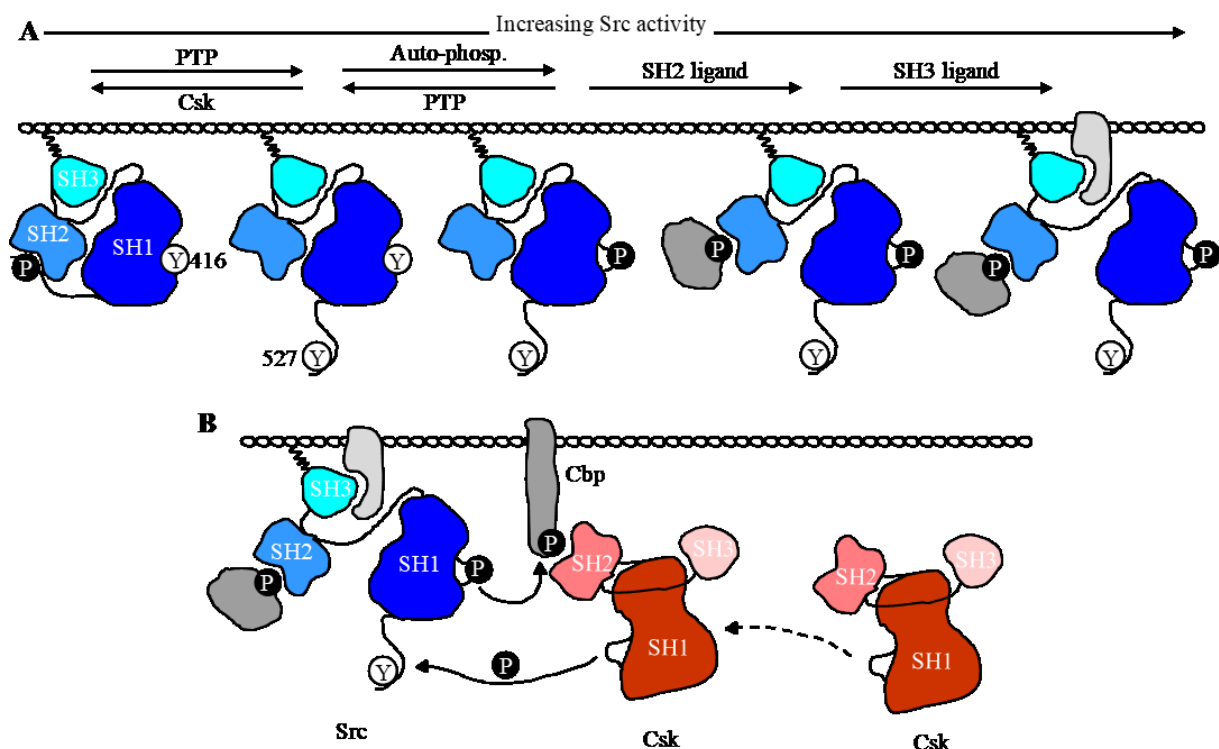


Figure 1.9: Regulation of Src and Csk kinases

(A) Src kinase has multiple levels of activation (from left to right). In the resting state, the phosphorylation of Src Y527 by Csk induces intramolecular interactions that leave Src fully inactive. This can be reversed by the action of phosphatases (PTP), and further activation comes from the autophosphorylation of Src at Y416. Src is maximally activated when Y527 is dephosphorylated, Y416 phosphorylated, and the SH2/SH3 domains bound to target proteins through phosphotyrosine or polyproline motifs respectively. (B) Csk is constitutively active but under basal conditions Csk is cytoplasmically localised. The phosphorylation of membrane bound Cbp by Src enables recruitment of Csk through its SH2 domain. This interaction increases Csk activity, but importantly brings Csk to sites of Src activity where it can phosphorylate Src Y527, inhibiting its activity. Phosp. = phosphorylation.

2001). While Src hyperactivity is regularly observed in cancers there are no reports of somatic mutations in *src* or *csk*. However, there are many observations of Cbp downregulation in cancers, and this may be one key mechanism that leads to Src activation (Masaki et al., 1999; Oneyama et al., 2008; Sirvent et al., 2010).

SFKs regulate numerous cellular events including cell proliferation and differentiation, survival, adhesion, migration and cytoskeletal dynamics. They couple with an extensive list of receptor pathways: i) adhesion related receptors - integrins, cell-adhesion molecules (CAMs), cadherins and selectins; ii) numerous RTKs – PDGFR, EGFR, the insulin receptor; iii) immune recognition receptors - FcRs, and T- and B-cell antigen receptors; iv) G-protein coupled receptors (GPCR); v) cytokine receptors – IL2-receptor; vi) voltage gated channels; vii) ligand gated channels and viii) GPI-linked receptors. Known SFK targets include focal adhesion proteins Fak, paxillin and p130^{cas}, cytoskeletal cortactin and ezrin, the tyrosine kinases Syk/Zap-70 and Tec kinases, several serine/threonine kinases, and numerous RTKs. Dissecting the exact roles of specific SFKs and other NRTKs is made very difficult by several factors: i) the vast range of targets described for SFKs, ii) the complicated expression patterns and spatiotemporal regulation, iii) the functional redundancy between SFK family members in numerous cellular processes iv) the lack of specific small molecule inhibitors and v) that SFKs can activate each other and members of other tyrosine kinase families.

I. Src family kinase regulation of adhesion, migration, differentiation and apoptosis

SFKs play key roles in the regulation of cell adhesion, migration and apoptosis as well as the cell cycle control. The activation of Src by integrin engagement and its ability to phosphorylate β 1-integrin along with numerous focal adhesion complex proteins including paxillin, vinculin, talin, tensin, p130^{cas} and Fak, strongly links SFKs to modulation of focal adhesion dynamics (Thomas and Brugge, 1997). As is the case for many Src regulated processes, protein tyrosine kinase inhibitors block focal adhesion formation. However, due to the involvement of other

tyrosine kinases such as Fak and Abl, it is difficult to dissect the exact roles of SFKs. To this end, Csk has been used as a tool to more specifically study SFKs. Focal adhesion proteins such as Fak and paxillin are able to recruit Csk upon SFK activation, creating a negative feedback loop and highlighting the need for tight control of SFK activity (Howell and Cooper, 1994). Csk overexpression employed to inhibit SFKs causes HeLa cells to adopt a spherical, loosely adhered form, strengthening the evidence for Src involvement in adhesion and spreading (Bergman et al., 1995). The integrin-Src-Fak relationship has been extensively studied and appears to also be involved not only in cell adhesion, but cell spreading in the form of filopodia, lamellipodia and stress fibres, cell migration and regulating membrane composition (Mitra and Schlaepfer, 2006; Mitra et al., 2005).

Another hallmark of SFK activity is the induction of cellular proliferation downstream of a number of integrin receptors and growth factor RTKs including the epidermal growth factor (EGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) receptors (Thomas and Brugge, 1997). SFKs are activated by these RTKs enabling the downstream activation of proteins involved in the induction of DNA synthesis like MAPK and PI3K. In line with the pro-proliferative roles of SFKs, Src has been attributed anti-apoptotic qualities through multiple mechanisms (Johnson et al., 2000). These include inhibition of the transforming growth factor (TGF)- β 1 induced apoptosis (Park et al., 2004), by inducing increased degradation of the death accelerator protein Bik through activation of Erk1/2 and phosphorylation of Bik (Lopez et al., 2012), and through phosphorylation of Ku70, preventing its pro-apoptotic acetylation (Morii et al., 2016). Finally, SFKs are crucial in the regulation of cellular differentiation, though their impacts appear to be cell type specific. In colorectal Caco2 cells, Src is activated during differentiation and seems to inhibit the expression of transcription factors involved in promoting cell polarisation. Accordingly SFK inhibitors increase the rate of this differentiation (Seltana et al., 2013). In fact, *in vivo* the ablation of Csk results in

increased proliferation of IECs but decreased differentiation of Paneth and goblet cells suggesting pro-proliferative but anti-differentiative roles for SFKs (Imada et al., 2016). In contrast, SFKs are key to enabling the differentiation of lymphocytes, neurons and osteoclasts.

1.5.2 NRTKs in immune cell signalling

Innate immune cells are key to the defence against microbial pathogens, and the SFKs along with other NRTKs are integral to the functioning of these cells that include macrophages, dendritic cells, mast cells and granulocytes. The SFKs predominantly expressed in immune cells are Hck, Fgr and Lyn; while Src is expressed at a much lower level. The Syk/Zap-70 family of tyrosine kinases work in conjugation with, but downstream of SFKs to orchestrate signalling from a vast range of immune cell receptors to downstream Tec-family tyrosine kinases (Lowell, 2011). Aside from this SFKs in immune cells are still crucial to the regulation of integrin/FAK signalling as described in the previous section. These three families of tyrosine kinases function primarily in activating pathways regulated by receptors with ITAMs. While most SFKs and Syk can perform ITAM phosphorylation, it is thought that Lyn is the predominant force at play in immune cell ITAM activation (Bewarder et al., 1996; Ghazizadeh et al., 1994; Jankowski et al., 2008). However, Lyn is also able to act in inhibitory pathways via the phosphorylation of ITIM motifs found in receptors including the Fc γ RIIb which dampens the activating signals through ITAM encoding Fc γ Rs, B- and T-cell receptors (Ravetch and Lanier, 2000). The role of SFKs/Syk/Tec in ITAM mediated signalling with respect to the Fc γ RIIa has been described in the prior section about phagocytosis. Similar principles can be applied to the ITAM motifs found within the CD3 and ζ chains associated with the T-cell receptor, and the Ig α and Ig β chains associated with B-cell receptors (Love and Hayes, 2010) and these kinases are therefore integral to the proper functioning of lymphocytes.

1.5.3 NRTKs and pathogen recognition receptors

The relationship between NRTKs and TLR signalling is complex, though most receptors have a reliance upon tyrosine phosphorylation (reviewed in (Chattopadhyay and Sen, 2014)). Tyrosine kinase activity is required for both TLR4 signalling branches in response to LPS detection, as TLR4 induced genes can be blocked by tyrosine kinase inhibitors (Chen et al., 2003; Smolinska et al., 2008) TLR4 tyrosine mutants are defective in inflammatory signalling and interactions of TLR4 with Src, Btk, Lyn and Syk have been reported (Chattopadhyay and Sen, 2014). Interestingly Syk has been shown to inhibit the MyD88-Mal proinflammatory branch of signalling but activate the TRIF-TRAM interferon branch (Lin et al., 2013). There is direct evidence of Src and Btk phosphorylation of TLRs, however the RTK EGFR is also important in regulation of both TLR3 and TLR4. Similarly, there is multitude of contrasting data surrounding the roles of NRTKs in regulation of intracellular NLRs and inflammasomes. Recent reports have independently shown Syk, Btk, Fak and Ptk2 to have roles in NLRP3 induced IL1 β release during pyroptosis (Chung et al., 2016; Hara et al., 2013; Ito et al., 2015). However, it has also been demonstrated that tyrosine dephosphorylation of NLRP3 by PTPN22 is required for robust activation of IL1 β secretion (Spalinger et al., 2016).

1.5.4 Bacterial utilisation of NRTKs during infection

NRTK signalling has been utilised by bacteria within their infection strategies, for example allowing *Salmonella* and *Yersinia* invasion of mammalian cells. Invasive signalling resulting from the interaction between mammalian β -integrins and *Yersinia* proteins YadA and invasin is Src dependent (Eitel et al., 2005; Uliczka et al., 2009). For *Salmonella* invasiveness, Src is required for phosphorylation of the brush border microvilli actin-modifying protein villin, promoting membrane ruffling and *Salmonella* internalisation (Lhocine et al., 2015), and is integral to invasion dependent on the *Salmonella* outer membrane kinase Rck (Wiedemann et al., 2012). As well as being a GEF, the *Salmonella* T3SS effector SptP has phosphatase activity

which tightly regulate Src (Lhocine et al., 2015). Src and Abl have been demonstrated to be necessary for the invasive strategies of *Shigella flexneri* where it is recruited to and activated by the *Shigella* T3SS translocator component IpaC at the site of bacterial invasion. Src/Abl/Btk facilitate the phosphorylation of cortactin necessary for actin polymerisation and downstream *Shigella* invasion and actin comet tail formation (Dehio et al., 1995; Dragoi et al., 2013; Mounier et al., 2009). Phosphorylation of *Helicobacter pylori* CagA by Src and Abl results in drastic cell elongation and increased motility (Krisch et al., 2016). As previously described EPEC requires redundant tyrosine kinases Src/Abl/Tec family kinases signalling for actin pedestal formation during their extracellular lifestyle, through Tir phosphorylation (Phillips et al., 2004; Swimm et al., 2004a, 2004b). Alternatively, these same kinases can orchestrate the anti-inflammatory function of Tir through phosphorylation of ITIM tyrosines (Yan et al., 2012, 2013). As EPEC pedestal formation can be prevented by SFK inhibitors or the overexpression of the ART EspJ (Phillips et al., 2004; Young et al., 2014) it has been proposed that aside from preventing phagocytosis EspJ may serve to control pedestal dynamics throughout EPEC/EHEC infection.

1.6 ADP-ribosylation

ADP-ribosylation (ADPr) is the covalent attachment of single units (mono-ADPr (MAR)) or polymeric chains (poly-ADPr (PAR)) of ADP-ribose to proteins. Catalysed by an ever-increasing list of prokaryotic and eukaryotic ARTs this PTM utilises the substrate β -nicotinamide adenosine dinucleotide (NAD^+), and can be targeted to a range of amino acids including lysine, arginine, glutamic acid, aspartic acid, cysteine and serine (Daniels et al., 2015; Leidecker et al., 2016) (Figure 1.10). Historically radioactively labelled NAD has been utilised to characterise the activities of bacterial ARTs (bART), but its applications are restricted due to a lack of enrichment methodologies. Biotin-tagged NAD has been used to identify targets after enrichment of ADP-ribosylated proteins utilising streptavidin/Neutravidin pulldown

(Wang et al., 2010; Zhang, 1997). However, its bulky form is has been proposed to interrupt catalysis, and some report non-specific modification of proteins by this analogue (Haag and Buck, 2015). More recently, alkyne tagged NAD analogues have been developed which are almost identical to native NAD. Additional functionalities can be attached to this small alkyne group after ADPr, by means of an azide-containing capture reagent and a copper catalysed cycloaddition reaction (click chemistry) (Rostovtsev et al., 2002). Capture reagents can incorporate a range of tags for enrichment and visualisation and are often compatible with denaturing conditions eliminating the possibility of pulling down complexes.

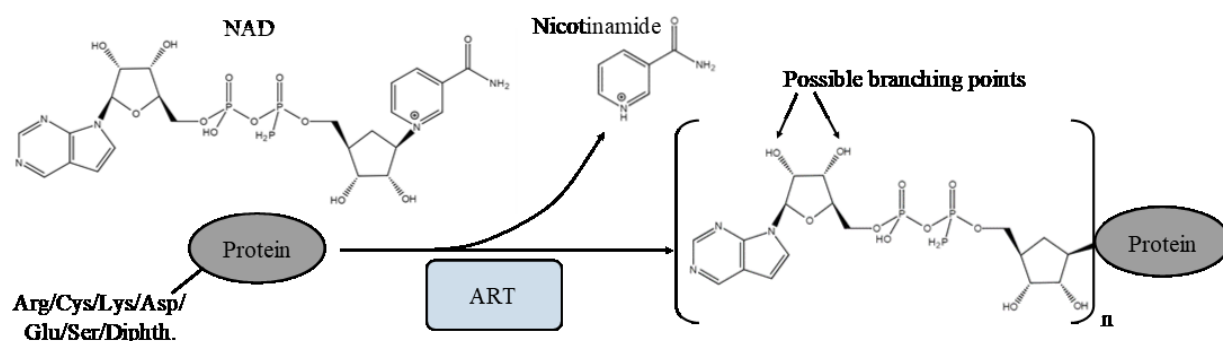


Figure 1.10: ADP-ribosylation reactions

ADP-ribosyltransferases utilise NAD^+ for ADP-ribosylation. Nicotinamide is first cleaved from NAD^+ before conjugation of ADP-ribose onto target protein Arg/Cys/Lys/Asp/Glu/Ser/Diphthamide residues as either mono-ADP-ribose or in polymeric branched chains.

The use of any of these analogues to study ADPr in cell culture or *in vivo* systems is hampered by their cell-impermeability. Attempts have been made to utilise transient cell permeabilisation, (Bakondi et al., 2002; Davis et al., 1998). However, superior methodologies have been developed for the proteomic study of ADPr in cell and *in vivo*. The first utilises a clickable alkyne analogue of adenosine, shown to be up taken by cells where it is metabolically incorporated into NAD (Westcott et al., 2017) enabling the enrichment of ADP-ribosylated proteins via a similar click chemistry workflow as described above. The second uses an ADP-ribose-binding protein ‘macro-domain’ such as Af1521 from thermophilic organism *Archaeoglobus fulgidus* to enrich ADP-ribosylated from unadulterated cell or tissue samples

(Martello et al., 2016). These tools are already being utilised to understand the growing roles of ADPr, and the underappreciated occupancy of ADPr in the human proteome.

1.6.1 Mammalian ADP-ribosyltransferases and hydrolases

Three groups of mammalian ARTs have been categorised; 1) the arginine-specific ecto-enzymes, 2) sirtuins (SIRTs) and 3) the PAR polymerases (PARPs). As most PARP family members have MAR not PAR activity it has been suggested that the nomenclature be redesigned for ARTs into two sub-categories. The PARP/Tankyrase subfamily would be named ARTDs based upon homology to the bacterial diphtheria toxin and the ecto-enzymes as ARTCs owing to their homology to clostridial C2 and C3 toxins (Hottiger et al., 2010) (see following section for catalytic details). This new nomenclature will be used within this thesis.

Originally presumed to only control gene regulation, ADPr has since been described as central to the maintenance of numerous cell signalling pathways. ARTDs are intracellular enzymes with diverse roles in the nucleus and cytoplasm including gene transcription, DNA repair, apoptosis, WNT signalling, response to bacterial and viral pathogens, and oxidative stress to name a few (Liu and Yu, 2015). Contrastingly, ARTC1-5 are extracellular mono-ARTs, catalysing ADPr of extracellular or cell-surface proteins regulating cell communication and signal transduction (Seman et al., 2004). ADP ribosylation is a reversible PTM, with its removal catalysed by several enzymes including ADP-ribosyl hydrolase 3 (ARH3) (Oka et al., 2006), PAR-glycohydrolase (PARG) (Slade et al., 2011), MacroD1 and MacroD2 (Jankevicius et al., 2013). Each contain a ADPr-binding macro-domain but the nature hydrolysis varies; for example while PARG and ARH3 catalyse the degradation of PAR chains, MacroD1 and MacroD2 can hydrolyse the ester linkage between acceptor residues and ADP-ribose removing MAR (Hottiger, 2015; Jankevicius et al., 2013).

1.6.2 Bacterial ARTs

bARTs have mainly been characterised in the context of pathogenic toxins and effector proteins (Simon et al., 2014), the first to be identified being diphtheria toxin (DT) of *Corynebacterium diphtherium*. A single DT molecule can be fatal to a mammalian cell via the ADPr of eukaryotic elongation factor 2 (eEF2) at a diphthamide residue (modified histidine), which inhibits its function and protein synthesis (Chung and Collier, 1977; Collier, 2001; Yamaizumi et al., 1978). With over 35 known bARTs, the magnitude of their importance for bacterial pathogenicity is now fully appreciated (Simon et al., 2014). Within this group some bARTs are delivered via secretion systems, but many are modular AB toxins that possess catalytic (A) domains and binding (B) domains for interacting and internalisation into target cells. Like the recent categorisation of mammalian ARTs, bARTs can be classified as DT-like or cholera toxin (CT)-like, which can be further divided into C2-, C3-like groups, based upon conserved active site motifs and domain organisation. While DT-like bARTs are single polypeptide chain AB toxins, CT-like enzymes are AB₅ toxins with the catalytic domain non-covalently linked to an oligomer of five B subunits, binary C2-like toxins have A and B subunits on separate polypeptide chains, and C3-like toxins only possess the catalytic domain. Recently several toxins including the A₂B₅ typhoid toxin have emerged as bARTs which fit into none of these categories. DT- and CT-toxin groups differ in their key active site residues; DT-like toxins possessing an essential HYE catalytic triad (Bell and Eisenberg, 1996), and CT-like toxins with an RSE motif (residues used to classify mammalian ARTCs and ARTDs) (Han et al., 2001; Tsuge et al., 2008). A conserved glutamic acid (DT E148) between these families has been proposed as essential for both their toxicities (Hottiger et al., 2010). However, *Pseudomonas syringae* HopF2 and *Mycobacterium* Arr have been shown to rely on a conserved aspartic acid residue instead (Baysarowich et al., 2008; Wang et al., 2010).

A variety of substrates have been described for these bARTs (reviewed in (Simon et al., 2014)). Broadly speaking, DT-like toxins ADPr of EF-2 for the inhibition of protein synthesis, CT-like toxins ADPr of G-proteins leading to adenylyl cyclase activation, C2-like toxins (*Clostridium botulinum* C2, *Clostridium perfringens* Iota-toxin, *Clostridium difficile* toxin) ADPr G-actin (Popoff et al., 1988; Vandekerckhove et al., 1988) and C3-like toxins ADPr cytoskeletal regulators including Rho GTPases (*Clostridium botulinum* C3) (Han et al., 2001; Vogelsang et al., 2007). The ADPr of G-actin R177 destroys the actin cytoskeleton by inhibiting actin polymerisation and stimulating F-actin depolarisation. This is accompanied by the formation of microtubule-based protrusions and can increase the adherence and colonisation by bacteria (Schwan et al., 2009). Contrastingly, the *Photorhabdus luminescens* toxins TccC3 and TccC5 which ADPr actin and Rho GTPases respectively, activate actin polymerisation and GTPase activity resulting in phagocytosis inhibition (Lang et al., 2010, 2016).

Many T3SS-secreted bARTs have been described, lacking the typical binding (B) domain of other modular AB toxins due to their alternative delivery mechanism. *Salmonella* SpvB is part of the C2-like toxin sub family, ADP-ribosylating G-actin and is required for virulence (Lesnick et al., 2001; Roudier et al., 1992; Tezcan-Merdol et al., 2001). The *Pseudomonas syringae* effector HopF2 inhibits MKK5 resulting in suppression of the plant immune response (Wang et al., 2010), and *Pseudomonas aeruginosa* ExoS and ExoT inhibit phagocytosis via the ADPr of Ras, Erm and vimentin or CrkI/II proteins respectively, despite sharing ~76% sequence identity (Barbieri et al., 2001; Ganesan et al., 1999; Maresso et al., 2007; Sun and Barbieri, 2003). In 2014 the ART activity of the EPEC T3SS effector EspJ was also defined (Young et al., 2014)

1.6.3 The T3SS-secreted ADP-ribosyltransferase EspJ

Originally identified by transcriptomics analysis and later shown to be translocated by the T3SS (Dahan et al., 2004, 2005), EspJ is a 217 residue non-LEE encoded EPEC/EHEC effector

protein. The *espJ* gene has been found in the vast majority of tested EPEC and EHEC strains whilst being absent from avirulent *E. coli* (Bugarel et al., 2011; Garmendia et al., 2005). Contrasting data surrounds the association of *espJ* with virulence. While one study found that *espJ* was more prevalent in EPEC strains from non-lethal infections (Donnenberg et al., 2015), another associated it more frequently with lethal infections (Hazen et al., 2016). EPEC *espJ* is found downstream of Cif and NleH within prophage 2, EHEC O157:H7 *espJ* is located on prophage CP933U within a *tccP* containing operon, while CR *espJ* is encoded along with NleH, NleF and NleG7 on genomic island 14 (Garmendia and Frankel, 2005; Iguchi et al., 2009; Petty et al., 2010). It has been reported that the clearance of a CR Δ *espJ* mutant was delayed, suggesting that EspJ may have roles in bacterial colonisation (Dahan et al., 2005). It has since been identified that EspJ is able to inhibit Fc γ R- and CR3-mediated phagocytosis via the inhibitory ADPr of Src kinase (Marchès et al., 2008; Young et al., 2014). This inhibition prevents the phosphorylation of the Fc γ RIIa, thus preventing downstream phagocytosis signalling. Interestingly, EspJ modified a universally conserved catalytic residue within the Src kinase domain, E310, which ordinarily forms a crucial salt bridge to K295M in the active conformation of Src (Hanks and Hunter, 1995). Further, the ADPr event was accompanied by a glutamic acid to glutamate switch by amidation, an event which could not be uncoupled from ADPr. Further studies have suggested that EspJ is mitochondrially localised and unstable within the host cell (Kurushima et al., 2010), and that it has multiple binding partners including Synmembrin-8 (RIC8A), intraflagellar transport protein 20 homolog (IFT20), centromere protein H (CENPH) (Blasche et al., 2014). A role in pedestal modulation has been suggested due to the prevention of EPEC but not EHEC pedestal formation by overexpression of EspJ prior to bacterial infection. This disparity may be owed to the lack of tyrosine phosphorylation in Tir_{EHEC} downstream signalling (Young et al., 2014).

While the role of EspJ during the EPEC/EHEC interaction with macrophages is reasonably well characterised, the consequences of endogenous EspJ *in vivo* within IECs is currently unknown. The large number of roles for SFKs as eluded to in this introduction, means that there is great potential for EspJ to modulate an array of cellular processes. Furthermore, the modification of such a conserved residue within the kinase domain raises questions regarding the potential promiscuity of EspJ ADPr activity for the perturbation of other kinases.

1.7 Aims and objectives

This study aims to further the understanding of the role of EspJ during infection. EspJ can ADP-ribosylate and inhibit Src kinase, impacting upon phagocytosis and pedestal dynamics. However, the repertoire of EspJ substrates and the impact of this *in vivo* is unclear. This, along with the characterisation of EspJ homologues from *Salmonella* and *Yersinia* will be the focus of this thesis.

Specific objectives include:

- Investigate the biochemical and functional properties of EspJ homologues predicted in *Salmonella* and *Yersinia* – do they possess ADP-ribosyltransferase activity, can they modify Src and inhibit phagocytosis, do they affect *Salmonella* invasiveness?
- Determine the range EspJ substrates – Can EspJ modify other proteins and is there specificity within these targets?
- Analyse the role of EspJ during the *in vivo* infection of mouse intestinal epithelial cells to determine processes aside from phagocytosis which it may modulate.

Chapter 2: Materials and methods

2 Chapter 2: Materials and methods

2.1 Cells and strains

2.1.1 Bacterial strains

Bacterial strains and sources are listed in table 2.1. Strains were stored at -80 °C using Microbank™ cryovials and recovered by streaking on Luria-Bertani (LB)-agar plates overnight at 37 °C. Bacteria were routinely cultured in broth at 37 °C with shaking at 200 rpm. Selection was provided by addition of antibiotics ampicillin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (34 µg/ml), tetracycline (6 µg/ml), gentamycin (10 µg/ml), streptomycin (50 µg/ml), or nalidixic acid (50 µg/ml), where appropriate. *Salmonella* and CR strains were similarly cultured, but within using Lennox LB broth unless otherwise stated.

2.1.2 Mammalian cell culture

HeLa/A549/Caco2 cell lines were maintained in Dulbecco's Modified Eagle's Media (DMEM, Sigma Aldrich) containing 1000/1000/4500 mg/l glucose, 1% (v/v) Glutamax™ (Life Technologies), and 10% (HeLa/A549) or 15% heat inactivated foetal calf serum (FCS). Thp1 macrophages were maintained in RPMI supplemented with 10% FCS (+0.02% PMA for differentiation). Cell lines were grown in tissue culture-treated flasks at 37 °C and 5% CO₂, except Caco2 cells which were kept at 10% CO₂. Cells were maintained by passaging three times per week, detaching cells by trypsinisation (PBS, 1 mM EDTA, 2 mM trypsin) (Sigma), or using cell scrapers (J774.A1 only), and seeding at between 1:5 and 1:10 dilution. For polarisation Caco2 cells were seeded at 1×10^5 cells per well, in 6 well plates, and media replaced at days 2, 4 and 6 and daily from day 7 to 14. Thp1 differentiation was performed by seeding at 2×10^7 cells per 10 cm dish, with addition of 0.02% phorbol-12-myristate-13-acetate (PMA). 48h later media was replaced with media lacking PMA and FCS.

Table 2.1: Bacterial strains

Strain	Frankel ICC	Name	Description	Source
<i>E. coli</i>	N/A	Top10	F ⁻ mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 nupG recA1 araD139 Δ (ara-leu)7697 rpsL(StrR) endA1 λ -	Invitrogen
<i>E. coli</i>	N/A	DH5 α	F ⁻ Φ 80lacZ Δ M15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK ⁻ , mK ⁺) <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	ThermoFisher Scientific
<i>E. coli</i>	N/A	BL21 STAR (DE3)	F ⁻ <i>ompT hsdS_B</i> (rB ⁻ , mB ⁻) <i>gal dcm rne131</i> (DE3)	ThermoFisher Scientific
<i>E. coli</i>	N/A	BL21(DE3) pLysS	F ⁻ <i>ompT hsdS_B</i> (rB ⁻ , mB ⁻) <i>gal dcm</i> (DE3) pLysS(CamR)	ThermoFisher Scientific
<i>E. coli</i>	N/A	CC118 λ pir	Δ (ara-leu) araD Δ lacX74 galE galK <i>phoA20 thi- rpsE rpoB argE</i> (Am) recA1, <i>lpir</i>	Herrero 1990
<i>E. coli</i>	N/A	CC1047		Kaniga 1991
<i>E. coli</i>	ICC481	E2348/69	EPEC O127:H6	Levine 1978
<i>E. coli</i>	ICC608	85-170	EHEC O157:H7	Tzipori 1987
<i>E. coli</i>	ICC188	85-170 Δ <i>espJ</i>	EHEC O157:H7 <i>espJ</i> deletion mutant	Dahan 2005
<i>C. rodentium</i>	ICC168	WT	<i>C. rodentium</i> O152, nalR	Shauer 1993
<i>C. rodentium</i>	ICC1470	Δ <i>espJ</i>	ICC168 <i>espJ</i> deletion mutant	Pollard 2018
<i>C. rodentium</i>	ICC1472	Δ ART	ICC168 <i>espJ</i> deletion mutant complemented with <i>espJ</i> -R79A on the genome	Pollard 2018
<i>C. rodentium</i>	ICC1471	Δ <i>espJ</i> comp- <i>espJ</i>	ICC168 <i>espJ</i> deletion mutant complemented with WT <i>espJ</i> on the genome	Pollard 2018
<i>S. enterica</i> subsp. <i>salamae</i>	ICC1301	3558/07	O13,22:z10:z6	Pollard 2016
<i>S. enterica</i> subsp. <i>salamae</i>	ICC1334	Δ <i>seoC</i>	<i>S. enterica</i> subsp. <i>salamae</i> <i>seoC</i> deletion mutant, Kn-R	Pollard 2016
<i>S. enterica</i> subsp. <i>salamae</i>	ICC1366	Δ <i>escN</i>	<i>S. enterica</i> subsp. <i>salamae</i> <i>escN</i> deletion mutant Kn-R	Pollard 2016
<i>S. enterica</i> subsp. <i>salamae</i>	ICC1367	Δ <i>invA</i>	<i>S. enterica</i> subsp. <i>salamae</i> <i>invA</i> deletion mutant Kn-R	Pollard 2016
<i>S. enterica</i> subsp. <i>salamae</i>	ICC1368	Δ <i>ssaV</i>	<i>S. enterica</i> subsp. <i>salamae</i> <i>ssaV</i> deletion mutant Kn-R	Pollard 2016
<i>S. enterica</i> subsp. <i>arizonae</i>	ICC340	SARC6	O62:z36:-	Boyd 1996
<i>S. bongori</i>	ICC325	CEIM46082	O48:z36:-	Giammanco 2002

2.1.3 Animals

Animal experiments were performed in accordance with the Animals Scientific Procedures Act 1986 and approved by the local Ethical Review Committee and UK Home office guidelines. Experiments were designed in agreement with the ARRIVE guidelines (Kilkenny et al., 2010), for the reporting and execution of animal experiments, including sample randomization and blinding. Pathogen-free female C57BL/6 mice (18 to 20 g, 6 mice per group) (Charles River,

UK) were housed in HEPA-filtered cages with sterile bedding (Processed corncobs grade 6), nesting (LBS Serving technology) and free access to sterilized food (LBS Serving technology) and water.

2.2 Molecular biology

Plasmid constructs used in this study, and their sources are summarised in table 2.2, oligonucleotide primers and associated restriction enzymes in table 2.3.

2.2.1 Polymerase chain reaction (PCR)

DNA sequences were amplified by PCR using KOD polymerase (Merck) with 10-100 ng of template DNA, 0.2 mM dNTPs, 1.5 mM MgSO₄ and 0.3 µM primers (Sigma) in a 50 µl volume. Colony PCR was performed by touching one colony into 1x OneTaq master mix (NEB) with 0.2 µM primers in 25 µl reaction volume. 10 min at 95 °C for bacterial lysis (colony PCR only) was followed by 2 mins at 95 °C for initial nucleotide denaturation, and then 30 cycles of 95 °C DNA melting, 15 seconds annealing at typically at 56-58 °C (primer melting temperature minus 2-5 °C) and extension at 72 °C (KOD - 20 sec/kb, OneTaq 60 sec/kb). A final extension was performed for 2 min at 70 °C.

2.2.2 Agarose gel electrophoresis

DNA amplicons mixed with 6 x loading dye (NEB) prior to separation at 100-150 V using 1% w/v agarose dissolved in 40 mM Tris-acetate, 1 mM EDTA (TAE), with addition of 1:10000 dilution of SYBR-safe DNA stain (Invitrogen). SafeImage blue light trans-illuminator (Invitrogen) was used for visualisation. 2-log DNA ladder (NEB) was used for estimation of amplicon sizes.

2.2.3 PCR and plasmid purification

PCR-products were purified using Qiaquick PCR purification or gel extraction kits (Qiagen), and genomic DNA using DNeasy blood and tissue kit (Qiagen) according to manufacturer's

instructions. PeqLab miniprep kit was used to purify plasmid DNA from 5 ml overnight bacterial cultures according to instructions. DNA concentrations were estimated using NanoDrop NS-1000 (Thermo Scientific).

2.2.4 Restriction enzyme digestion and ligation

PCR amplicons and target vectors were digested at 37 °C for 1-2 h using restriction enzymes (NEB) (Table 2.3) in 50 µl. Vectors were next dephosphorylated using calf intestinal phosphatase (CIP) for 30 min at 37 °C. Products were purified by gel extraction (Qiagen) before being ligated using T4 DNA ligase (NEB), 100 ng vector, at 1:3 molar ratio of vector:insert, for 30 min in 1 x ligase buffer (NEB).

2.2.5 Site directed mutagenesis (SDM)

SDM was performed by inverse PCR using either overlapping primers or end-to-end primer approaches (see table 2.3). PCR was followed by a 1 h DpnI digest of template vector. For the overlapping primer strategy DpnI-digested PCR product was directly used for bacterial transformation, according to Quikchange II mutagenesis kit (Aglient). Alternatively, DpnI-digested PCR products were purified by gel extraction (Qiagen), before simultaneous phosphorylation and ligation using T4 polynucleotide kinase (T4 PNK) and T4 DNA ligase in ligase buffer (NEB) for 30 min at 37 °C. Ligation products were used for bacterial transformation.

Table 2.2: Plasmids used in this study

Plasmid/pICC	Description	Source / Reference
pMALXE	Expression MBP tagged fusion proteins in <i>E. coli</i> expression host	Moon <i>et al</i> , 2000
pICC2446	pMALXE- expression of MBP tagged EPEC EspJ	Pollard <i>et al</i> , 2016
pICC2447	pMALXE—expression of MBP tagged EHEC EspJ	Pollard <i>et al</i> , 2016
pICC2448	pMALXE- expression of MBP tagged <i>C. rodentium</i> EspJ	Pollard <i>et al</i> , 2016
pICC2449	pMALXE - expression of MBP tagged <i>S. salamae</i> SeoC	Pollard <i>et al</i> , 2016
pICC2450	pMALXE - expression of MBP tagged <i>S. arizonae</i> SeoC	Pollard <i>et al</i> , 2016
pICC2451	pMALXE - expression of MBP tagged <i>S. bongori</i> SboC	Pollard <i>et al</i> , 2016
pICC2452	pMALXE - expression of MBP tagged <i>Y. enterocolitica</i> YspJ	This study
pICC2453	pMALXE – expression of MBP-EPEC EspJ R79A	Pollard <i>et al</i> , 2016

pICC2552	pMALXE – expression of MBP-EHEC EspJ R79A	This study
pICC2553	pMALXE – expression of MBP-EHEC EspJ D187A	Pollard <i>et al.</i> , 2018
pICC2554	pMALXE – expression of MBP-EHEC EspJ R79A/D187A	This study
pETM11	Expression of His tagged fusion proteins in <i>E. coli</i> expression host	GE Healthcare
pICC2440	pETM11- expression of MBP tagged EPEC EspJ	This study
pICC2441	pETM11—expression of MBP tagged EHEC EspJ	This study
pICC2442	pETM11- expression of MBP tagged <i>C. rodentium</i> EspJ	This study
pICC2443	pETM11- expression of MBP tagged <i>S. salamae</i> SeoC	This study
pICC2446	pETM11- expression of MBP tagged <i>S. arizonae</i> SeoC	This study
pICC2444	pETM11- expression of MBP tagged <i>S. bongori</i> SboC	This study
pICC2445	pETM11- expression of MBP tagged <i>Y. enterocolitica</i> YspJ	This study
pGEX-KG	Expression GST tagged fusion proteins in <i>E. coli</i> expression host	GE Healthcare
pICC1615	pGEX-KG– expression of GST tagged Src FL	Young <i>et al.</i> , 2014
pICC1617	pGEX-KG– expression of GST tagged Src ₂₅₀₋₅₃₃	Young <i>et al.</i> , 2014
pICC2368	pGEX-KG– expression of GST tagged Src ₂₅₀₋₅₃₃ -K295M	Young <i>et al.</i> , 2014
pICC2454	pGEX-KG– expression of GST tagged Src ₂₅₀₋₅₃₃ -K295M/Y416A	Pollard <i>et al.</i> , 2016
pICC2455	pGEX-KG– expression of GST tagged Src ₂₅₀₋₅₃₃ -K295M/Y416F	This study
pICC2486	pGEX-KG– expression of GST tagged Src ₂₅₀₋₅₃₃ -K295M/Y416A/E310A	Pollard <i>et al.</i> , 2016
pET28a	Expression of N-terminally hexahistidine tagged proteins in <i>E. coli</i> expression host	GE Healthcare
pICC1611	pET28a – expression of His tagged EspJ ₂₈₋₂₁₇	Young <i>et al.</i> , 2014
pICC451	pET28a – expression of His tagged NleH1	Hemrajani <i>et al.</i> , 2010
pICC2550	pET28a – expression of His tagged Csk	This study
pICC2551	pET28a – expression of His tagged Csk E236Q	This study
pCX340	Expression vector for TEM-1 fusion protein,	Charpentier and Oswald, 2004
pICC526	pCX340- expression of <i>S. bongori</i> SboI-TEM-1 fusion protein	Fookes <i>et al.</i> , 2011
pICC522	pCX340- expression of <i>S. bongori</i> FabI-TEM-1 fusion protein	Fookes <i>et al.</i> , 2011
pICC524	pCX340- expression of <i>S. bongori</i> SboC-TEM-1 fusion protein	Fookes <i>et al.</i> , 2011
pICC2390	pCX340- expression of <i>S. salamae</i> SeoC-TEM-1 fusion protein	Pollard <i>et al.</i> , 2016
pRK5-myc	Eukaryotic expression vector of Myc tagged protein.	BD Pharmingen
pICC2271	pRK5- expression of myc tagged EPEC EspJ	Young <i>et al.</i> , 2014
pICC2275	pRK5- expression of myc tagged EPEC EspJ R79A/D187A	Young <i>et al.</i> , 2014
pICC2304	pRK5- expression of myc tagged EHEC EspJ	Pollard <i>et al.</i> , 2016
pICC2426	pRK5- expression of myc tagged <i>C. rodentium</i> EspJ	Pollard <i>et al.</i> , 2016
pICC2427	pRK5- expression of myc tagged <i>S. salamae</i> SeoC	Pollard <i>et al.</i> , 2016
pICC2428	pRK5- expression of myc tagged <i>S. arizonae</i> SeoC	Pollard <i>et al.</i> , 2016
pICC2429	pRK5- expression of myc tagged <i>S. bongori</i> SboC	Pollard <i>et al.</i> , 2016
pICC2430	pRK5- expression of myc tagged <i>Y. enterocolitica</i> YspJ	This study
pICC2275	pRK5- expression of myc tagged EPEC EspJ-R79A/D187A	Pollard <i>et al.</i> , 2016
pWSK29	Expression vector for bacteria	(Wang and Kushner, 1991)
pICC2438	pWSK29 - expression of <i>S. salamae</i> seoC	Pollard <i>et al.</i> , 2016
pICC2466	pWSK29 - expression of <i>S. salamae</i> seoC R79A	Pollard <i>et al.</i> , 2016
pCB6-GFP	Expression of GFP tagged proteins in mammalian cells	Newsome <i>et al.</i> , 2006
pICC2286	pCB6- expression of GFP tagged Src	Newsome <i>et al.</i> , 2006
pICC2347	pCB6- expression of GFP tagged Src R175K	Newsome <i>et al.</i> , 2006

pICC2287	pCB6- expression of GFP tagged Yes1	Newsome <i>et al.</i> , 2006
pICC2285	pCB6- expression of GFP tagged Fyn	Newsome <i>et al.</i> , 2006
pICC2288	pCB6- expression of GFP tagged Abl	Newsome <i>et al.</i> , 2006
pICC2549	pCB6- expression of GFP tagged Csk	Pollard <i>et al.</i> , 2018
pSEVA612S	Plasmid for mutation of EspJ in <i>C. rodentium</i>	Herrero <i>et al.</i> , 1990
pICC2562	pSEVA612S – (+)300bp-(-)EspJ-(-)300bp - no espJ	This study
pICC2560	pSEVA612S – (+)300bp-EspJ-(-)300bp	This study
pICC2561	pSEVA612S – (+)300bp-EspJ-R79A-(-)300bp	This study
pKD46	Arabinose inducible expression of λ -red recombinase	Datsenko and Wanner, 2000
pKD4	Kanamycin resistance cassette	Datsenko and Wanner, 2000
pGEMT	High copy cloning plasmid	Promega
pICC2439	pGEMT-Kn resistance cassette flanked by 500 bp up/downstream of <i>S. salamae</i> SeoC coding sequence—for knocking out SeoC on chromosome	Pollard <i>et al.</i> , 2016
pFPV25.1	Constitutive GFP expression used for visualisation of <i>Salmonella</i> during infection	(Valdivia, 1997)
pEGFP-Fc γ RIIa	Expression of EGFP tagged Fc γ RIIa in mammalian host	(van Zon <i>et al.</i> , 2009)
pMXs-IP	Retroviral plasmid for viral package signal, transcription and processing, puromycin selectable	Invitrogen
pICC2437	pMXs-IP-GFP-Fc γ RIIa	Pollard <i>et al.</i> , 2016
pCMV-VSV-G env	Envelope glycoprotein of Vesicular stomatitis virus	Walther Mothes, Yale University
pCMV-MMLV-gag-pol	Capsid, reverse transcriptase and insertase	Walther Mothes, Yale University

Table 2.3: Primers used in this study

#	Plasmid/use	Primer Sequence (5'-3') (Restriction site underlined)	Restriction site
Classical restriction enzyme-based cloning			
1 2	pMXs-IP_GFP_Fc γ RIIa (pICC2437)	F-ggcctc <u>gag</u> atgtctcagaatgtatgtccc R-caagcggccgcttactgtacagctcgtccatg	XhoI NotI
3 4	pMALXE_EPEC_EspJ (pICC2446)	F- catgccatgggtccaatcataaagaactgcttcatc R- cacaagctttcatttttgagtggtggatattaac	NcoI HindIII
5 6	pMALXE_EHEC_EspJ (pICC2447)	F-cacggatccatgtcaattataaaaaactgcttcatc R-cacaagctttatttttgagaggatataatgtcaac	BamHI HindIII
7 8	pMALXE_ <i>C. rodentium</i> _EspJ (pICC2448)	F-cacggatccccaattataaaggtcctgtttatcatc R-cacaagctttattttttaaatgggtatatgtcaac	BamHI HindIII
9 10	pMALXE_ <i>S. salamae</i> _SeoC (pICC2449)	F-cacggatccaatgtataaagaactgttttcatc R-gataagcttctatttttacttacaggataaatatc	BamHI HindIII
11 12	pMALXE_ <i>S. arizonae</i> _SeoC (pICC2450)	F-cacggatccaatattatcaagaactgttttcattcctcaac R-gccaagcttctattttgttactggataaatatc	BamHI HindIII
13 14	pMALXE_ <i>Y. enterocolitica</i> _YspJ	F-caagcggccgcaaatttataaaaaagcggttttcatc R-cacggatccctattttgttgcactggataaatatc	NotI BamHI
15 16	pMALXE_ <i>S. bongori</i> _SboC (pICC2451)	F-cacggatccaatgtataaaaaactgttttcatc R-cacaagcttctattttgttactggatagatatac	BamHI HindIII
17 18	pETM11_EPEC_EspJ (pICC2446)	F- catgccatgggtccaatcataaagaactgcttcatc R- cacaagctttcatttttgagtggtggatattaac	NcoI HindIII
19 20	pETM11_EHEC_EspJ (pICC2447)	F- catgccatgggtccaatcataaaaaactgcttcatcattaatt R- catgccatgggtccaatcataaaaaactgcttcatcattaatt	NcoI HindIII

21	pETM11_ <i>C. rodentium</i> _EspJ (pICC2448)	F- catgccatgggtccaattataaggtcgtgtttatc	NcoI HindIII
22		R- cacaagctttttatttttaaatgggtatatgtcaac	
23	pETM11_ <i>S. salamae</i> _SeoC (pICC2449)	F- catgccatgggtaatgttataaagaactgttttcac	NcoI HindIII
24		R- gcttctgcagctatttgttgcactggataaatatc	
25	pETM11_ <i>S. arizonae</i> _SeoC (pICC2450)	F- catgccatgggtaatatatcaagaactgttttcattcctcaacag	NcoI HindIII
26		R- gccaaagcttctatttttgttgcactggataaatatc	
27	pETM11_ <i>Y. enterocolitica</i> _YspJ	F- catgccatgggtaattttataaaaagcgggttttcac	NcoI EcoRI
28		R- cacgaattcctatttgttgcactggataaatatc	
29	pETM11_ <i>S. bongori</i> _SboC (pICC2451)	F-ctgggatcccatgaatgttataaagaactgttttc	BamHI PstI
30		R-tggcggccaagcttctgcagctatttttacttacaggataaatatc	
31	pRK5_ <i>S. arizonae</i> _SeoC (pICC2428)	F-cacggatccaatattatcaagaactgttttcattcctcaac	BamHI HindIII
32		R-gccaagcttctatttttgttgcactggataaatatc	
33	pRK5_ <i>S. bongori</i> _SboC (pICC2429)	F-ctgggatcccatgaatgttataaaaactgtctttc	BamHI PstI
34		R-gcttctgcagctatttttgttgcactggatagatc	
35	pRK5_ <i>C. rodentium</i> _EspJ (pICC2426)	F-ctgggatccccaattataaggtcctgtttatc	BamHI PstI
36		R-gcttctgcagttatttttaaatgggtatatgtcaac	
37	pRK5_ <i>Y. enterocolitica</i> _YspJ	F-ctgggatcccatgaattttataaaaagcggttttc	BamHI PstI
38		R-gcttctgcagctatttgttgcactggataaatatc	
39	pWSK29_ <i>S. salamae</i> _SeoC (pICC2438)	F-tagtggatccaagaaggagatatacctacgtaatgaatgttataa	BamHI HindIII
40		gaactgttttc R-gataagcttctatttttacttacaggataaatatc	
41	pET28-Csk (pICC2550)	F- ggggaatttccatgtgcagcaatacaggccgcct	NdeI BamHI
42		R- cacggatcctcacagggtgcagctcgtggg	
43	pCB6-Csk (pICC2549)	F- ttcagatctggtaccatgtcagcaatacaggccgc	BglII NotI
44		R- ataagaagcggccgcgccgccgcccagggtgcagctcgtgggt	
45	<i>C. rodentium</i> EspJ -300 bp flank	F- ctagtctagagcaaaataatataagaagttagaatg	XbaI NdeI
46		R- ggaattccataggataaactcctgtgcgccatatt	
47	<i>C. rodentium</i> EspJ +300 bp flank	F- ggaattccatgtcttaatatacttagatataataaggtg	NdeI SacI
48		R- ctggagctcgcagcaacacatccgggatc	
Site directed mutagenesis by overlapping (o) or blunt ended (b) inverse PCR			
51	EPEC EspJ R79A (o)	F- tataaaacgggatttctgtgctgtagcaatccaaagtaatcagttactgat	
52		R- atcagtaaaactgattactttggattgctacagcaacgaaatcccgtttata	
53	EPEC EspJ D187A (o)	F- ggagccaaagtatatccagctatatcatgctctctgaga	
54		R- tctcagagagcatgatatactggatatactttggctcc	
55	EHEC EspJ R79A (o)	F- gagattttgtgctgtagcaatccaaacaatcagtttac	
56		R- gtaaaactgattgttttggattgctacagcaacaaaatctc	
57	EHEC EspJ D187A (o)	F-ggagcaaaagtatatcccgtacatcatgctctctgagac	
58		R-gtctcagagagcatgatgtagcgggatatacttttgcctc	
59	<i>S. salamae</i> SeoC R79A (b)	F- gcaattcaggaagataaatttacagatttaaaaag	
60		R- tacagcaacaaattttcagtaatattaaaag	
61	<i>C. rodentium</i> EspJ R79A (b)	F- gcagttcaagacaatcaattcactgat	
62		R- tacagcaacgaaattgcgtttata	
63	Src Y416A (b)	F- gcaacagcacggcaaggtgc	
64		R- ctcgttgcctcgtgatgagc	
65	Src Y416F (b)	F- ttcacagcacggcaaggtgc	
70		R- ctcgttgcctcgtgatgagc	
71	Src E310A (b)	F-ggaggccctcctgcaggcacgccaagtg	
72		R-cctcatcacttgggtgcctgcaggaag	
73	Csk E236Q (b)	F- caagcctcagtcacgcaac	
74		R- agccaggaaggcctggg	
Other (<i>S. salamae</i> and <i>C. rodentium</i> mutagenesis)			
75	PCR amplification of <i>S. salamae</i> <i>seoC</i> ± 500bp	F-aactttttgtgtaattcttataaaacag	
76		R- cgctcaatgcgggtattatc	
77	Inverse PCR removal of <i>seoC</i> from pGEMT	F-atatctctcatctggatggggac	
78		R-ttttaactaatcctgcttaaaataatg	
79	PCR check Kanamycin insert for <i>S. salamae</i> Δ <i>seoC</i>	F-catagtctgatactcttagatcatc	
80		R-gttatttttcatggcgggaag	

81	PCR amplification of pKD4 kanamycin resistance cassette	F-catatgaataatcctccttag
82		R-tgtgtaggctggagctgcttcg
83	PCR amplification of Kn-R cassette $\pm escN$ 50 bp flanks	F- gtttgattcctatcatgatctcattttggcatttttttcttatggccgtgtgt
84		aggctggagctgcttcg R- gcattaaagtcattaactgtccccaacacttttaattctatattattacccat atgaataatcctccttag
85	PCR amplification of Kn-R cassette $\pm invA$ 50 bp flanks	F- acttaacagtgcctggttacgacctgaattactgattctgggtactaatgggtgtaggctggag
86		ctgcttc R- gctatctgctatctcaccgaaagataaaacctccagatccggaaaacgacccatatgaata tctcct tag
87	PCR amplification of Kn-R cassette $\pm ssaV$ 50 bp flanks	F- gttcacgttttaggtgagagaatcagagcgcaacagtggctcaatgtatgctgtgtaggctg
88		gagctg ctctg R- cctgaattcggcccatcgccgatagccatcggggggaggatatttcagccatatgaatat cctccttag
89	PCR check Kanamycin insert for <i>S. salamae</i> $\Delta escN$	F- caattaaccaagtctcaaagc
90		R- gtacttttccccactccaga
91	PCR check Kanamycin insert for <i>S. salamae</i> $\Delta invA$	F- gacgccagctgttcgc
92		R- caattccgcctcaataatgg
93	PCR check Kanamycin insert for <i>S. salamae</i> $\Delta ssaV$	F- ccagataactgttttaacgatgaa
94		R- gaactcgcggacttctcg
95	PCR screen <i>C. rodentium</i> <i>espJ</i> mutants (+/- 300 bp)	F- gttgctttgttctctgccac
96		R- ggcggtttttgtaaaccggagc

2.2.6 Competent bacterial cell preparation

Chemically competent cells (*E. coli* BL21 STAR, BL21 pLysS, Top10, DH5 α) were generated by first inoculating a 100 ml culture of LB with 1 ml of a bacterial overnight culture and growing to an optical density (OD)_{600nm} of 0.45-55 (2-4h). Sub-cultured bacteria were cooled for 15 min on ice before harvesting bacteria at 4500g for 20 min and discarding the supernatant. Bacteria were resuspended in 20 ml of cold buffer RF1 (Table 2.4) and incubated on ice for 10 min before centrifugation at 4500g, 20 min, removing supernatant and resuspending in buffer RF2 (Table 2.4). Electrocompetent cells (*S. salamae* 3558/07 or EHEC 85-170 or CC118 λ pir) were similarly prepared only using sterile 15% glycerol in place of RF1/RF2. Chemically/electro-competent cells were aliquoted, flash frozen using liquid nitrogen and stored at -80 °C.

2.2.7 Bacterial transformation

1 – 5 µl of DNA was gently mixed with 80 µl of chemically/electro-competent cells and incubated on ice for 15 min. Chemically competent cells were heat-shocked in a 42 °C water bath for 60 seconds, then placed on ice for 2 mins before adding 50 µl SOC medium (Sigma) and incubating for at least 1h at 37 °C with shaking. Electrocompetent cells were electroporated at 2.5 kV, 200Ω and 25 µF before immediate addition of 1 ml LB broth and recovery at 37 °C for at least 1h. 200 µl of transformation suspension was plated on LB-agar (Lennox for *Salmonella*) plus the appropriate antibiotic.

2.3 Bacterial strain manipulation

2.3.1 *Salmonella* mutagenesis (λ -red recombinase)

S. salamae $\Delta seoC$, $\Delta escN$, $\Delta invA$, $\Delta ssaV$ mutants were generated using the lambda red recombinase method (Datsenko and Wanner, 2000). PCR with primers 75/76 was used to amplify *S. salamae* *seoC* $\pm$ 500 bp from genomic DNA, which was inserted into linearised pGEMT vector by blunt ended ligation. Inverse PCR and primers 77 and 78 were used to remove *seoC* and generate pGEMT encoding only the $\pm$ 500bp flanking regions of *seoC*. A kanamycin-encoding cassette (Kn^R) was amplified from pKD4 with primers 81/82, which was inserted into the pGEMT backbone containing the *seoC* flanks. A product encoding the Kn^R between *seoC* flanking regions was amplified by PCR. The Kn^R from pKD4 was amplified with $\pm$ 50 bp flanking regions of *S. salamae* $\Delta escN$, $\Delta invA$ and $\Delta ssaV$ using primer pairs 83/84, 85/86, 87/88. Purified PCR products for Kn^R - $\pm$ 500bp-(*seoC*) or Kn^R - $\pm$ 50bp (*escN/invA/ssaV*) were electroporated into electrocompetent *S. salamae* 3558/07 pre-transformed with pKD46 and expressing lambda-red genes from an arabinose inducible promoter (Datsenko and Wanner, 2000), and plated on 50 µg/ml kanamycin LB-agar. Selected colonies were grown at 42 °C to induce the loss of pKD46 and screened by PCR using primer pairs 79/80 (*seoC*), 89/90 (*escN*), 91/92 (*invA*), 93/94 (*ssaV*) before sequencing PCR products (GATC).

2.3.2 *C. rodentium* mutagenesis

CR *espJ* deletion ($\Delta espJ$) was created without the insertion of a resistance cassette, and this strain used to complement on the genome with catalytically inactive *espJ*-R79A or WT *espJ* using the same methodology. EspJ flanking regions (± 300 bp) were amplified (primers 45/46 and 47/48). Amplicons and pSEVA612S were purified (Qiagen) and restriction enzyme-digested (Table 2.3). Digestion products were triple ligated before transformation into CC118 λ pir *E. coli*.

Tri-parental conjugation was used to delete *espJ* from CR encoding the endonuclease I-SceI within the pre-transformed pACBSR (Ruano-Gallego et al., 2015). *E. coli* CC118 λ pir carrying the pSEV612s derivatives (donor) was grown together on LB-agar for 6 h with *E. coli* CC1047 carrying pRK2013 (helper), and CR harbouring pACBSR (receiver). This patch of bacteria was harvested and plated on LB-agar supplemented with gentamycin and streptomycin overnight. Single colonies were grown in LB-broth-streptomycin with 0.4% arabinose to induce I-SceI expression for the excision of the pSEVA612s backbone. After plating overnight, single colonies were screened for the loss of *espJ* (primers 95/96) and single cloned passages multiple times to allow the spontaneous loss of pACBSR. PCR-products were sequenced to confirm positive clones (GATC).

For complementation on the genome, *espJ* was cloned with its flanking regions into pSEVA612S and mutated by SDM (primers 61/62), before transformation into CC118 λ pir and repeating the protocol using this strain as the donor and CR $\Delta espJ$ as the receiver.

2.4 Protein biochemistry

2.4.1 SDS-PAGE

Protein samples were denatured using Laemmli buffer (60 mM Tris-HCl pH6.8, 1% SDS, 5% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) and boiling for 5 min at 100 °C.

Polyacrylamide gels were used to separate proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 180 V in Tris-Glycine-SDS buffer (Geneflow). For home-made gels, proteins were compressed using a 4% stacking layer (4% polyacrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS) and separated with between 10% and 15% polyacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS. BioRad 4-20% PROTEAN® TGX Gels were also occasionally used for separation. Gels were either stained using InstantBlue stain (expedeon) or transferred to Polyvinylidene fluoride (PVDF) membrane (GE Healthcare) for western blotting analysis.

2.4.2 Western blotting and in gel fluorescence

PVDF was activated in methanol before protein transfer in either BioRad transfer buffer or Tris-Glycine buffer (Geneflow), using a Trans-Blot® Semi-Dry transfer cell at 15 V for 30 min. Membranes were blocked in 5% skimmed milk/PBS/0.02% Tween-20 (PBST) 3% bovine serum albumin (sigma) (BSA)/PBST before incubation for 1-2 h with primary or secondary antibodies in either 1% milk/PBST or 3% BSA/PBST with 3 x 5 min PBST washes in between. After secondary antibody incubation membranes were washed 3 x 5 min with PBST before detection using EZ-ECL (biological industries) and a Fuji LAS3000 imager. In gel fluorescence was visualised directly using Typhoon Imager (GE Healthcare Life Sciences) at between 750 V and 1000 V.

2.4.3 Protein overexpression

Single colonies of BL21 STAR or BL21 pLysS carrying expression plasmids (see table 2.2) were grown in 5 ml LB overnight before diluting 100-fold into the desired volume of LB. Sub cultures were grown OD_{600nm} of 0.6-1.0 before cooling on ice for 30 min and inducing protein expression with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). Proteins were expressed overnight at 18 °C before harvesting bacteria at 4500g for 20 min. Whole cell samples were taken pre-induction and the next morning, to assess protein expression by SDS-PAGE.

2.4.4 Protein purification

Protein purifications were all performed at 4 °C. Bacterial pellets were resuspended in 40 ml per litre of expression culture of GST/MBP/His lysis buffers (Table 2.4). Lysis buffers were supplemented with 2 x Roche cOmplete protease inhibitor cocktail tablets, 5 µl benzonase nuclease per 40 ml (Sigma), and 1 mg/ml of hen's lysozyme (Sigma). Bacterial suspensions were lysed by sonication, according to culture volume, and clarified by centrifugation at 40000g for 45 min. For small scale solubility tests, equivalent soluble and insoluble fractions samples were taken for assessment by western blotting. For the refolding of His-EspJ₂₈₋₂₁₇ insoluble pellets were washed 2 x with lysis buffer + 0.2% (v/v) Triton-X100 and 2 x with lysis buffer + 100 mM GuHCl. Washed pellets were resuspended in 6 M GuHCl-containing lysis buffer by rotation overnight at 4 °C before clarification at 45000g for 45 mins.

II. Protein concentration

Protein sample concentrations were continuously monitored throughout purifications using the NanoDrop NS-1000 (Thermo Fisher, Scientific) and sample concentrations increased using Amicon Ultra 10000 MWCO centrifugal filter units at 4500g.

III. Affinity chromatography

GST-Src constructs were AKTA purified using GSTrap 4B sepharose columns (GE Healthcare), and His-Csk/NleH/EspJ using HiTRAP Talon columns (GE Healthcare), while MBP-EspJ was purified by gravity flow using amylose resin (NEB). For GST and MBP purifications, after loading for 1 h resin was washed with at least 20 column volumes (CV) of lysis buffer, followed by elution using 20 mM reduced glutathione or 50 mM maltose supplemented buffer. His-tagged proteins were similarly washed but eluted over a 20 ml gradient of imidazole from 20 to 500 mM. For refolding, His-EspJ in 6M GuHCl-buffer was bound to the Talon column and washed with 6M GuHCl-buffer before 10 CV of 1 M GuHCl,

and 10 CV of 0M GuHCl based buffer prior to elution over a similar imidazole gradient. GST-Af1521 was purified using sepharose-4B resin in a batch mode.

IV. Gel filtration and anion exchange chromatography

Affinity-purified proteins were concentrated down to 500 µl before separation by size using a Superdex 200 10/300 (Invitrogen) chromatography column, collecting 1 ml fractions. After protein concentration, samples were diluted 10-fold to 50 mM NaCl before separating based upon charge using a Resource Q 6 ml column over a 40 ml gradient from 50 mM to 1000 mM NaCl.

2.4.5 GST-Src cleavage

GST was cleaved from Src at 4°C overnight with 10 units of thrombin per mg of fusion-protein before removing GST and un-cleaved GST-Src by incubating with glutathione sepharose resin

2.4.6 GST-Af1521 resin crosslinking

GST-Af1521 was crosslinked to sepharose resin using the dimethyl pimelimidate (DMP) as described by Abcam (<http://www.abcam.com/protocols/cross-linking-antibodies-to-beads-protocol>). Freshly prepared 13 mg/ml of DMP in 0.2 M triethanolamine/PBS was incubates 1:1 v/v with resin for 30 min at RT before aspirating supernatant and repeating twice. DMP was then quenched by washing 2 x with 50 mM ethanolamine/PBS followed by washing with 1 M glycine pH 3 for 10 min each. Crosslinked resin was washed in 2 x in Af1521 lysis buffer and stored for up to 2 weeks at 4 °C.

2.4.7 Protein complex pulldowns

For pulldowns 500 µL of lysates, or a known 10 µg of purified MBP-EspJ were incubated with 50 µl of amylose resin for 1 h and washed 3 x with 1 ml of lysis buffer. 10 µg of GST-cleaved Src-₂₅₀₋₅₃₃K295M/Y416A was added to the MBP-EspJ-bound resin for 1 h at 4 °C with rotation

before 3 x 5 min washes in MBP-lysis buffer. Addition of 5 x Laemmli buffer and boiling allowed analysis of pulldowns by SDS-PAGE/western blotting.

2.4.8 *In vitro* ADP-ribosylation and kinase reactions

ADPr reactions using 6-biotin-17-NAD⁺ (AMSBIO) were performed for 1 h at room temperature in ADPr buffer (Table 2.4) plus 10 μ M NAD-biotin unless otherwise stated. Reactions were stopped by the addition of Laemmli buffer and boiling for 5 min.

For the *in vitro* ADPr of Src or Csk by the panel of EspJ homologues, 500 μ l of expression lysate was incubated with 50 μ l of amylose resin for 1 h at 4°C with rotation before washing 3x with MBP-lysis buffer. 4 μ g of GST-Src₂₅₀₋₅₃₃ or His-Csk was added to EspJ-bound resin, or 4 μ g of purified MBP-EspJ_{EHEC} in a 40 μ l reaction volume. Dynabead-bound GFP-tagged tyrosine kinases were incubated with 2 μ g of purified MBP-EspJ_{EHEC} in ADPr buffer for 1 h at room temperature in a total volume of 20 μ l.

For testing the inhibition of Csk by EspJ, MBP-EspJ_{WT}/EspJ-D187A/TssF1 were incubated at 100 x molar excess with Csk in ADPr buffer with 10 μ M His-Csk. After 2h GST-Src₂₅₀₋₅₃₃ K295M/Y416A/E310A or poly(glu₄, tyr) (Sigma) were added in 100-fold excess to Csk, along with 10x kinase reaction buffer to an end concentration of 100 mM KCl, 100 mM MOPS, 10 mM DTT, 10 mM MgCl₂, 100 μ M ATP. At 1, 5, 15 and 30 mins reactions were stopped by addition of Laemmli buffer and 2 min boiling.

For the *in vitro* chemical proteomics screen of EspJ targets, 400 μ g of HeLa/A549/Caco2/Thp1 cell lysate in ADPr buffer was incubated with 20 μ g of MBP-EspJ_{EHEC} and 2-ethynyl-adenosine-NAD (Jena Biosciences) for 3 h at room temperature. Proteins were then precipitated with 4 x volume methanol, 1.5 x volume chloroform, 2 x volume water, and washed 2x with ice cold methanol before resuspension in 25 μ l of 2% SDS/PBS by vortex mixing for 30 min.

2.4.9 Click chemistry

Resuspended proteins from cell lysate ADPr with eNAD were diluted to 0.5% SDS/PBS by addition of 75 μ l PBS. Cell lysates in 4% SDS buffer from EHEC infections HeLa/Caco2 in presence of 2YnAd, were also subjected to click chemistry. Protein concentrations were adjusted to around 1 mg/ml for optimal reaction efficiency. Azide-biotin was conjugated onto proteins by click chemistry reactions containing 100 μ M AzRB (structure 3, figure 1 from (Broncel et al., 2015)), 1 mM CuSO₄, 1 mM TCEP and 100 μ M TBTA for 2 h with vortex mixing. Reactions were quenched by chloroform/methanol precipitation and washed 2x with methanol, before resuspension in 50 μ l 2% SDS/PBS as described above.

Table 2.4: Buffer compositions

Buffer/media	Composition
Protein purifications	
MBP lysis/gel filtration	500 mM NaCl, 50 mM CAPS, 5 mM DTT, 10% glycerol, pH 7.4 or 10
GST lysis/gel filtration	300 mM NaCl, 50 mM Na ₂ HPO ₄ , 5 mM DTT, 5% glycerol, pH 7.4
His lysis/gel filtration	50 mM Tris pH 8, 150 mM NaCl, 10% glycerol, 2 mM DTT
Afl521 lysis buffer	50 mM Tris, 150 mM NaCl, 1 mM MgCl ₂ , 1 mM DTT
Enzyme buffers	
1 x ADP-ribosylation	50 mM Tris pH 7.4, 1 mM DTT, 60 μ M ATP, 5 mM MgCl ₂
1 x kinase buffer	100 mM KCl, 100 mM MOPS, 10 mM DTT, 10 mM MgCl ₂ , 100 μ M ATP
Competent cell production	
RF1	100 mM ClRb, 50mM Cl ₂ Mn, 30 mM C ₂ H ₃ KO ₂ , 10 mM CaCl ₂ , 15% w/v glycerol
RF2	10 mM MOPS, 10 mM ClRb, 75 mM CaCl ₂ , 15% w/v glycerol
Mammalian cell lysis	
Kinase IP	20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na ₃ VO ₄ , 1 mM dithiothreitol (DTT), 0.5% NP-40, 2 cOmplete mini EDTA-free protease inhibitor tablets (Roche), 5 μ l of Benzonase nuclease/10 ml
“10 X” RIPA	100 mM HEPES, 150 mM NaCl, 10 mM EDTA, 1% Na-deoxycholate, 10% NP40
4% SDS	4% SDS, 50 mM HEPES pH 7.5, 150 mM NaCl
6 M GuHCl	6 M GuHCl, 100 mM HEPES, 10 mM DTT pH 8.5
8M urea	8 M urea, 100 mM HEPES, 10 mM DTT pH 8.5
Other	
Enterocyte dissociation buffer	1 x Hank’s balanced salt solution w/o Mg and Ca, plus 10 mM HEPES, 1 mM EDTA and 5 μ l/ml 2 β mercaptoethanol

Table 2.5: Antibodies and reagents used in this study

Reagent/resource (dilution)	Use (dilution)	Source	Identifier
Primary antibodies and conjugates			
GST, mouse monoclonal [3G10/1B3]	WB (1:2000)	Abcam	Cat#ab92, RRID:AB_307067
MBP, mouse monoclonal [MBP-17] HRP conjugate	WB (1:2000)	Abcam	Cat#ab49923, RRID:AB_881602
His, mouse monoclonal (HRP)	WB (1:2000)	Sigma-Aldrich	Cat# A7058, RRID:AB_258326
Phosphotyrosine, mouse monoclonal [PY20] (1:2000)	WB (1:2000)	Sigma-Aldrich	Cat# P4110, RRID:AB_477342
Csk, rabbit monoclonal [C74C1] (1:2000)	WB (1:2000)	Cell Signalling Technology	Cat# 4980S, RRID:AB_2276592
c-Myc, chicken, polyclonal (1:100)	IF (1:100)	Bethyl	Cat# A190-103A, RRID:AB_66662
BacTrace® Anti-Salmonella CSA-1, goat	IF (1:200)	Insight biotechnology	Cat# 01-91-99
BSA, mouse monoclonal	IF/opsonisation (1:100)	Sigma-Aldrich	Cat# B2901, RRID:AB_258533
GFP, mouse monoclonal [9F9.F9]	IP (1:50)	Abcam	Cat# ab1218, RRID:AB_298911
GFP, rabbit monoclonal	WB (1:2000)	Abcam	Cat# ab290, RRID:AB_303395
Intimin b, purified chicken antibody IgY	IF (1:200)	John Morris Fairbrother (Mundy et al., 2007)	N/A
Ki-67, rabbit monoclonal antibody (Clone SP6)	IF (1:20)	ThermoFisher Scientific	Cat# RM-9106-F0; RRID: AB_721371
Secondary antibodies			
Peroxidase-AffiniPure Goat Anti-Mouse IgG, Fc gamma Fragment Specific	IF (1:10000)	Jackson ImmunoResearch	Cat# 115-035-008, RRID:AB_2313585
Peroxidase-AffiniPure Goat Anti-Rabbit IgG, Fc Fragment Specific	IF (1:10000)	Jackson ImmunoResearch	Cat# 111-035-008, RRID:AB_2337937
Cy3 AffiniPure Goat Anti-Chicken IgY (IgG) (H+L) J	IF (1:200)	Jackson ImmunoResearch	Cat# 103-005-155; RRID: AB_2337379
Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	IF (1:200)	Jackson ImmunoResearch	Cat# 711-545-152; RRID: AB_2313584
Rhodamine Red-X-AffiniPure Donkey Anti-Mouse IgG (H+L)	IF (1:500)	Jackson ImmunoResearch	Cat# 715-295-150, RRID:AB_2340831
AMCA-AffiniPure Donkey Anti-Mouse IgG (H+L)	IF(1:200)	Jackson ImmunoResearch	Cat# 715-155-150, RRID:AB_2340806
Alexa Fluor 647-AffiniPure Goat Anti-Chicken IgY (IgG)	IF(1:100)	Jackson ImmunoResearch	Cat# 103-605-155, RRID:AB_2337392
Alexa Fluor 488-AffiniPure Donkey Anti-Mouse IgG (H+L)	IF(1:200)	Jackson ImmunoResearch	Cat# 715-545-150, RRID:AB_2340846
Rhodamine Red-X-AffiniPure Donkey Anti-Goat IgG (H+L)	IF(1:200)	Jackson ImmunoResearch	Cat# 705-295-147, RRID:AB_2340423
Chemicals			
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)		ThermoFisher Scientific	Cat# D3571, RRID:AB_2307445
Phalloidin iFluor-647	IF(1:10000)	AAT Bioquest	Cat# 23127-AAT
Streptavidin-HRP	WB (1:5000)	Dako	Cat# P0397

2.5 Mammalian cell techniques

2.5.1 Cos-7 GFP-FcγRIIa stable cell generation

GFP-FcγRIIa was sub-cloned from pEGFP- FcγRIIa (van Zon et al., 2009) into pMXs-IP (Invitrogen). HEK293 cells were seeded in 6 well plates at 4.8×10^4 cells per well. 24 h later Lipofectamine® 2000 (Invitrogen) was used for transfection with 500 ng pMXs-IP-GFP- FcγRIIa, plus 100 ng of pCMV-VSV-G plasmid and 400 ng of pCMV-MMLV packaging plasmids (Shenoy et al., 2012). HEK293 cells were left for 48 h to produce Vesicular Stomatitis pseudo-typed retrovirus encoding pEGFP- FcγRIIa. The supernatant buffered to a final concentration of 20 mM HEPES before filtering through a non-PVDF 0.45 μm membrane and adding the filtered virions to a T25 flask of 70-90% confluent Cos-7 cells. Transduction was monitored by fluorescence microscopy after 24 h and cells then selected for 1 week using 0.3 μg/ml puromycin. Finally, cells were fluorescence activated cell sorting (FACS) sorted using BDFACS Aria III and assessed using BDFACS FortessaIII.

2.5.2 Transfection of Cos-7 GFP-FcγRIIa cells

Cos-7 GFP-FcγRIIa cells were seeded on glass coverslips in 24 well tissue culture plates (BD biosciences) at 5×10^4 cells per well 24 h prior to transfection. Genejuice Transfection Reagent (Novagen) was used at a 3:1 Genejuice:DNA ratio according to manufacturer's instructions for the transfection of pRK5-Myc-EspJ/SeoC/SboC/YspJ. 0.5 μg/well of plasmid DNA was transfected for a 14 to 16 h before subjecting to the opsono-phagocytosis protocol (see below).

2.5.3 Cell culture infection

I. *S. salamae* infection of HeLa/J774.A1 cells

HeLa/J774.A1 cells were seeded in 24 well plates on glass coverslips (immunofluorescence only) at 7.5×10^4 / 1.5×10^5 cells per well 24 h pre-infection. *S. salamae* overnight cultures were diluted 1:33 into 5 ml Lennox-LB, grown to an OD_{600nm} of 1.8 (2.5 h) at 37 °C before infecting

at an MOI of 100 (9 μ l per well). *seoC* complementation from the pWSK29 plasmid was induced 30 min before infection using 0.05 mM IPTG which was maintained throughout the infection. Infected cells were washed after 30 mins and challenged with opsonised beads as described below. *S. salamae* expressing GFP from the pFPV25.1 plasmid (Valdivia, 1997) was used for infections to visualise bacterial invasion/internalisation, and external bacteria stained pre-permeabilisation using the BacTrace® goat anti-CSA-1 antibody (KPL).

II. *EHEC* infection of HeLa/Caco2 cells

Caco2 cells were differentiated as described above and HeLa cells seeded at 1×10^5 cells per well in 6 well plates, 48 h prior to infection. The day before infection, EHEC 85-170 strains were grown for 8 h in LB-broth and then diluted 1:500 into 1000mg/l glucose DMEM. DMEM static sub cultures were incubated overnight at 37 °C, 5% CO₂ to induce T3SS expression. The next morning cells were infected at an MOI of 10 (HeLa) or 100 (Caco2). Bacteria were sedimented onto monolayers at 500g (HeLa) or 200g (Caco2) for 5 min. Infections were washed 3 x with PBS after 2.5 h. At specified time points cells were again washed 3 x with PBS before either immunofluorescence staining or lysis. Cells lysed using 200 μ l (HeLa) or 500 μ l (Caco2) per well of either RIPA, urea, GuHCl or SDS-based buffers (Table 2.4) for 10 min on ice (except SDS buffer) before scraping cells into 1.5 ml microcentrifuge tubes and lysing further by pulse-sonication. After clarification by centrifugation at 20000g for 10 min, supernatants were subjected to either GST-Afl521 pulldowns, or prepared by click chemistry for Neutravidin pulldowns to enrich ADP-ribosylated proteins. See later sections for details.

2.5.4 Gentamycin protection assay

HeLa cells were infected as described above but after 60 min cells were washed with PBS before incubating with gentamycin (200 μ g/ml) supplemented media. After another 60 min cells were washed 5 x with PBS before lysis with 0.1% Triton X-100 and plating on LB-agar in serial dilutions.

2.5.5 Translocation assay

The β -lactamase (TEM1)-translocation assay was performed as previously described (Fookes et al., 2011). 8.0×10^3 HeLa/ 4.0×10^3 J774.A1 cells were seeded per well of a 96 well plate (BD Falcon) 48 h prior to infection. *S. salamae* were prepared as described above and the expression of TEM fusion proteins from pCX340 (Charpentier and Oswald, 2004) using 0.05 mM IPTG 30 min prior to infection. At an OD_{600nm} of 1.8 *S. salamae* was added to monolayers at an MOI of 100 for 1 h, and bacterial samples were taken for assessment of fusion protein expression by western blotting. Next, cells were washed with 100 μ L Hanks' Buffered Salt Solution (Gibco), supplemented with 20 mM HEPES and 3 mM Probenecid (Sigma) pH7.4 (HBSS-HP), and 20 μ L of the β -lactamase substrate CCF2-AM added (Invitrogen) (Fookes et al., 2011). Cells were incubated for 105 min at RT in the dark before washing 5 x with HBSS-HP. Fluorescence emission at 450 nm and 520 nm was measured using a Fluostar Optima plate reader (excitation wavelength 410 nm, 10-nm band-pass). The translocation rate was calculated as recommended in the LiveBLAzer™ FRET-B/G Loading Kit manual. Expression of the TEM1 fusion proteins was analyzed by Western blot using a mouse anti- β -lactamase antibody (QED Bioscience Inc)

2.5.6 Lactate dehydrogenase (LDH) release assay

J774.A1 cells were infected with *S. salamae* as described above, and infection supernatants harvested at 30 and 60 min. Mammalian and bacterial cells were removed by sequential centrifugation at 4000g and 20000g and the resulting supernatants assayed for LDH release using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. The net absorbance was calculated from the absorbance at 490 nm in a media only control, and readings then normalized to uninfected cell supernatants to allow calculation of fold change.

2.5.7 Bead opsonisation and phagocytosis assay

Prior to bead addition transfected cells were incubated for 2 h with serum-free DMEM, and macrophages infected with *S. salamae* as described above. Per coverslip, 10 μ l (Cos-7 GPF-Fc γ RIIa) and 2.5 μ l (J774.A1) of 3.4 μ m SPHERO™ BSA coated polystyrene bead (Spherotech) slurry was washed in 1 mL of 20 mM MES, 8 mM HEPES. Washed beads were incubated with mouse anti-BSA antibody (Sigma) at RT for 1 h. Antibody-bound beads were then washed and resuspended in 1 ml of in serum free DMEM ((Cos-7 GPF-Fc γ RIIa) or serum containing DMEM (J774.A1 cells) per coverslip, before adding to transfected Cos-7 GPF-Fc γ RIIa cells or infected J774.A1 macrophages.

Beads were sedimented onto cell monolayers at 500g for 5 min. Beads were allowed to bind Cos-7 cells for 15 min at 4 °C before replacing washing and replacing with serum containing DMEM and incubating for 90 min at 37°C with 5% CO₂. Infected J774.A1 cells were challenged for 30 min at 37°C with 5% CO₂. J774.A1/Cos-7 cells were washed 1 x with cold tissue culture grade PBS on ice before staining extracellular beads for 7 min with donkey anti-mouse Alexa-488/RRX antibody in 0.2% BSA/PBS. Cells were then washed 2 x with cold PBS prior to fixation.

2.5.8 Immunofluorescence staining and visualisation

Infected/transfected cells were fixed with 3.7% paraformaldehyde (PFA)/PBS for 15 min before neutralisation with 50 mM NH₄Cl/PBS for 15 min and permeabilization with 0.2% Triton X-100/PBS for 2 min. Fixed cells were blocked for 15 min using 0.2% BSA/PBS before sequential incubation with primary and secondary antibodies in 0.2% BSA/PBS, separated by 3 x PBS washes. Stained coverslips were washed 3 x with PBS and 2 x with ddH₂O before mounting on microscope slides using ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific). Immunofluorescence images were acquired using a Axio Z1 Observer microscope

(Zeiss) and processed using AxioVision software and Fiji Image J software (Schindelin et al., 2012).

2.5.9 Immunoprecipitation of GFP-NRTKs

HeLa cells were seeded in 6 well plates at 1.5×10^5 cells per well and transfected 24 h later using 2.5 μ g plasmid DNA and 5 μ l Lipofectamine 2000 (Thermo Fisher Scientific) per well. 2 wells were transfected per pCB6-GFP-NRTK construct. 14-16 h post transfection cells were washed 2 x with PBS and lysed on ice using kinase IP buffer (table 2.4) followed by pulse-sonication. Lysates were clarified by centrifugation at 20000g for 10 min, pre-cleared for 15 min with Dynabeads™ Protein G (Thermo Fisher Scientific). 30 μ l of Dynabeads per condition were prepared by washing once with PBS, followed by incubation with rabbit anti-GFP (9F9.F9) (Abcam) for 30 min. Antibody-bound beads were washed with PBST (0.02% Tween-20), before addition to pre-clared lysates for 1 h for immunoprecipitation. Beads were sequentially washed with (i) PBS/0.5% Triton X-100, (ii) PBS/0.05% Tween-20, (iii) 150 mM NaCl/20 mM Tris pH 8 and (iv) kinase IP buffer, before ADPr by recombinant MBP-EspH_{EHEC} (see earlier section)

2.6 *C. rodentium* infection of mice

2.6.1 Infection and colonisation

CR was cultured in 2 x 15 ml of LB-broth overnight from two individual -80 °C microbank beads. Cultures were combined, and bacteria harvested at 4500g for 15 min. Bacterial pellets were resuspended in 3 ml PBS and 200 μ l of this or PBS (uninfected) used to inoculate mice by oral gavage. The inoculum was determined by plating serial dilutions on LB-agar and counting colony forming units (cfu).

Colonisation was similarly monitored on days 3, 6, 7 and 8 by calculating cfu/gram of stool after serial dilutions. Harvested stools were weighed and 10 ml PBS added per gram of stool,

incubated at RT for 1 h and then homogenised. Supernatants after a quick pulse centrifugation were used for serial dilutions in PBS.

2.6.2 Histology and immunofluorescence

0.5 cm of distal colon was washed with PBS, before fixing with 1 ml of buffered formalin, paraffin embedding and sectioning at 5 μ m. Colon sections were stained with haematoxylin and eosin (H and E) (Lorraine Lawrence, ICL). Alternatively, sections were prepared for immunofluorescence. Slides were de-waxed using 2 x 10 min washes in HistoClear (National Diagnostics) before step-wise rehydration in 2 x 100% ethanol, 2 x 95% ethanol, 1 x 80% ethanol, 2 x PBS + 0.1% Tween-20 + 0.1% saponin (PBSTS). Epitope retrieval was achieved by steaming in sodium citrate for 20 min and finally washing again in PBSTS. Slides were blocked using 10% normal donkey serum (NDS)/PBSTS for 20 min, before sequential incubation with primary/secondary antibodies in NDS/PBSTS for 1 h, separated by 2 NDS/PBSTS washes. Antibodies used are in Table 2.5. Immunofluorescence images were acquired using a Axio Z1 Observer microscope (Zeiss) and processed using AxioVision software and Fiji Image J software (Schindelin et al., 2012).

2.7 Label free LC-MS/MS

2.7.1 Enrichment and preparation of ADP-ribosylated proteins

All centrifugation steps for pulldowns were performed for 1 min at 1500g. After pulldowns and washing, Neutravidin resin and sepharose-GST-Af1521 resin were processed in the same way.

III. Neutravidin pulldowns of proteins post-click chemistry

Resuspended proteins were diluted with PBS to 0.2% SDS/PBS and incubated with 30 μ l of pre-equilibrated Neutravidin resin (Thermo Fisher Scientific) per sample. After a 1 h RT pulldown with end over end rotation, resin was washed 3x 1% SDS/PBS, 2 x 4M urea/PBS, 3x

50 mM ammonium bicarbonate/H₂O pH 8 to remove non-specific binders. 5% input, flow through and pulldown samples were taken for analysis by western blotting/in gel fluorescence.

IV. Af1521 pulldowns

Infection cell lysates in 10 x RIPA buffer were diluted 10-fold in 100 mM HEPES, 150 mM NaCl. GST-Af1521 (WT/G42E) crosslinked sepharose resin was equilibrated by washing 3 x in 1 x RIPA. Each infection condition was split in two. Half was added to Af1521_{WT} and half to Af1521G42E for 2 h at 4 °C with end over end rotation. Resin was washed 3 x with 1 x RIPA, 3 x with dilution buffer and 1 x with 50 mM ammonium bicarbonate/H₂O pH 8.

V. Trypsinisation and desalting

0.4 µg of sequencing grade modified trypsin (Promega) was added to resin in 50 µl of 50 mM ammonium bicarbonate pH 8. Proteins were digested overnight at 37°C with vortex mixing. The next morning digested peptides supernatants were combined with supernatants from subsequent washes using 80 µl of 50 mM ammonium bicarbonate and 80 µl of 0.1% trifluoroacetic acid (TFA). Combined peptide eluates were desalted using home-made stop and go extraction (stage) tips consisting of 3 layers of SDB-XC (Empore) solid phase extraction medium. All centrifugation steps were performed at 1500g. Stage tips were activated with methanol and pre-equilibrated with 0.1% TFA/H₂O. Peptides were loaded onto stage tips, desalted with 0.1% TFA/H₂O and eluted with 79% acetonitrile (ACN)/H₂O. Eluted peptides were speed-vacuum dried and then stored at -80°C. Before analysis samples were resuspended in 15 µl of 0.5% TFA 2% ACN and transferred to LC-MS vials.

2.7.2 Mass spectrometry

Mass spectrometry was performed by Kate Hadavizadeh (Tate lab, ICL). Analysis used an EASY-Spray LC Column of 50 cm by 75 µm inner diameter (Thermo Fischer Scientific), with a 2h acetonitrile gradient in 0.1% aqueous formic acid (FA), at a flow rate of 250 nl/min. Easy

nLC-1000 was coupled to a Q Exactive mass spectrometer via an easy-spray source (Thermo Fisher Scientific). The Q Exactive was operated in a data-dependent acquisition mode with survey scans acquired at a resolution of 75,000 at m/z 200 (transient time 256 ms). Up to ten of the most abundant isotope patterns with charge +2 or higher from the survey scan were selected with an isolation window of 3.0 m/z and fragmented by higher-energy collisional dissociation with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17,500 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10^6 and for MS/MS to 10^5 , and the intensity threshold was set to 8.3×10^2 .

2.7.3 Data processing

Data were processed using MaxQuant (Cox and Mann, 2008) (version 1.5.7.4) and peptides identified by matching MS/MS spectra against the human proteome (Uniprot) using Andromeda search engine (Cox et al., 2011). No fixed modifications were set. Methionine oxidation and N-terminal acetylation were set as variable modifications. Reference proteomes were digested *in silico* using the trypsin/P setting with up to three missed cleavages. Standard settings for label free quantification were selected, the false discovery rate set to 0.01, and peptides matched between runs. Other parameters were used as pre-set in MaxQuant. Datasets can be found as PRIDE project PXD008533.

Protein groups were analysed using Perseus (version 1.5.6.0). Potential contaminants, 'reverse', 'identified by site', and proteins with only one unique peptide were removed and data logarithmised ($\log_2$). Replicates were grouped and filtered for proteins with at least three valid values across three (Caco2/THP1) or four (A549/HeLa) replicates in one group. Missing values were imputed using a downshifted normal distribution (1.8 downshift, 0.3 width) for each sample, to allow statistical analysis by a two-sided T-test within the volcano plot function

(permutation-based FDR-corrected p-value of 0.01, s0 value of 1). Profile plots were also produced within Perseus software.

2.8 IEC proteome mass spectrometry

2.8.1 Digest and TMT labelling

Performed by Lu Yu (Choudhary lab, ICR). Enterocytes were dissolved in 100 μ l of 0.1 M triethylammonium bicarbonate (TEAB), 0.1% SDS and lysed by pulse probe sonication. 80 μ g proteins/sample were SpeedVac dried then resuspended in 100 μ l of 4%SDS/100mM TEAB/15mM TCEP, assisted with ultrasonic bath. After reduction at 56°C for 20 min, samples were cooled to 25°C and alkylated with IAA for 30 min. Proteins were purified by 20% TCA precipitation followed by one wash with ice-cold acetone before resuspending in 100 mM TEAB and digesting with 3 μ g trypsin (Pierce MS grade) at 37°C for 18 h. 40 μ g protein digest were taken for labelling with 0.4 mg of TMT10plex as instructed by the manufacturer (Thermo Fisher).

2.8.2 Mass spectrometry

Performed by Lu Yu (Choudhary lab, ICR).

VI. *Basic reverse-phase peptide fractionation*

Ten samples were mixed and SpeedVac dried, before fractionation on a U3000 HPLC system (Thermo Fisher) with an XBridge BEH C18 column (2.1 mm id x 15 cm, 130 Å, 3.5 μ m, Waters) at pH 10, flow rate 200 μ l/min in 30 min linear gradient from 5 - 35% acetonitrile /NH₄OH. Fractions were collected every 30 sec into a 96-wellplate then concatenated (equally by retention time interval to 24 fractions) and SpeedVac dried.

VII. *LC-ESI-MS/MS analysis*

Peptides were resuspended in 0.5% FA and 1/3 were injected on the Orbitrap Fusion Tribrid mass spectrometer coupled to U3000 RSLCnano UHPLC system (Thermo Fisher). Peptides

were first loaded to a PepMap C18 trap (100 μ m i.d. x 20 mm, 100 Å, 5 μ m) for 10 min at 10 μ l/min with 0.1% FA/H₂O, then separated on a PepMap C18 column (75 μ m i.d. x 500 mm, 100 Å, 2 μ m) at 300 nl/min and a linear gradient of 4-33.6% ACN/0.1% FA in 120 min /cycle at 150 min per fraction. The data acquisition used the SPS10-MS3 method with Top Speed at 3s per cycle time. The full MS scans (m/z 380-1500) were acquired at 120,000 resolution at m/z 200 with a lock mass at 445.12003, and the AGC was set at 4e5 with 50 ms maximum injection time. The most abundant multiply-charge ions ($z = 2-6$, above 5000 counts) were subjected to MS/MS fragmentation by CID (35% CE) and detected in ion trap for peptide identification. The isolation window by quadrupole was set m/z 1.0, and AGC at 1e4 with 35 ms maximum injection time. The dynamic exclusion window was set ± 7 ppm with a duration at 60 sec, and only single charge status per precursor was fragmented. Following each MS2, the 10-notch MS3 was performed on the top 10 most abundant fragments isolated by Synchronous Precursor Selection (SPS). The precursors were fragmented by HCD at 60% CE then detected in Orbitrap at m/z 110-400 with 50K resolution for peptide quantification data. The AGC was set 1e5 with maximum injection time at 86 ms.

VIII. Database searching and quantification

The LC-MS/MS data were processed in Proteome Discoverer 2.1 (Thermo Fisher Scientific) using the SequestHT search engine to search against a combined protein database comprised of the mouse protein database (17,555 entries, Swiss-prot, December 2015), a *Citrobacter rodentium* (5020 entries, Uniprot, December 2015) and the in-house common contaminate database. The precursor mass tolerance was set at 30 ppm and the fragment ion mass tolerance was set at 0.5 Da. Spectra were searched for fully tryptic peptides with maximum 2 miss-cleavages. Carbamidomethyl (C) and TMT6plex (Peptide N-terminus and K) were set as static modifications, and Deamidation (N, Q) and Oxidation (M) were set as dynamic modifications. Peptides were validated by Percolator using the Decoy database search and only peptides at

high confidence were used in the protein identification. The search result was further filtered where the protein FDR was set at 0.01 (strict) and 0.05 (relaxed). The TMT10plex reporter ion quantifier used 30 ppm integration tolerance on the most confident centroid peak at the MS3 level. Reporter abundance used S/N and the co-isolation threshold is set 75%. Both unique and razor peptides were used for quantification. Only master proteins at high or medium FDR were exported for further filtering/processing.

2.8.3 Bioinformatics

Perseus (version 1.5.6.0) was used to process protein groups. Absolute intensities were logarithmised (log2) and those with only one unique peptide were removed. Ratios between conditions were created and proteins with a fold change of more than 1.5 further analysed. Venn diagrams were created in Venny 2.1 and heat maps in Perseus.

GO terms were first analysed using ClueGO, Cytoscape (version 3.6.0) (Bindea et al., 2009; Shannon et al., 2003). Min/max GO levels were set to 3 and 7, respectively. GO terms with a p value < 0.01 were selected with a minimum of 5 proteins and 5% of the GO term proteins. GO term grouping and fusion were allowed and the most significant GO term used for visual representation (Bonferroni corrected p-value). KEGG pathway mapper and Ingenuity Pathway Analysis (IPA, Qiagen) were also used for further analysis of canonical pathways, and upstream analysis.

The kinase prediction function (KEA) of Expression 2 Kinase (X2K) (Chen et al., 2012) was used in isolation to predict upstream regulatory kinases from the group of 457 proteins upregulated in the Δ ART vs WT samples. This was compared to the kinase enrichment analysis of ten randomly generated sets of 457 proteins from the total 7400 proteins identified.

**Chapter 3 – The EspJ homologues from *E. coli*,
Salmonella and *Yersinia***

3 Results – The EspJ homologues from *E. coli*, *Salmonella* and *E. coli*

3.1 Bioinformatics analysis of the EspJ homologues

The NCBI basic local alignment search tool (BLAST) revealed that aside from in EPEC, EHEC and CR, EspJ has homologues predicted in *Salmonella bongori*, *Salmonella enterica* subsp. *salamae* (*S. salamae*), *S. enterica* subsp. *arizonae* (*S. arizonae*), and *Yersinia enterocolitica*. Accordingly, homologues were not identified in *Yersinia pestis*, or any other *Salmonella enterica* subspecies (*diarizonae*, *houtenae*, *indica*, *enterica*). We named these homologues *S. salamae* and *S. arizonae* *seoC*, *S. bongori* *sboC*, and *Y. enterocolitica* *yspJ*. Sequence alignment using Multalin server (Corpet, 1988) showed that the predicted EspJ homologues have sequence identity between 56% and 92%, with most homology shared beyond the first 50 residues (Figure 3.1A, Table 3.1). Phylogenetic analysis clustered the homologues into two clades (Figure 3.1B); one containing EspJ from the A/E pathogens EPEC, EHEC and CR which shared identity between 83% and 92%, and another clade containing the *seoC*, *sboC* and *yspJ* which shared between 74% and 83% identity (Table 3.1). Tertiary structure predictions were created using Phyre2 server (Kelley and Sternberg, 2009), before aligning predictions to the known structure of the diphtheria toxin (DT) (1TOX, PDB) using Dali pairwise comparison tool (Hasegawa and Holm, 2009). This revealed that EspJ R79 and D187 aligned structurally to the NAD-binding and catalytic DT residues H21 and E148 respectively, and this is confirmed by the sequence alignment (Figure 3.1A/C).

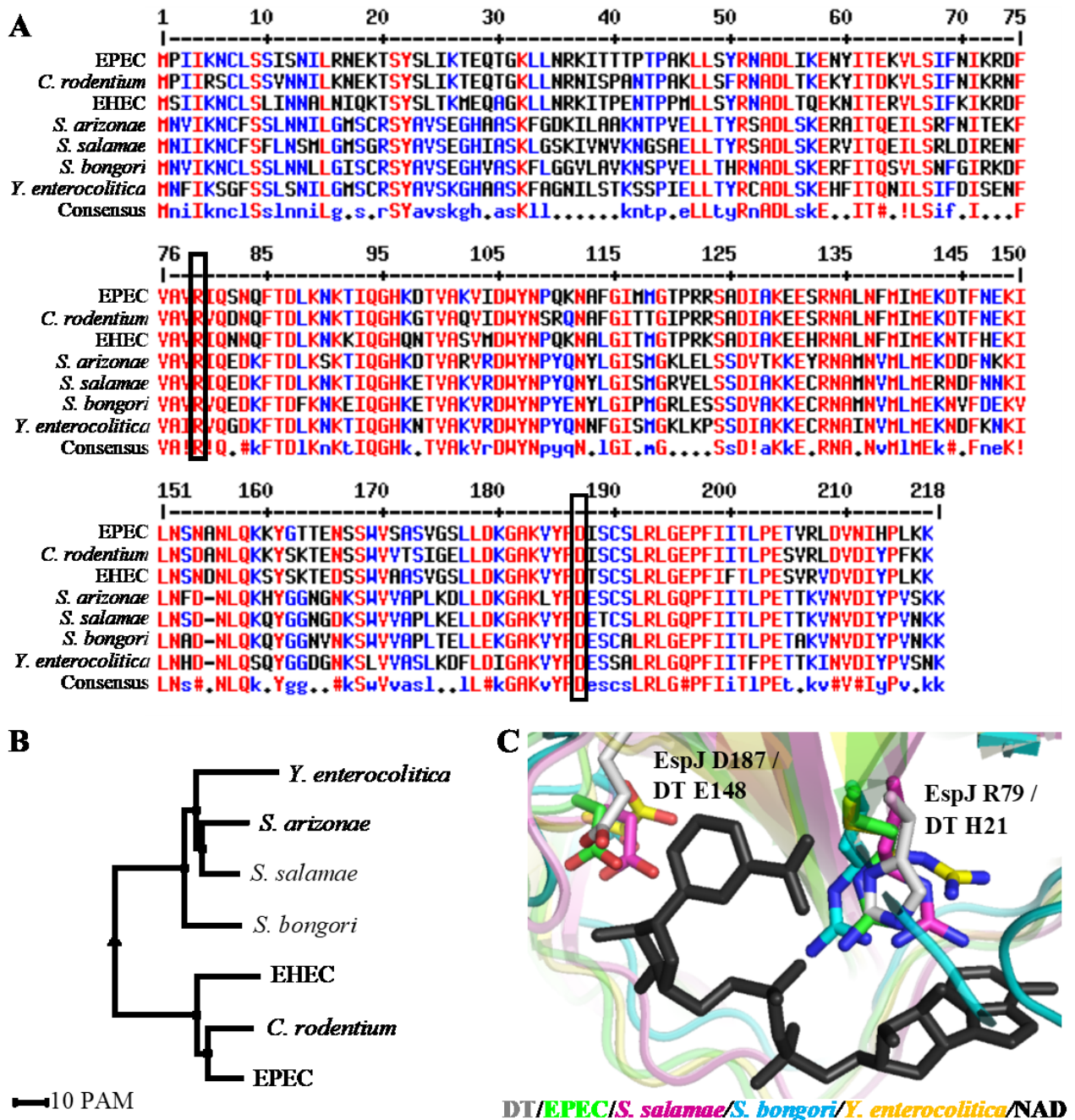


Figure 3.1 - Bioinformatic analysis of EspJ homologues

(A) Sequence alignment of the EspJ homologues. Conserved residues R79 and D187 crucial for EPEC EspJ activity are highlighted with a black box. Red - high consensus, blue - low consensus, black - no consensus, ! - I/V, # - N/D/Q/E/B/Z. (B) Phylogenetic analysis of the EspJ homologues showing separation of EPEC, EHEC and *C. rodentium* homologues from the *Salmonella* homologues. A scale for Point Accepted Mutations (PAM) is displayed. (C) Structural alignment of key catalytic residues H21 and E148 from Diphtheria Toxin (DT, PBD 1TOX grey) compared to EspJ R79 and D187 from EPEC (green), *S. salamae* (magenta), *S. bongori* (cyan), *Y. enterocolitica* (yellow). NAD backbone is displayed in black. EspJ predicted structures were generated using Phyre2 and aligned using Dali alignment software. (A) and (B) adapted from Pollard *et al*, 2016. Sequence alignment and phylogenetic tree were generated using Multalin version 5.4.1.

Table 3.1: Percentage sequence identity within the EspJ/SeoC/SboC/YspJ homologues

Homologue (full length)	EPEC	EHEC	<i>C. rodentium</i>	<i>S. salamae</i>	<i>S. arizonae</i>	<i>S. bongori</i>
EHEC	83					
<i>C. rodentium</i>	83	92				
<i>S. salamae</i>	58	57	56			
<i>S. arizonae</i>	57	56	56	83		
<i>S. bongori</i>	57	57	56	78	77	
<i>Y. enterocolitica</i>	55	55	55	76	74	77
N-terminal 50 residues	EPEC	EHEC	<i>C. rodentium</i>	<i>S. salamae</i>	<i>S. arizonae</i>	<i>S. bongori</i>
EHEC	69					
<i>C. rodentium</i>	65	80				
<i>S. salamae</i>	35	37	35			
<i>S. arizonae</i>	31	35	31	71		
<i>S. bongori</i>	35	41	35	80	63	
<i>Y. enterocolitica</i>	36	30	32	74	58	66

3.2 EspJ homologue small scale expression tests

To test their *in vitro* characteristics a favourable protein expression and purification system for each EspJ homologue was needed. The EspJ homologues were cloned into pETM11 and pMALXE vectors for the IPTG-inducible bacterial expression of N-terminally (his) tagged or maltose binding protein (MBP) tagged proteins respectively. The pMALX(E) vector encodes non-cleavable MBP with reduced surface entropy and a rigid linker to the protein of interest, originally designed for improving protein crystallisation (Moon et al., 2010). Small scale (5 ml) expression and solubility tests showed that while each homologue was well expressed from the pETM11 vector in BL21 STAR *E. coli*, not all the homologues were fully soluble based upon anti-his western blotting of the lysate supernatant and pellet fractions. In fact, *S. bongori* SboC showed no sign of solubility, *S. salamae* SeoC was less than 50% soluble and around 50% solubility was displayed by *S. arizonae* SeoC and *Y. enterocolitica* YspJ. In contrast, homologues were all well expressed and appeared to be 100% soluble after expression from

the pMALXE vector (Figure 3.2). For this reason, it was decided to use the pMALXE vector for future protein production of EspJ homologues.

3.3 Large scale expression and purification of the EspJ homologues

Each EspJ homologue was expressed from 1 L of bacterial expression culture and purified in parallel to compare the suitability of proteins for future functional studies. SDS-PAGE showed

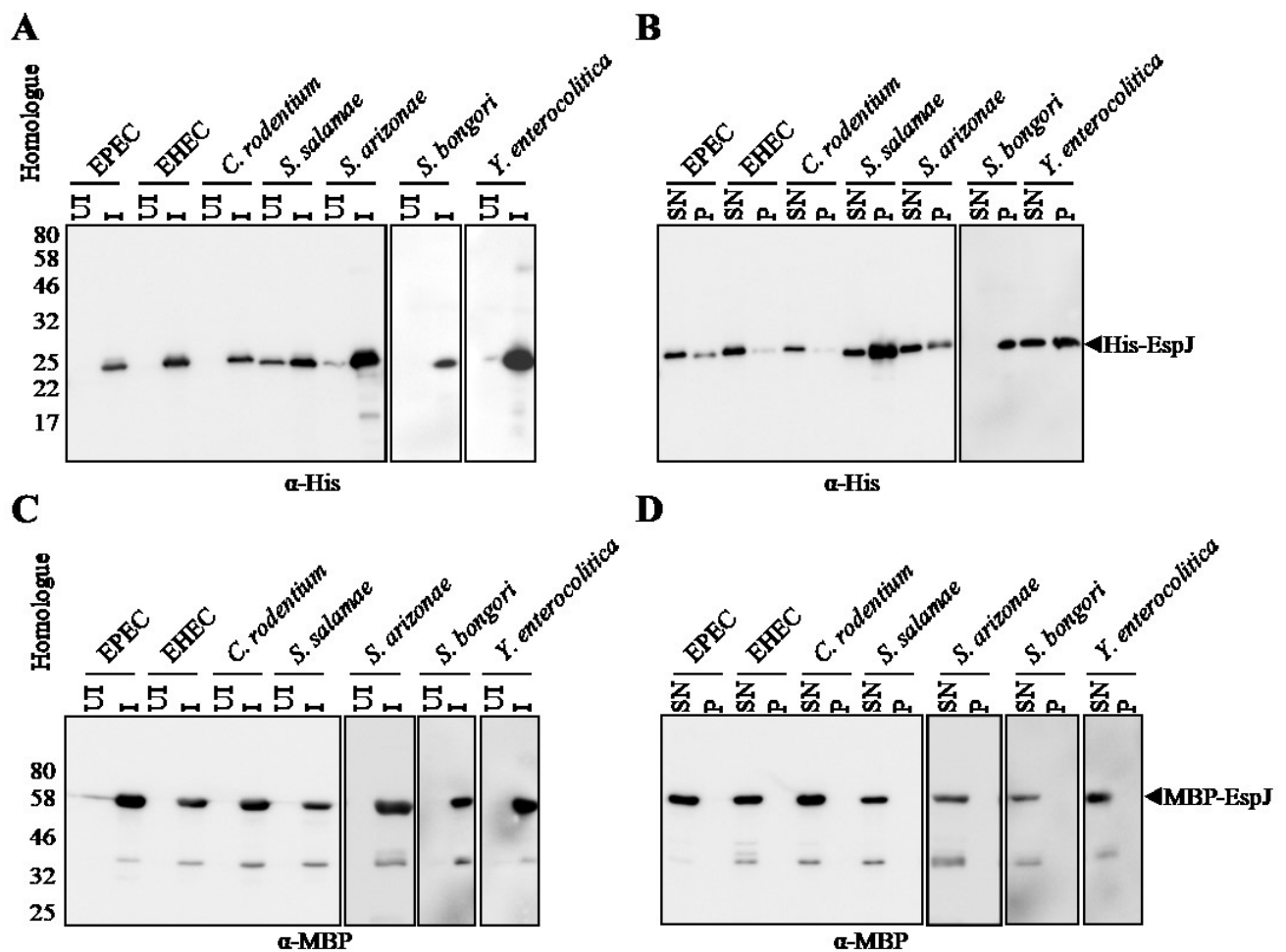


Figure 3.2- Small scale expression and solubility tests for the EspJ homologues

BL21 STAR *E. coli* were used to express fusion proteins from pETM11 or pMALX(E) vectors to produce hexa-histidine (his) or maltose binding protein (MBP) N-terminally tagged proteins respectively. Samples were taken before (UI) and after an overnight induction at 18 °C (I). After lysis the soluble supernatant (SN) and insoluble pellet (P) samples were also taken. Western blotting revealed protein expression and solubility. (A) and (C) show protein expression from pETM11 or pMALX(E) by comparing induced and uninduced samples showing decent expression levels for each homologue in both vectors. (B) and (D) show supernatant and pellet samples displaying a varying solubility of his tagged homologues (B) but consistently soluble MBP-tagged EspJ homologues.

that each protein was expressed at this large scale, but to varying degrees, with the highest level of expression from the EPEC, CR, *S. salamae* and *S. arizonae*. Accordingly, SDS-PAGE analysis of homologues affinity purified using amylose resin showed that SboC and YspJ yielded lower amounts of protein (Figure 3.3). Proteins were then further purified and analysed by gel filtration chromatography, which separates proteins by their globular size. SDS-PAGE and absorbance at 280nm shows varied profiles for the elution of each homologue, with four major peaks. Known protein standards were analysed by gel filtration to enable back-calculation of the theoretical molecular weights of these EspJ protein species (Table 3.2). The retention volume of 8 ml displays soluble but aggregated proteins that are too large to be separated by the column. Two further peaks at around 12 ml and 14 ml contain MBP-tagged EspJ homologues with calculated molecular weights of about 180 kDa and 70 kDa, respectively. As the MBP-EspJ fusion protein is about 65 kDa these peaks most likely represent homodimers/homotrimers and monomers respectively. Finally, a peak at 15-16 ml represents MBP which has dissociated from the fusion protein (Figure 3.4, Table 3.2). This gel filtration clearly indicates that EspJ of EPEC and CR are the most stable homologues under these conditions.

As there were three different gel filtration peaks containing EspJ_{EPEC} it was important to test the enzymatic activities of each EspJ isoform. After incubating each EspJ species with GST-Src and NAD-biotin, western blotting revealed that while the apparent multimeric and monomeric MBP-EspJ_{EPEC} isoforms were able to ADP-ribosylate Src, the soluble aggregates eluted at 8 ml were inactive (Figure 3.5).

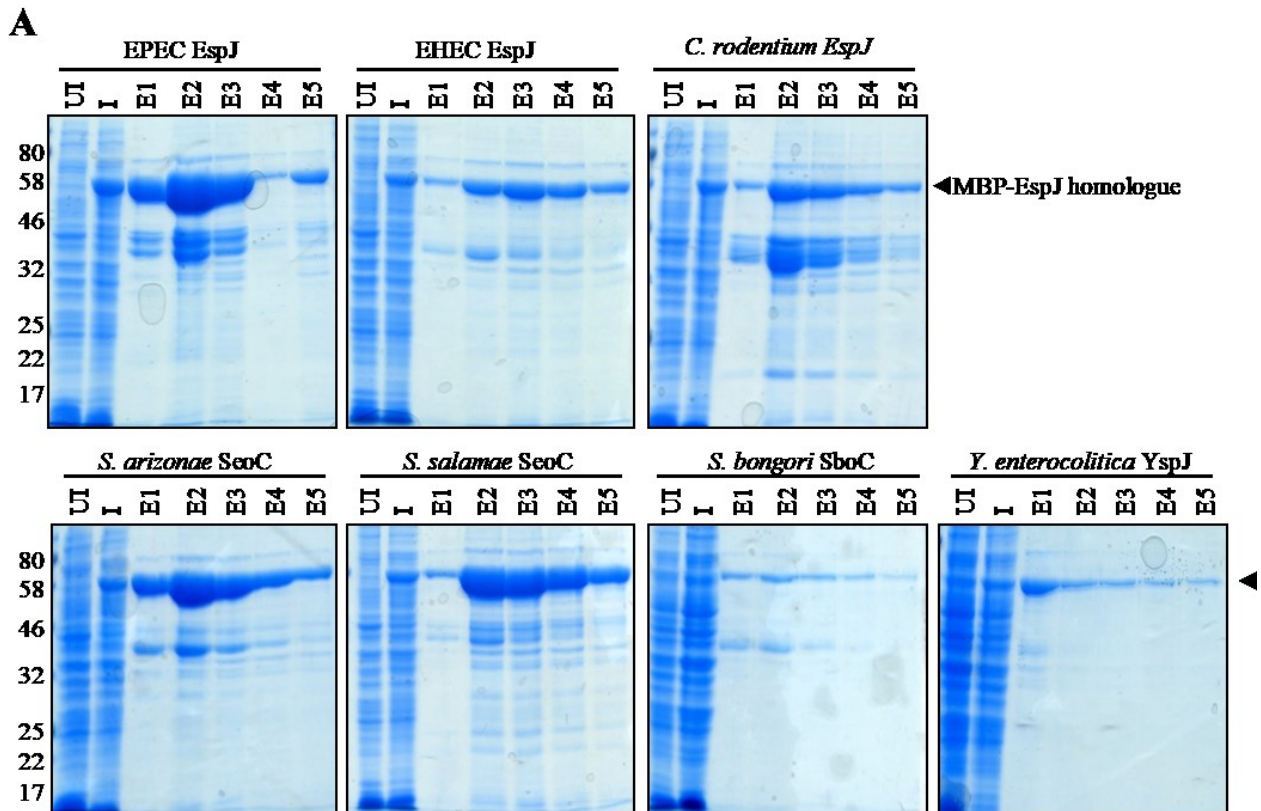


Figure 3.3 - Large scale purification of the EspJ homologues from 1 L of bacterial expression culture

Representative SDS-PAGE gels for the affinity chromatography purification of MBP tagged EspJ homologues by gravity flow using amylose resin. UI = uninduced, I = induced, E = amylose affinity resin elution. arrow refers to the protein band for MBP-EspJ/SeoC/SboC/YspJ. Numbers on the left show protein standard markers (kDa).

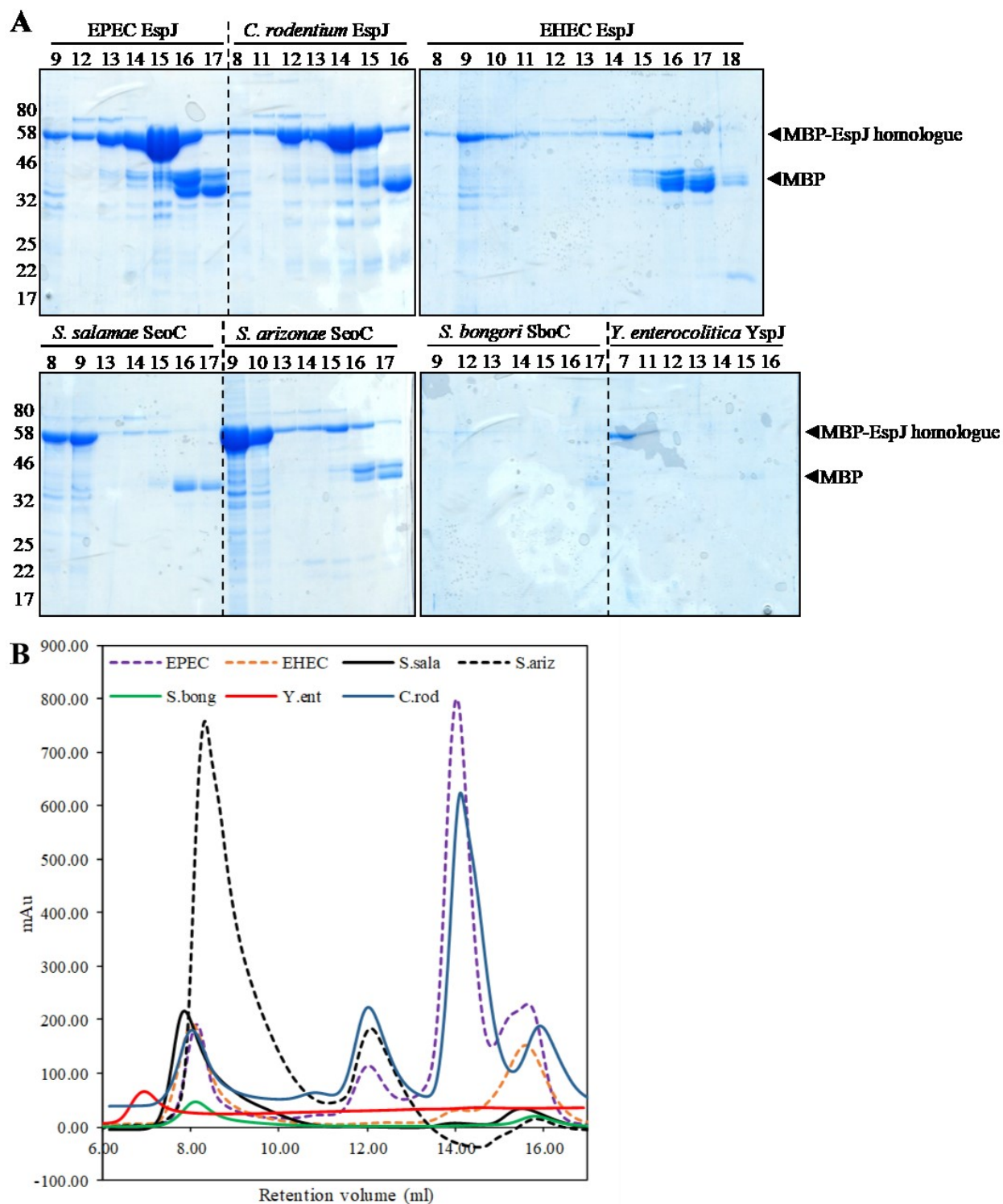


Figure 3.4 - Gel filtration purification of MBP-EspJ homologues

(A) Representative SDS-PAGE gels and (B) absorbance traces of elution from size exclusion chromatography of MBP-EspJ homologues using an S200 10/300 Superdex column. Peaks at a retention volume of 8-10 ml represent soluble aggregates of EspJ, peaks at 12 ml are predicted to be EspJ homodimers, 14 ml monomers and 16 ml free MBP. mAu—milli absorbance units at 280 nm. Dashed purple—EPEC EspJ, dashed orange- EHEC EspJ, solid black—*S. salamae* SeoC, dashed black—*S. arizonae* SeoC, solid green—*S. bongori* SboC, solid blue—*C. rodentium* EspJ.

Table 3.2 - Gel filtration standards and calculated molecular weights for EspJ homologue purifications

Standards				
Protein standard	Mw (KDa)	Elution volume	Elution/ void volume	Log Mw
Blue dextran	2000	7.72	N/A	N/A
Amylase	200	11.65	1.509067	5.30103
Alcohol dehydrogenase	150	12.64	1.637306	5.176091
Albumin	66	14.09	1.82513	4.819544
Carbonic Anhydrase	29	16.32	2.11399	4.462398
Cytochrome C	12.4	17.75	2.299223	4.093422
EspJ homologues				
Homologue	Theoretical Mw	Elution volume	Calculated MW (KDa)	Protein form
EPEC	65.4	8.14	1050.4	Aggregate
		12.01	181.5	Multimer
		14.03	72.6	Monomer
		15.66	34.6	MBP
EHEC	65.6	8.04	1099.1	Aggregate
		14.02	71.1	Monomer
		15.6	35.6	MBP
<i>C. rodentium</i>	65.4	8.02	1109.1	Aggregate
		12.02	180.7	Multimer
		14.11	70.0	Monomer
		15.92	30.8	MBP

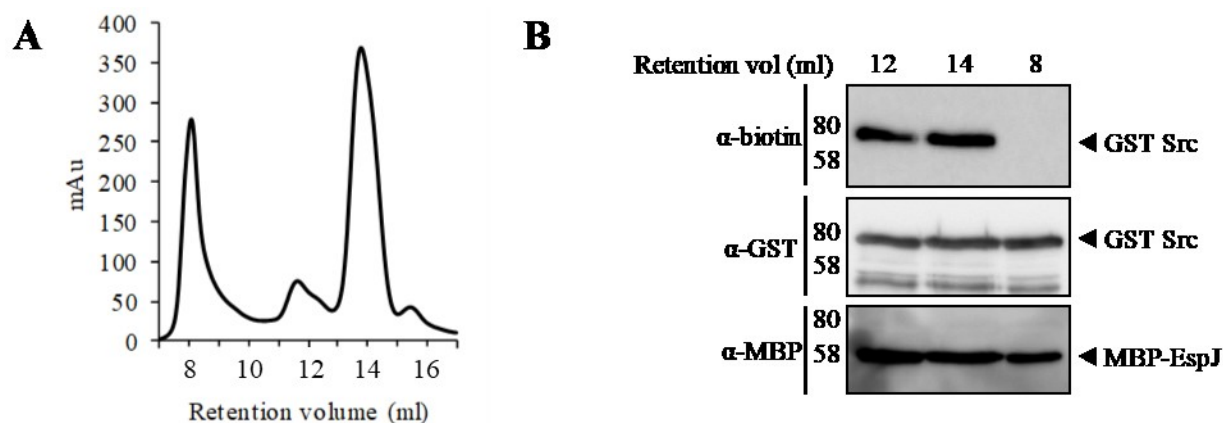


Figure 3.5 – ADP-ribosyltransferase of different MBP-EspJ proteoforms

(A) Shows a representative gel filtration trace for the purification of EPEC MBP-EspJ (mAU = milli absorbance units at 280 nm). (B)

Western blotting and anti-biotin reagents shows that MBP-EspJ eluted at 12 ml and 14 ml, but not 8 ml, can ADP-ribosylate of

GST-Src using NAD-biotin.

3.4 Optimising the purification of the EspJ homologues

As the purification of several EspJ homologues did not yield soluble monomeric or dimeric protein it was necessary to further optimise the purification conditions (Figure 3.4). In isolation the isoelectric points (pI) for (pMALXE)-MBP and EspJ are 5.26 and 9.67. Under the current purification conditions at pH 7.4 the net charge for MBP will be negative and EspJ positive. It was proposed that this may result in aggregation of the fusion protein as observed by gel filtration and that raising the pH may prevent this aggregation. For this optimisation, EPEC and EHEC were selected as representatives for well-behaved and poorly-behaved EspJ homologues respectively. Affinity chromatography from a 250 ml bacterial expression cultures lysed in pH 7.4 or pH 10 clearly demonstrates the increased yield both for EspJ_{EPEC} and EspJ_{EHEC} reflecting either an increased solubility or an improved interaction with the amylose resin (Figure 3.6). Consistently, the comparison of MBP-EspJ_{EHEC} gel filtration traces from a 1 L purification at pH 7.4 to a 250 ml preparation at pH 10 shows a dramatic increase in the yield of monomeric MBP-EspJ_{EHEC}, and direct comparisons of 1 L purifications at pH 10 show that MBP-EspJ_{EHEC} is much more comparable to MBP-EspJ_{EPEC} than at pH 7.4 (Figure 3.6). Crucially at pH 10 MBP-EspJ_{EPEC} was still able to ADP-ribosylate Src using NAD-biotin, indicating that this form is active and suitable for further functional studies (Figure 3.6C).

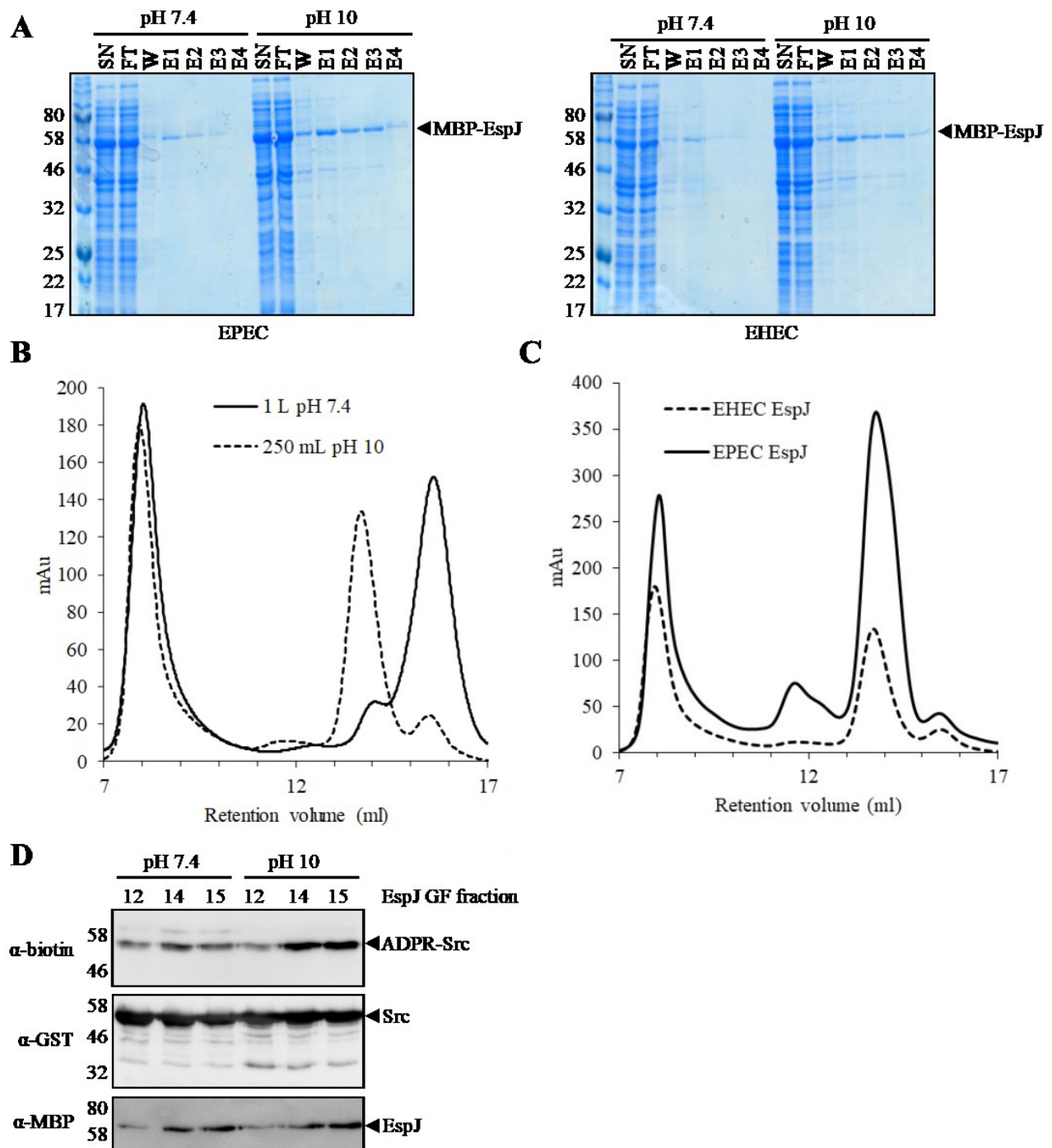


Figure 3.6: The effect of pH on the purifications of EPEC/EHEC EspJ and activity

(A) SDS-PAGE representing the amylose affinity purification of MBP-EspJ_{EPEC/EHEC} at pH 7.4/10 from 250 mL bacterial expression culture. L= ladder, SN = supernatant, FT= resin flow through, W = wash, E = elution fractions. (B) Gel filtration using a Superdex S200 10/300 comparing the purification of MBP-EspJ_{EHEC} from 1 L bacterial expression at pH 7.4 and 250 mL expression culture at pH 10. (C) Gel filtration trace comparing the purification of MBP-EspJ_{EPEC/EHEC} from 1 L of bacterial expression at pH 10. (D) Comparison of MBP-EspJ_{EPEC} ADP-ribosylation activity at pH 7.4/10. Dimeric (gel filtration fraction 12) or monomeric (fractions 14 or 15) MBP-EspJ_{EPEC} were incubated with GST-Src_{250-533K295M/Y416A} and NAD-biotin before detecting ADP-ribosylation using western blotting and streptavidin-HRP.

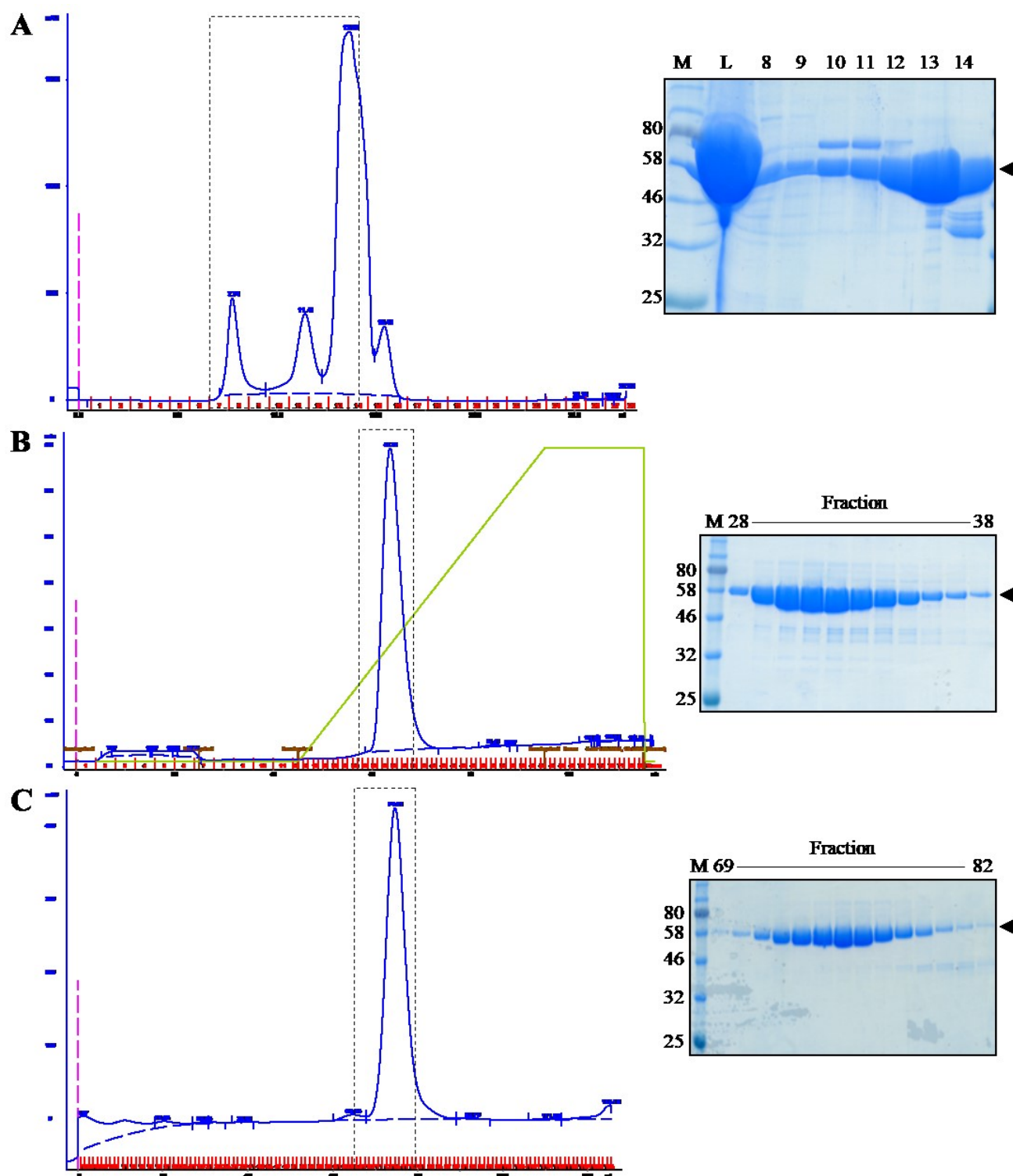


Figure 3.7- EPEC EspJ purification at pH 10

MBP-EspJ_{EPEC} was purified from 4 L of expression. (A) First gel filtration trace using S200 10/300 after amylose purification with SDS-PAGE (right) of the load sample, and fractions 8-14 (black dashed box on trace). (B) Anion Exchange chromatography trace with a NaCl gradient from 50 mM to 1 M NaCl (green line). Representative SDS-PAGE for boxed fractions is shown on the right. (C) Second gel filtration using S200 16/600 with SDS-PAGE for boxes fractions shown on the right.

With a view to performing structural studies of EspJ the stability of its oligomeric isoforms, monomeric EspJ_{EPEC} was further purified on a large scale (Figure 3.7). After amylose affinity purification, the fusion protein produced the expected gel filtration trace with some large aggregates, a homodimeric/homotrimeric species, some free MBP and the majority of the protein appeared monomeric (Figure 3.7A). The apparent monomeric peak (fractions 13 and 14) was taken forwards for further purification by anion exchange chromatography, using an elution gradient from 50 mM to 1000 mM NaCl (Figure 3.7B). Post anion exchange, a second gel filtration step on a larger column removed further impurities and revealed that the selected monomer peak had retained its homogeneous form as it eluted in one symmetrical peak (Figure 3.7C). This confirmed that after several rounds of protein concentration and exposure to different buffers, EspJ_{EPEC} was stable as a monomer. Purified protein from this and other similar preparations were subjected to crystallographic screening with multiple sparse matrix screens, at varying concentrations of protein and in the presence and absence of NAD. Unfortunately, there were no evident promising protein crystallisation conditions using this protein construct.

3.5 Attempts to isolate the EspJ-Src protein complex

It was proposed that the EspJ-Src protein complex may be favourable to protein crystallisation. In previous work the only evidence for an EspJ Src interaction was inferred via the ADPr of Src by EspJ. However, neither the yeast 2 hybrid nor co-immunoprecipitation was able to detect a stable complex (PhD thesis (Young, 2013)). Due to the enzymatic nature of the relationship between EspJ and Src it is likely that the interaction is only transient, and thus a method of trapping the complex may be necessary before the reaction is complete and Src released. Amylose pull down experiments using recombinantly purified EspJ homologues and Src constructs were performed to investigate this.

MBP-EspJ homologues from expression lysates were bound to amylose resin before incubating with Src-²⁵⁰⁻⁵³³K295M/Y416A (after cleavage of GST) under various conditions. Src was found in the resin and flow through fractions after incubation with MBP EspJ (Figure 3.8A), though the presence of EspJ also in the flow through means this cannot be classified as unbound Src. A significant amount of Src was detected in the bound fraction for all the MBP homologues compared to the MBP control, but none indicated obvious stronger binding (Figure 3.8A). Three bands were detected using an anti Src antibody, whereas only one band was seen before incubation with EspJ (load control). It was hypothesised that the three bands may represent reaction intermediates during Src modification. If true, then pull downs using the catalytic mutant EPEC EspJ R79A or an excess of NAD should change the band intensities as they should inhibit or promote the reaction respectively. However, no obvious change in the proportions of these Src bands was observed in these conditions or with the addition of ATP/MgCl₂, common constituents of ADP ribosylation buffers (Figure 3.8B). Western blotting of pull downs performed with NAD biotin suggested that the middle Src band was the ADP-ribosylated isoform of Src. Furthermore, when purified recombinant MBP-EspJ-EPEC/EHEC were used instead of expression lysate, the upper and lower bands detected by the anti Src antibody disappeared suggesting that they may be a result of antibody cross reactivity with bacterial cell lysate proteins. Promisingly, a 1:1 ratio of Src:EspJ (WT or R79A) increased the pull down of Src when compared to an excess of EspJ:Src. Further optimisations were attempted, although neither pH changes, stoichiometric alterations nor pre-incubation of the complex prior to pulldown could improve the pulldown. Previous studies have used a non-hydrolysable analogue of NAD, β -TAD, to stabilise the complex between ARTs and their targets (Tsuge et al., 2008). Unfortunately, this was not effective in the case of EspJ and Src, and as Src was pulled down to a similar extent in the presence or absence of EspJ

it was deduced that under these conditions it was not possible to form a stable EspJ-Src complex (Figure 3.8C).

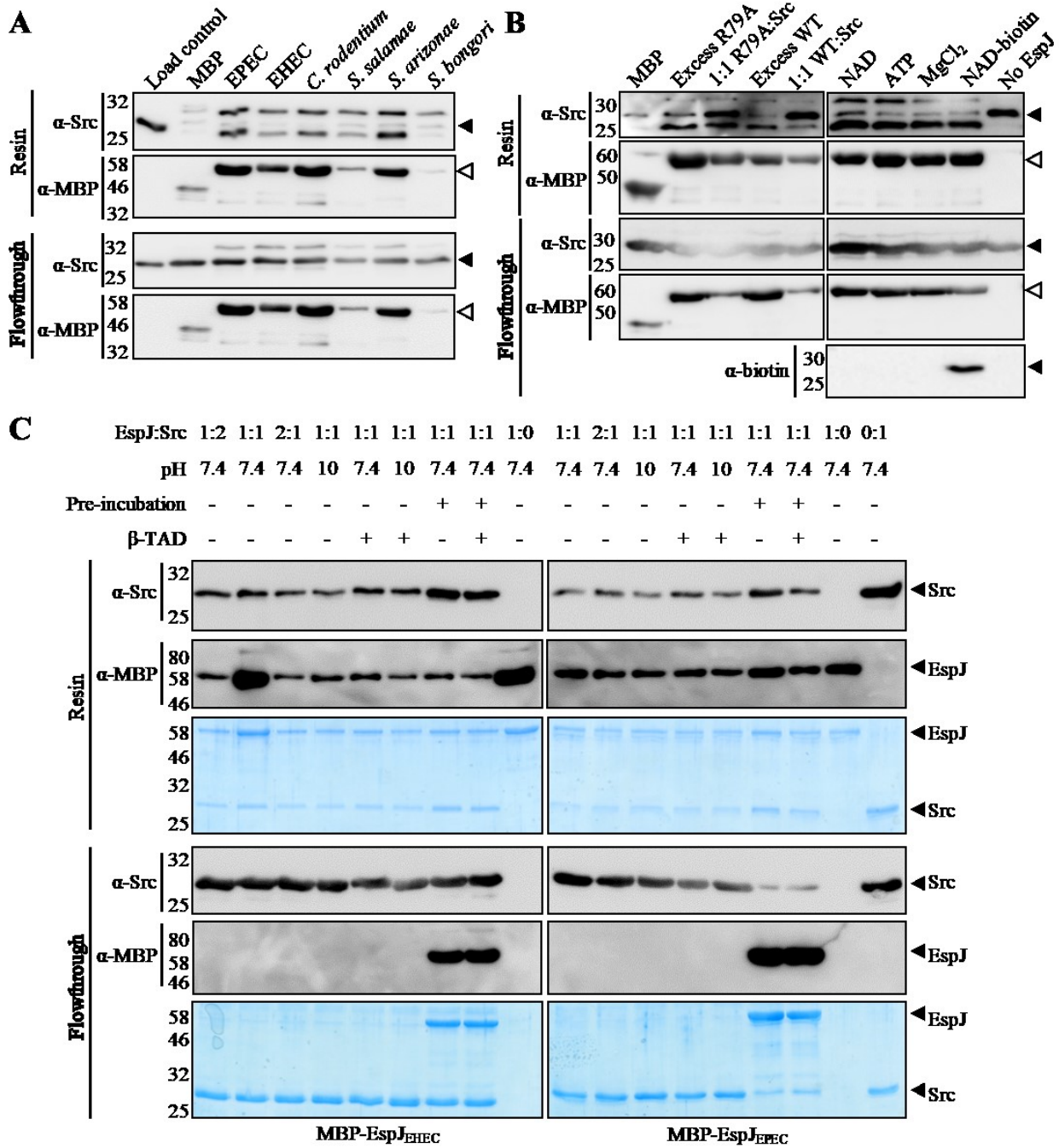


Figure 3.8- MBP pulldowns of Src

Either cell lysate (A and B) or purified recombinant (C) MBP-EspJ homologues were incubated with amylose resin prior to the addition of Src₂₅₀₋₅₃₃K295M/Y416A. After several washes, the flow through and resin fractions were analysed by SDS-PAGE and western blotting. (A) Comparison of the interactions between EspJ homologues (white arrows) from EPEC/EHEC/*C. rodentium*/*S. salamae*/*S. arizonae*/*S. bongori* and Src₂₅₀₋₅₃₃K295M/Y416A (black arrows). (B) Pulldowns using MBP-EspJ_{EPEC} /MBP-EspJ_{EPEC}-R79A with excess EspJ, 1:1 molar ratio of EspJ:Src, or addition of 10 μ M NAD/100 μ M ATP/5 mM MgCl₂ /10 μ M NAD-biotin. (C) Pulldowns using MBP-EspJ_{EHEC} (left panel) or MBP-EspJ_{EPEC} (right panel) at different stoichiometric ratios, pH 7.4/10, with/without the addition of 10 μ M β -TAD or pre-incubated with Src prior to binding to amylose resin. The absence of EspJ shows that Src still binds to the amylose resin.

3.6 Enzymatic activity of the EspJ homologues

Based upon the high level of sequence homology, predicted structural homology and the presence of conserved catalytic residues R79 and D187 in all seven EspJ homologues, the ART activity of each was assayed. Apparent monomeric MBP-EspJ fusion proteins were incubated with NAD-biotin and GST-Src₂₅₀₋₅₃₃K295M (catalytically inactive Src kinase domain). The western blot in figure 3.9 is representative of several repeats described in table 3.3, and highlights the varying activities of EspJ/SeoC/SboC/YspJ. All homologues, except YspJ, showed the ability to ADP-ribosylate Src across several repeats. EHEC EspJ seemed to be the most reliable enzyme, while EPEC and CR homologues displayed weaker and less reliable activities, taking reproducibility and the intensities of anti-biotin western blot bands into account. The same assay was performed with MBP-EspJ fusion proteins bound to amylose resin to minimise differences in protein stability. This confirmed the lack of YspJ activity and showed that all other homologues could ADP-ribosylate Src variants K295M and K295M/Y416A. His tagged EPEC EspJ has been shown to ADP-ribosylate the Src glutamic acid E310 (Young et al., 2014). Accordingly, the mutation of Src E310 prevented its ADPr by any EspJ homologue, indicating that they each modify the same residue (Figure 3.10).

Table 3.3 - ADP ribosylation of Src by purified recombinant EspJ/SeoC/SboC/YspJ

Ticks (P) = ADP-ribosylation, crosses (O) = no ADP-ribosylation.

	EspJ/SeoC/SboC/YspJ homologue						
Repeat	EPEC	EHEC	<i>C. rodentium</i>	<i>S. salamae</i>	<i>S. arizonae</i>	<i>S. bongori</i>	<i>Y. enterocolitica</i>
1	✓	✓	×	✓	✓	✓	×
2	×	✓	×	✓	✓	×	×
3	✓	✓	✓	✓	✓	✓	×
4	×	✓	✓	✓	✓	✓	×

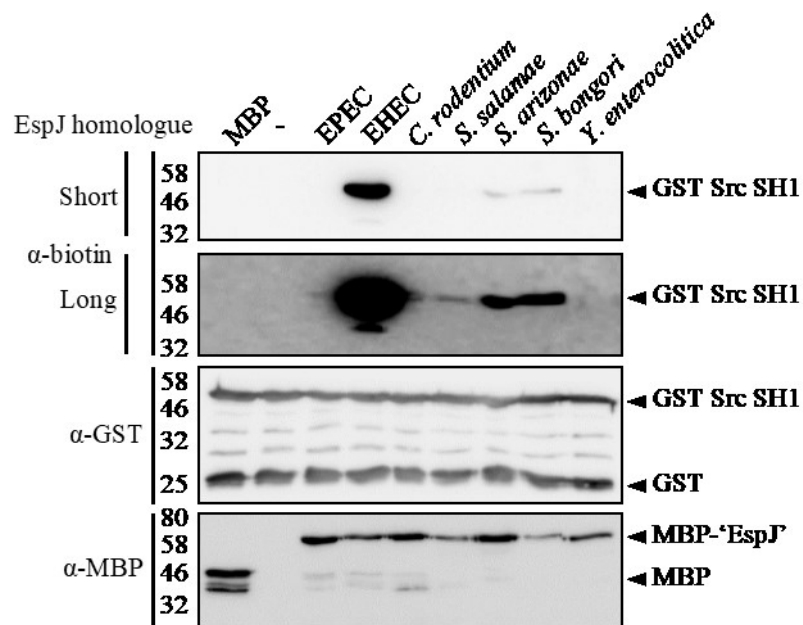


Figure 3.9 - ADP-ribosylation of Src by the EspJ homologues

Gel filtration-purified MBP-tagged EspJ homologues were incubated with GST-Src₂₅₀₋₅₃₃K295M and 10 μ M of 6-biotin-17-NAD⁺ (NAD-biotin) for 1 h before analysis by Western blotting. Antibodies specific for MBP revealed EspJ homologues, GST for Src, and streptavidin-HRP (α -biotin), revealed proteins ADP-ribosylated using NAD-biotin. Short and long exposures for the anti-biotin panel are displayed to show the range of ADP-ribosylation by the EspJ homologues.

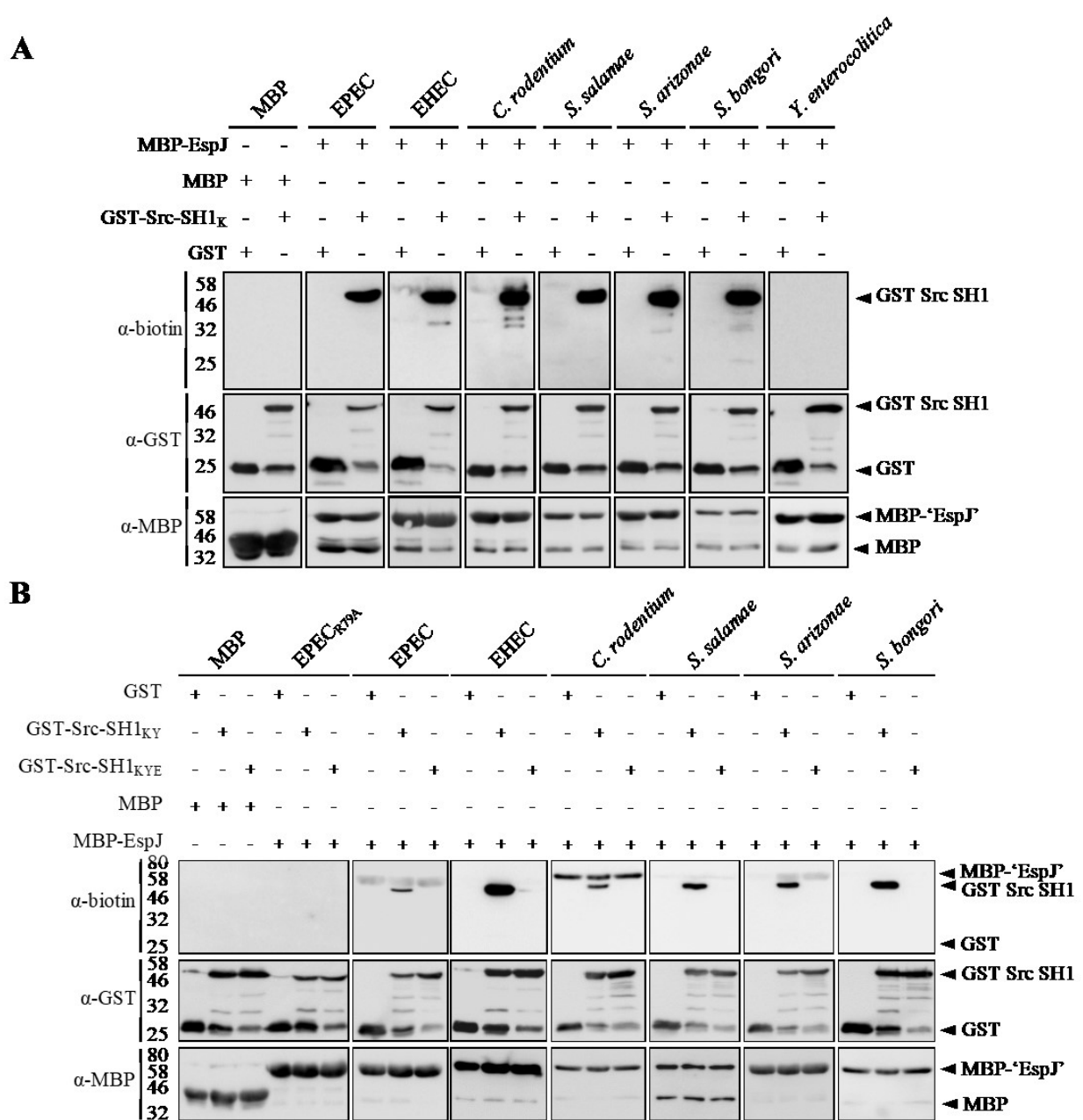


Figure 3.10 - On resin ADP-ribosylation of Src by the EspJ homologues at Src E310

MBP-tagged EspJ homologues bound to amylose resin were incubated with mutants of GST-Src₂₅₀₋₅₃₃, and 10 μ M of 6-biotin-17-NAD⁺ (NAD-biotin) for 1 h before analysis by Western blotting (K = K295M, Y = Y416A, E = E310A). Antibodies specific for MBP revealed EspJ homologues, GST for Src, and streptavidin-HRP (α -biotin), revealed proteins ADP-ribosylated using NAD-biotin. (A) All EspJ homologues apart from *Y. enterocolitica* YspJ were able to ADP-ribosylate GST-Src₂₅₀₋₅₃₃K295M while (B) a Src E310A mutation prevented modification by EspJ. (B) Adapted from Pollard *et al.*, 2016.

3.7 EspJ homologues localisation and activity in mammalian cells

After characterising the enzymatic activities of EspJ/SeoC/SboC/YspJ *in vitro* their properties were next investigated by ectopic expression within tissue culture cells. After transfection, myc-tagged EspJ homologues displayed similar localisation phenotypes with mostly diffuse cytoplasmic staining by immunofluorescence, but regularly seen examples of colocalisation to actin structures, or vesicular-like structures (Figure 3.11).

To enable the study of SeoC/SboC/YspJ impact on phagocytosis after transfection, a Cos-7 cell line stably expressing GFP-FcγRIIa was created (Figure 3.12) (van Zon et al., 2009). The cell line was generated by infection of Cos-7 cells with GFP-FcγRIIa-encoding MMLV virions, selection of transduced cells with puromycin, and FACS sorting based upon FITC-absorbance. The resulting Cos-7 cell was able to perform phagocytosis. This cell line phagocytosed around 60% of bound, IgG opsonised beads and the transfection of the catalytically inactive EPEC EspJ R79A/D187A did not affect this uptake. However, ectopic expression of each EspJ homologue reduced bead uptake to below 10%, implying an inhibition of FcγR-mediated phagocytosis as previously reported for EPEC and EHEC EspJ (Marchès et al., 2008). Interestingly, phagocytosis was also inhibited by *Yersinia enterocolitica* YspJ, which was unable to ADP-ribosylate Src *in vitro* (Figure 3.13).

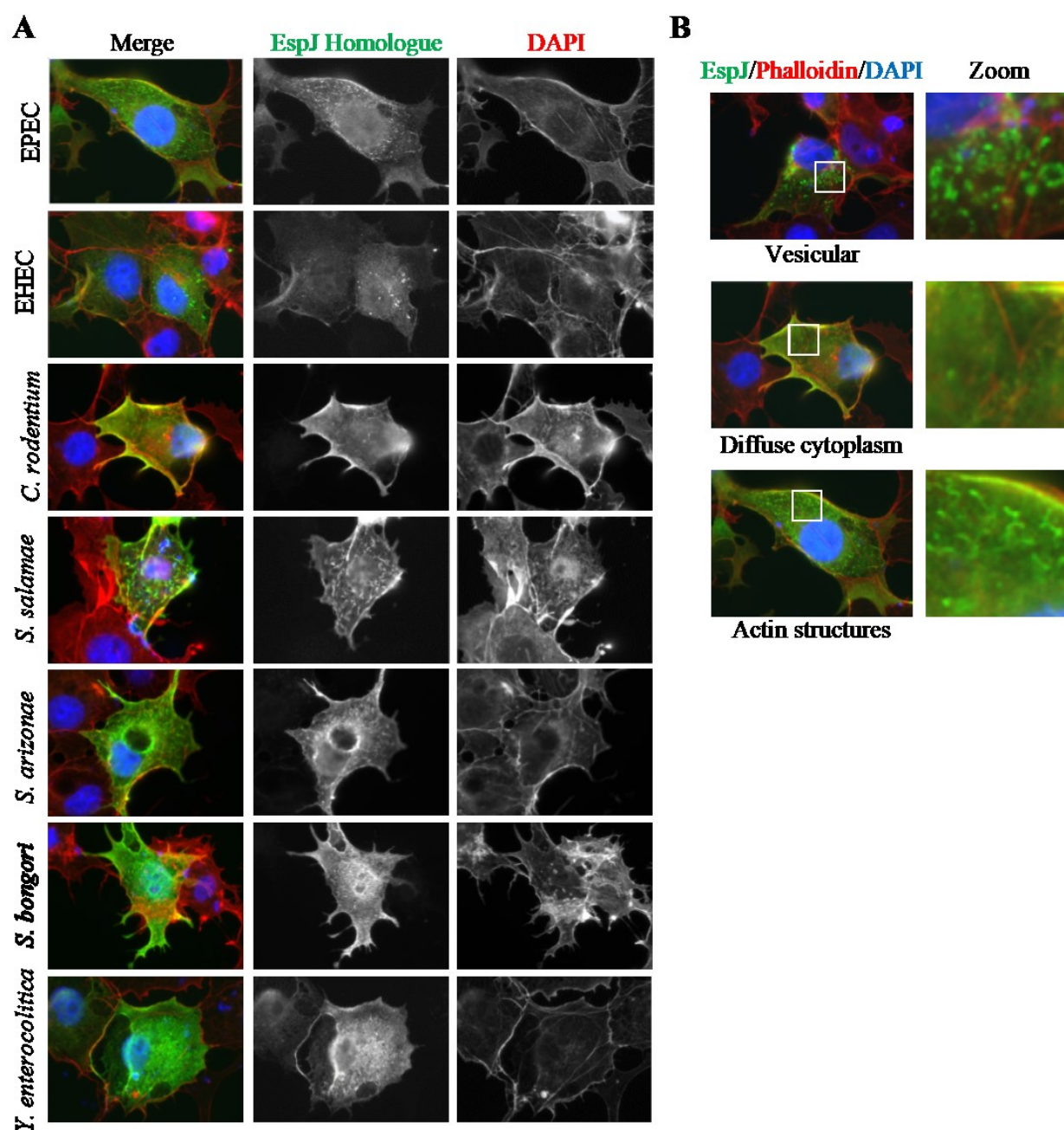


Figure 3.11 - The localisation of EspJ/SeoC/SboC/YspJ during ectopic expression

Cos-7 cells were transfected with Myc-tagged EspJ homologues, before staining for immunofluorescence. Panels show merged channels with EspJ/SeoC/SboC/YspJ in green, actin in red and DAPI in blue. (A) Shows representative images for the predominant cytoplasmic transfection phenotype for each homologue. (B) Shows the three phenotypes observed after transfection of each homologue—vesicular/mitochondrial, diffuse cytoplasmic, or co-localisation with actin structures.

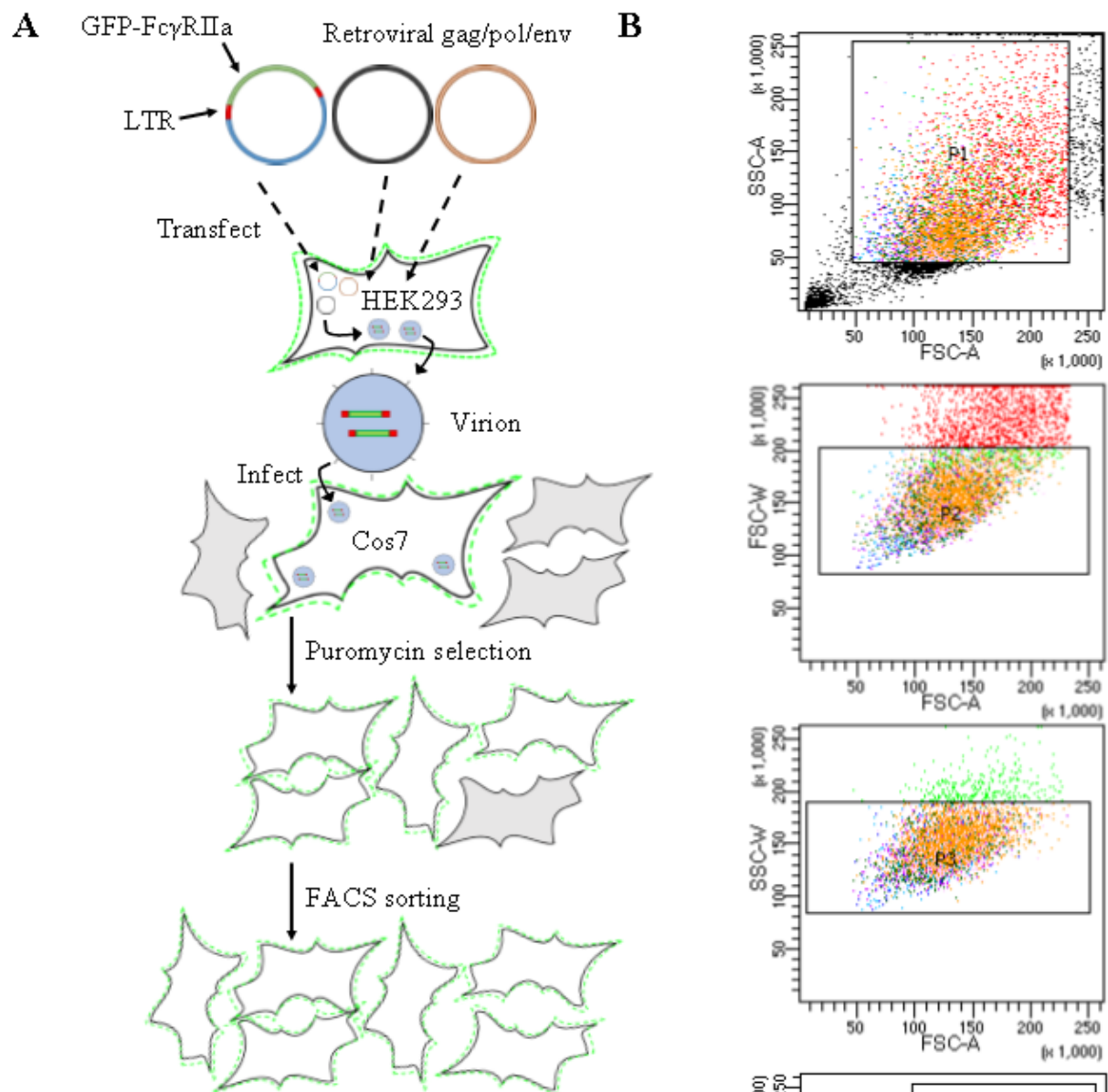


Figure 3.12 - Generating Cos-7 cell line stably expressing FcγRIIa

(A) pMXs-IP-GFP-FcγRIIa, pCMV-VSV-G (black circle) and pCMV-MMLV (brown circle) were transfected into HEK293 cells which were allowed to produce GFP-FcγRIIa-encoding virions for 48 h. These virions were used to infect Cos-7 cells and positive clones selected for with puromycin before FACS sorting. LTR – long terminal repeat. (B) Cells were gated based upon forward (FSC) and side scatter (SSC) to remove dead cells, cell debris and cell doublet/multipllets before gating based upon GFP expression FITC-absorbance. P5 to P8 gates were collected and expanded for analysis. Ultimately P7 cells were used for later experiments.

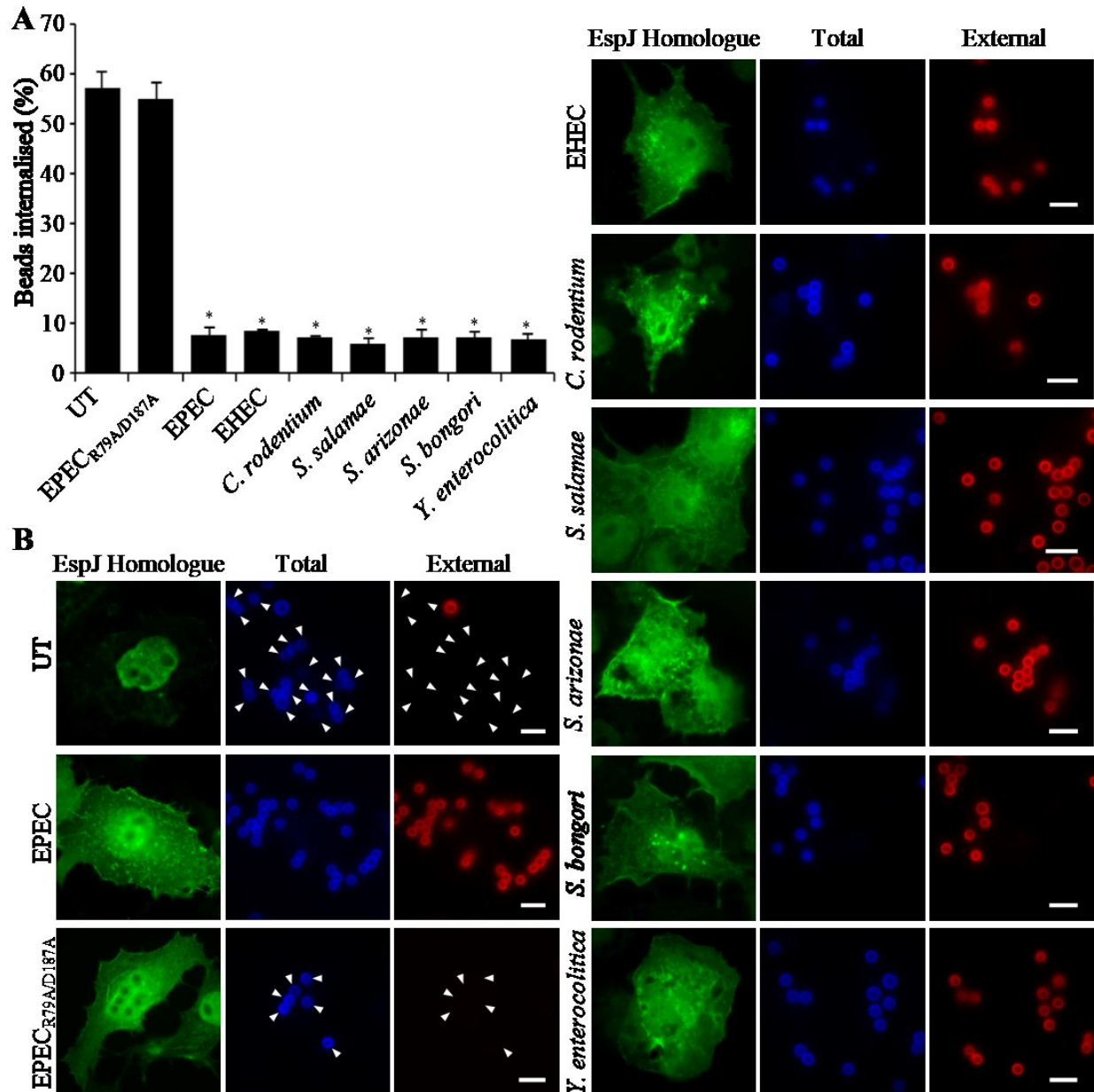


Figure 3.13 - Ectopic expression of EspJ homologues inhibits FcγRIIa-mediated phagocytosis

Cos-7 cells stably expressing GFP-FcγRIIa were transfected with plasmids encoding Myc-tagged EspJ homologues. 16 h later the cells were challenged with IgG opsonised 3 μm beads for 90 min. (A) Staining pre- and post-permeabilisation revealed external (red) and total (blue) beads which were counted for EspJ transfected cells (green), revealing percentage of internalisation. Results are the mean ±SEM of three independent experiments. (B) Representative immunofluorescence images of pRK5-EspJ transfected cells are shown. Arrows indicate the internalisation of beads by EspJ transfected cells. Scale bar, 10 μm. Statistics were performed with Graphpad Prism software using a OneWay Anova followed by Bonferonni post-test, * = $p < 0.01$. Figure adapted from Pollard *et al* 2016.

3.8 Translocation of *S. salamae* SeoC

EPEC and EHEC EspJ and *S. bongori* SboC are known to be translocated by the LEE and SPI-I T3SS respectively (Dahan et al., 2005; Fookes et al., 2011), but *seoC* is not a proven T3SS effector. As well as SPI-1 and SPI-2 T3SS, *S. salamae* has been reported to possess genes similar to the LEE pathogenicity island typically found in EPEC/EHEC/CR (Chandry et al., 2012; Desai et al., 2013), though its functionality has not been proven. Therefore, SeoC has the potential to be translocated by one or more of the SPI-1, SPI-2 and LEE T3SS. Translocation assays were performed using wild type *S. salamae* and SPI-1 ($\Delta invA$), SPI-2 ($\Delta ssaV$) or LEE ($\Delta escN$) T3SS mutants, expressing a SeoC-TEM-1 reporter fusion protein (Figure 3.14). In this assay a non-fluorescent substrate is passively up taken into mammalian cells where its esterification allows fluorescence energy transfer (FRET) and emission at 520 nm. The injection of a T3SS effector-TEM fusion can be measured by the β -lactam cleavage of the substrate which disrupts FRET and causes emission at 410 nm (Charpentier and Oswald, 2004). The *S. bongori* T3SS protein SboI-TEM fusion was successfully translocated by *S. salamae* whereas the negative control, housekeeping protein FabI, was not. SeoC-TEM was translocated into J774.A1 macrophages and HeLa cells during *S. salamae* infection. No translocation of SeoC-TEM was observed from the *S. salamae* $\Delta invA$ indicating that SeoC is a SPI-1 T3SS substrate. Translocation was still observed from $\Delta ssaV$ and $\Delta escN$ SPI-2 and LEE T3SS mutant strains, though at a reduced level compared to WT *S. salamae* (Figure 3.14).

3.9 Inhibition of macrophage phagocytosis by SeoC during infection

As SeoC was shown to be a SPI-1 T3SS substrate, its ability to inhibit phagocytosis during infection was next tested. J774.A1 mouse macrophages were infected with *S. salamae* WT or $\Delta seoC$ strains, or $\Delta seoC$ complemented with plasmid-encoded WT SeoC or inactive SeoC (R79A). Total and external staining revealed that while uninfected macrophages internalised around 60% of IgG opsonised beads, infection with WT *S. salamae* significantly inhibited bead

phagocytosis. This inhibition was reversed in the absence of *seoC* and could be restored by plasmid complementation with WT *seoC* but not *seoC*-R79A (Figure 3.15). This shows that SeoC is responsible for inhibition of phagocytosis by *S. salamae* and this is dependent on the ART activity of SeoC.

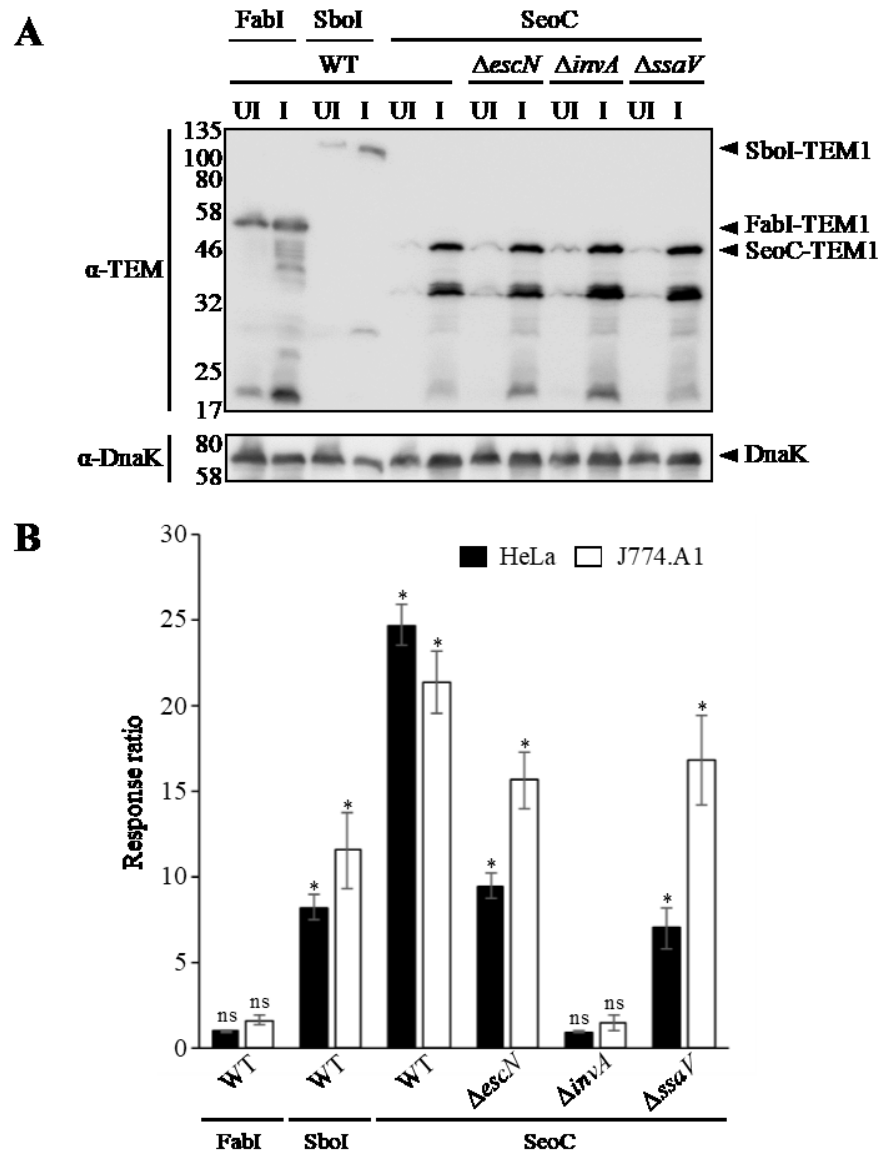


Figure 3.14 - SeoC is a SPI-1 T3SS secreted effector protein

S. salamae WT and mutant strains were used to infect HeLa cells and J774.A1 macrophages, with expression of either FabI, SboI or SeoC TEM-1 fusion proteins from the pCX340 vector before assaying TEM activity. (A) Anti-TEM-1 western blot shows the bacterial expression of fusion proteins before (UI) and after induction (I) with IPTG. (B) TEM-1 assay shows translocation of SeoC-TEM-1 from WT, $\Delta escN$ and $\Delta ssaV$ but not $\Delta invA$ *S. salamae*. Housekeeping protein FabI and T3SS effector SboI are used as negative and positive controls respectively. * = $p < 0.01$, compared to uninfected cells, ns = not significant. Figure adapted from Pollard *et al* 2016.

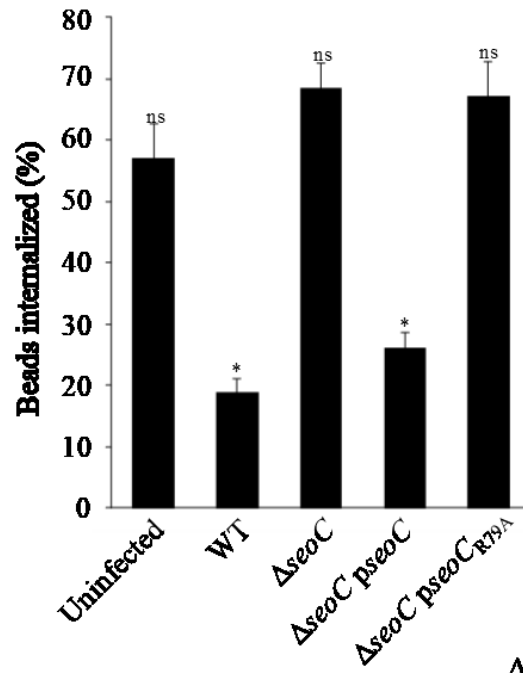
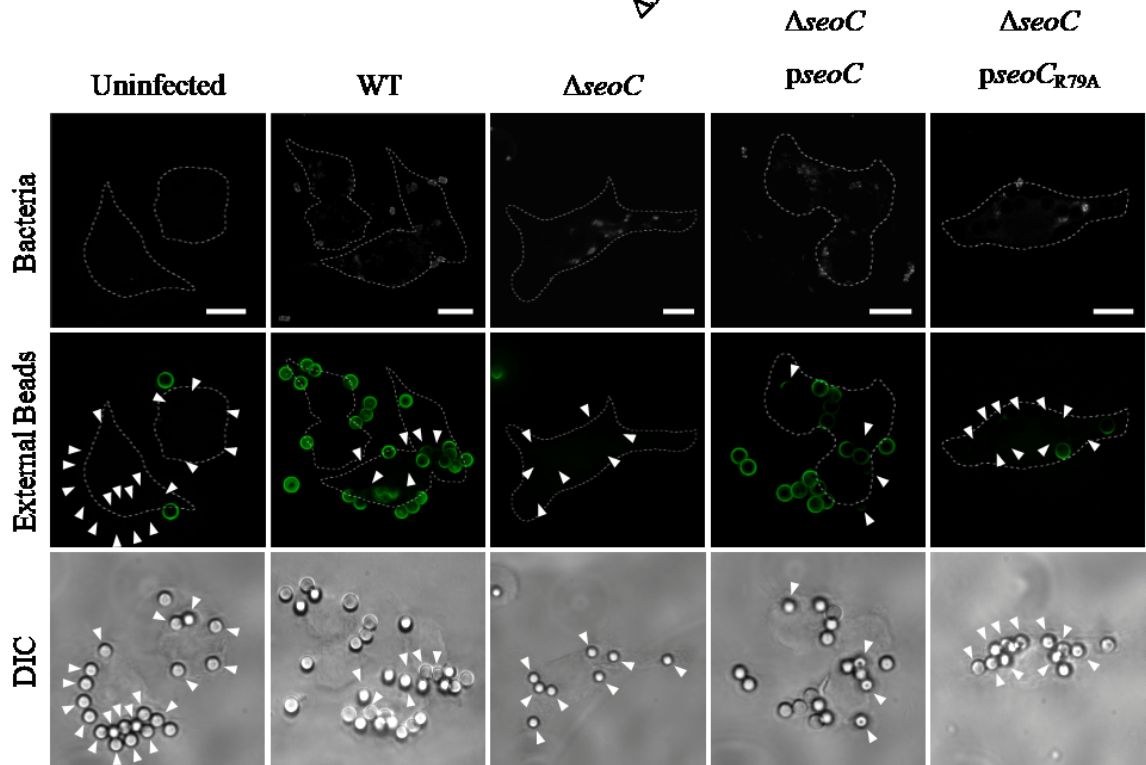
A**B**

Figure 3.15 - *seoC* is a SPI-1 T3SS that inhibits macrophage phagocytosis during infection

S. salamae WT, or $\Delta seoC$ with or without plasmid complementation by pWSK29-*SeoC*/*SeoC*-R79A (*pseoC*/*pseoC*-R79A) were used to infect J774.A1 macrophages before challenging with IgG-opsonised beads. (A) Staining of *S. salamae* pre- and post-permeabilisation was counted revealing that WT *S. salamae* infection significantly reduced bead internalisation. Infection with *S. salamae* $\Delta seoC$ removed this inhibition of internalisation, and inhibition could be restored by complementation with WT *seoC* but not *seoC*-R79A. * = $p < 0.01$, ns = not significant compared to uninfected cells. Figure adapted from Pollard *et al* 2016.

3.10 *S. salamae* invades host cells independently of *seoC*

S. enterica are invasive pathogens which control their entry into and replication within epithelial and phagocytic cells and *S. enterica* subsp. *salamae* are most commonly isolated from extra-intestinal infections. It may be unexpected for *S. salamae* to translocate an effector into host cells that can inhibit phagocytosis. GFP-expressing *S. salamae* were used to infect J774.A1 macrophages and HeLa epithelial cells. First, an lactate dehydrogenase (LDH) release assay was performed to check that different levels of cell death/lysis were not contributing to the calculations of bacterial internalisation. There was no significant increase in lactate LDH release, indicative of cell lysis, when comparing WT and $\Delta seoC$ *S. salamae* infected to uninfected cells (Figure 3.16). This shows that these strains are not cytotoxic at 30 and 60 min post infection. Gentamycin protection assays and external bacterial staining both suggested that *S. salamae* can invade HeLa and J774.A1 cells, and this is significantly inhibited the $\Delta invA$ SPI-1 T3SS mutant but not changed by the absence of *seoC* (Figure 3.16).

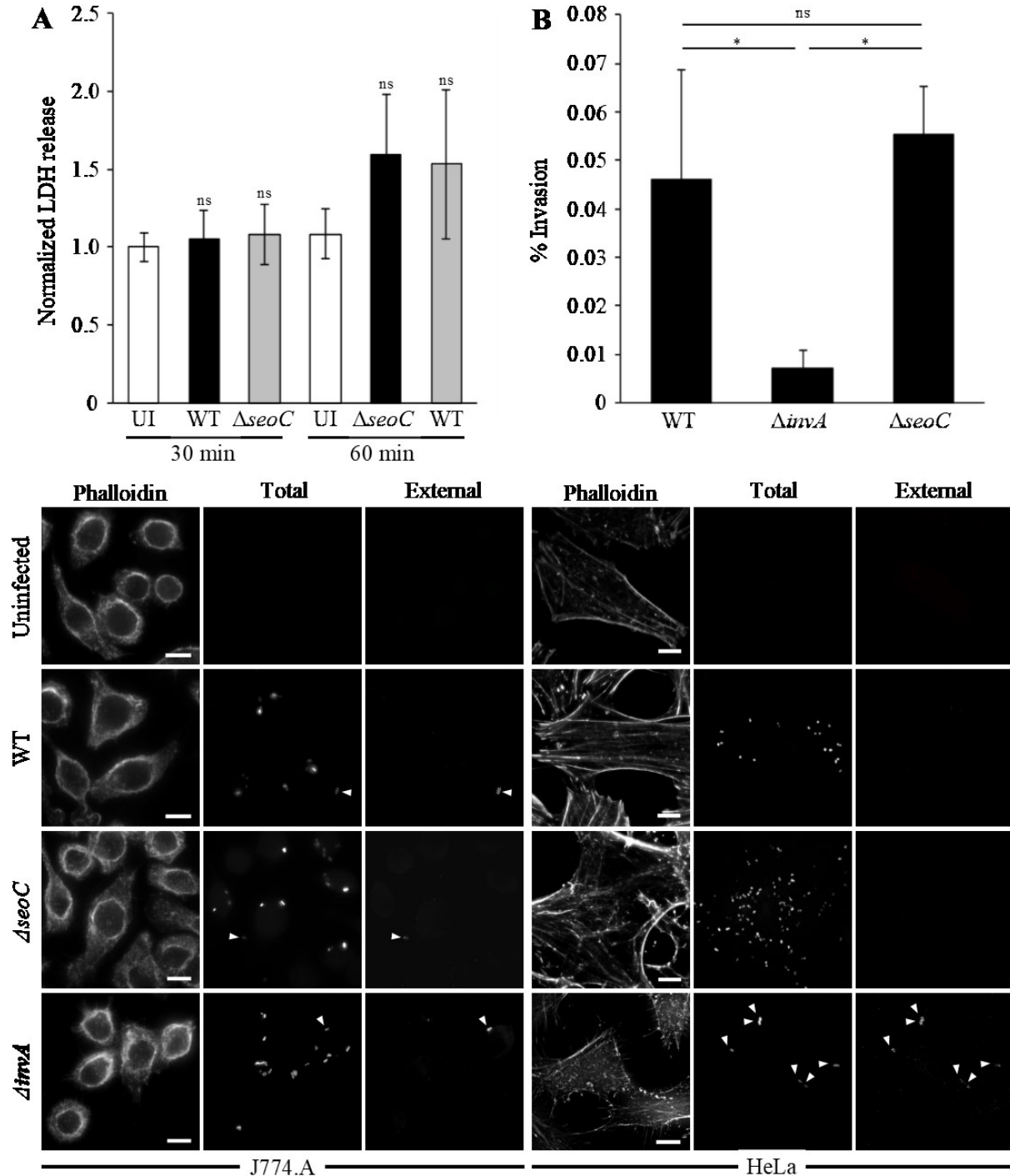


Figure 3.16 - *S. salamae* invades HeLa epithelial cells and J774.A1 macrophages, independently of *seoC*

(A) Lactate dehydrogenase (LDH) assay - *S. salamae* WT and $\Delta seoC$ do not cause increased cell lysis compared to uninfected J774.A1 cells 30 or 60 min post infection. (B) Gentamycin protection - the percentage of internalised cells from the infection inoculum. $\Delta invA$ was less invasive than WT and $\Delta seoC$, but $\Delta seoC$ had no impact on invasiveness. The mean $\pm$ SEM of three or more experiments each in triplicate is displayed. * = $p < 0.01$, ns = not significant. (C) J774.A1 macrophages and (D) HeLa cells were infected with *S. salamae* WT, $\Delta seoC$ and $\Delta invA$ strains expressing GFP, before staining external bacteria pre-permeabilisation. All strains were internalised by J774.A1 macrophages, while $\Delta invA$ was the only strain not able to invade HeLa cells. External bacteria are shown by arrows, scale bars = 10 μ M. Figure adapted from Pollard *et al* 2016.

3.11 Discussion

Prior to this study the EPEC and EHEC T3SS effector EspJ was demonstrated to inhibit FcγR- and CR3-mediated phagocytosis, dependent upon the presence of predicted catalytic residues R79 and D187. Further EspJ_{EPEC} was shown to inhibit Src kinase by ADPr *in vitro*. This chapter sought to investigate the functional characteristics of EspJ homologues.

A BLAST search predicted *espJ* to be encoded within CR, *S. bongori*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae* and *Y. enterocolitica*. The *espJ* gene was not predicted in any other *S. enterica* subsp. or other *Yersinia* species. This was confirmed elsewhere by a PCR screen for prevalence of the *Salmonella espJ* homologue, *seoC*, in 73 clinical isolates across *S. bongori* and *S. enterica* subsp. *enterica*, *salamae*, *arizonae*, *diarizonae* and *houtenae*. This identified *seoC* all tested *S. bongori*, 8 from 9 *S. arizonae*, and 4 from 7 *S. salamae* isolates but not in subspecies *enterica*, *diarizonae* or *houtenae* (Pollard et al., 2016). *E. coli*, CR, *Salmonella* and *Yersinia* are members of the *Enterobacteriaceae* family. Common ancestry between *E. coli* and *Salmonella* is estimated to be 140 million years and the divergence of *Yersinia* is predicted to predate this (Ochman and Wilson, 1987), yet *espJ* homologues have been identified in each. Pathogenicity islands are normally acquired by horizontal gene transfer but may also be carried within other integrative elements like cryptic prophages as is the case for *espJ* within EPEC and EHEC. Unfortunately, the localisation of *seoC* and *yspJ* within the *Salmonella* and *Yersinia* genomes is unknown, so it is not possible to comment further on their acquisition. The EspJ homologues were phylogenetically organised into two clades; one with the *Salmonella* and *Yersinia* homologues and one with the A/E pathogen homologues and sequence identity between the homologues agreed with this prediction.

To enable their functional characterisation *in vitro*, the EspJ homologues were purified from a bacterial expression system. Initial screens showed that using an MBP-tag improved the expression and solubility in comparison to his-tagged fusion proteins. Fusion proteins

including MBP, glutathione-S-transferase (GST), thioredoxin and antibody fragments are often used to improve protein solubility, as they can have a chaperone-like effect when fused to the N-terminus of the protein of interest, and enable subsequent affinity purification (Edwards et al., 2000). The MBP encoded within pMALXE includes a truncated, rigid linker in combination with MBP with surface entropy reduction mutations both maximise the probability of fusion protein crystallisation through reducing flexibility and encouraging the formation of crystal contacts (Moon et al., 2010). However, after extensive crystallographic screening of MBP-EspJ_{EPEC} it was not possible to obtain protein crystals.

EspJ_{EPEC} is a novel ART as it can simultaneously amidate ADP-ribosylate Src E310 resulting in ADP-ribose conjugated Q310. With a view to investigating the mechanism of this via structural methods, attempts were made to isolate the EspJ-Src complex, though these were unfortunately unsuccessful. EspJ and Src must interact to enable catalysis, but enzyme-substrate complexes are notoriously difficult to stabilise due to the often-transient nature of their interaction during catalysis. ADPr relies upon the initial hydrolysis of NAD releasing nicotinamide before conjugation of ADP-ribose onto target proteins. To prevent catalysis, non-hydrolysable analogues of NAD such as β -TAD have been used in structural studies of ARTs and other NAD-utilising enzymes (Marquez et al., 1986; Tsuge et al., 2008), but this was not effective for the EspJ-Src complex. In contrast to EspJ, the *P. syringae* T3SS effector HopF2 was shown to stably interact with its substrate MKK5 but mutations equivalent to EspJ R79A and D187A abolished the interaction, indicating a dependence upon a functional ART domain (Wang et al., 2010). The SH1 domain of Src was used in isolation for pulldowns, and perhaps the full length is required for a stable interaction, or it may be that the MBP-tag of EspJ destabilises the interaction. However, these two issues were difficult to avoid due to the poor solubility of full length Src or untagged-EspJ. Alternatively, it is possible that the stable

EspJ-Src interaction relies upon PTMs such as glycosylation only performed in mammalian expression systems.

Recombinantly purified MBP-EspJ homologue fusion proteins were present in three distinct peaks after gel filtration chromatography; large soluble aggregates, apparent monomeric or dimeric/trimeric fusion proteins according to calculated molecular weights. A hypothesis for the formation of large soluble aggregates could be that the MBP tag is keeping misfolded and otherwise insoluble EspJ in solution or that the fusion protein is forming electrostatic interactions between MBP and EspJ. In support of the prior theory, MBP-EspJ from this aggregated peak was unable to ADP-ribosylate Src indicating an incorrect fold. Raising the buffer pH was able to increase the proportion of MBP-EspJ_{EHEC} that was monomeric, though it is not known whether this is owed to an improved folding of EspJ, or by preventing fusion protein MBP to EspJ interactions. NMR spectroscopy or circular dichroism spectrometry could be used to assess the folding of these aggregates, and methods such as size exclusion chromatography multi angle laser light scattering (SEC-MALLS) or analytical ultracentrifugation (AUC) could be used to confirm the stoichiometry of EspJ monomers and dimers. *S. bongori* SboC and *Y. enterocolitica* YspJ were the least soluble EspJ homologues, though a large-scale comparison was not performed between all homologues at pH 10 which may improve their behaviour.

All the EspJ homologues except for YspJ were able to ADP-ribosylate Src and mutation of Src E310 prevented ADPr by EspJ suggesting a conserved target residue between the homologues. Src E310 protrudes into the kinase active site forming a salt bridge with K295 that is required for transfer of the phosphate moiety. Hence, as previously shown the modification of this residue inhibits Src kinase activity (Young et al., 2014). It is unclear whether the lack of YspJ activity was due to misfolding, a non-functional ART domain, or a difference in substrate specificity. However, YspJ was also the only homologue to not display evidence of auto-ADPr

suggesting that it may not be active. This could be investigated by testing the activity of YspJ purified with alternative affinity tags, or from other expression systems such as mammalian/insect cells or using baculovirus expression systems. This protein could be tested using recombinant Src kinase as a target or by incubation with cell lysate to find alternative targets. Human ARTD9 and ARTD13 each possess catalytically inactive ART domains. Furthermore, despite sharing high level of homology to the Pertussis Toxin which ADP-ribosylates G-proteins, the ART domain within the Typhoid toxin still has no confirmed target (Fowler et al., 2017; Miller and Wiedmann, 2016; Song et al., 2013). Finally, *P. syringae* HopF1 contains a non-functional ART domain while its neighbour HopF2 ADP-ribosylates MKK5 (Wang et al., 2010). HopF2 has also been shown to bind to and interrupt BAK1 (Zhou et al., 2014) and RIN4 (Wilton et al., 2010) signalling in a non-catalytic manner. It is therefore possible that YspJ perturbs host cell signalling through non-catalytic activity.

Auto-ADPr has been documented for many bacterial virulence factors including Diphtheria and Cholera toxins, *Streptococcus pyogenes* SpyA, *Helicobacter pylori* CagL and *Pseudomonas aeruginosa* ExoS (Coye and Collins, 2004; Riese et al., 2002; Talluri et al., 2017). While the functions of SpyA and CagL auto-ADPr is unclear, ExoS ADP-ribosylates an important arginine within its GAP domain inhibiting GAP activity, and the *Vibrio cholera* Cholix toxin also self-regulates by internal ADPr (M-H Sung et al., 2014). Human ARTD1 auto-modification has been reported on multiple sites and is proposed to impact upon its interactions with chromatin and histones (Chapman et al., 2013; Muthurajan et al., 2014). Mammalian Sirtuins are capable of a range of NAD⁺ dependent reactions including deacetylation, diacylation and ADPr, though their automodification is thought to lack functional relevance (Du et al., 2009; Van De Ven et al., 2017). To date the function of EspJ auto-ADPr is unknown, though within the EspJ homologues there appeared to be an inverse relationship between efficiency of Src modification and self-modification which may suggest

an auto-regulatory mechanism. To study this first the site of auto-modification must be identified, perhaps by mass spectrometry after auto-ADPr. From this point onwards, mutation of the site residue may alter the function and activity of EspJ.

Upon ectopic expression all the EspJ homologues showed similar localisation trends with either diffuse cytoplasmic staining, colocalization with actin-rich structures or in vesicular-like structures. EspJ has previously been reported to be mitochondrially targeted, which could explain the vesicle-like localisation, though a function of this observation has not been deduced (Kurushima et al., 2010). EspJ occupancy around sites of actin dynamics reflects its ability to perturb EPEC pedestal formation, and the actin polymerisation required for phagocytosis (Young et al., 2014). EspJ, SeoC, SboC and YspJ were all able to inhibit opsono-phagocytosis of IgG opsonised beads. This is surprising considering the apparent lack of YspJ catalytic activity *in vitro* and may imply that the recombinant inactive YspJ is mis-folded but retains the ADPr of Src in cells. Alternatively, despite sharing between 55% and 77% sequence identity to EspJ/SeoC/SboC, YspJ may target another protein in the phagocytosis pathway. This is supported by *Pseudomonas aeruginosa* ExoS and ExoT which share 76% sequence identity and both inhibit phagocytosis but ADP-ribosylate a distinct repertoire of proteins (Barbieri and Sun, 2004).

In this chapter it was proven that *S. salamae* *seoC* is a substrate of the SPI-1 T3SS, translocated into the cytosol of HeLa epithelial cells and J447.A1 macrophages, where it is able to inhibit opsono-phagocytosis. Several studies of *S. salamae* genomes have identified the presence of the LEE T3SS pathogenicity islands typical of A/E pathogens (Chandry et al., 2012; Desai et al., 2013; Pollard et al., 2016). There was also a decrease of SeoC translocation when comparing WT to LEE or SPI-2 T3SS mutant strains, which was statistically significant in HeLa but not J774.A1 cells. Though unclear, this slight dependency may be due to an attenuated infection in the Δ *ssaV* and Δ *escN* strains. Bacterial survival and replication during

infection would need to be monitored closely to determine this. The *Salmonella* effector SlrP can be translocated by both the SPI-1 and SPI-2 T3SS (Cordero-Alba and Ramos-Morales, 2014) so in theory *seoC* could be a substrate of the other T3SS, but this seems unlikely given the complete ablation of translocation in the $\Delta invA$ (SPI-1 mutant) strain. Complete understanding of these dynamics may require LEE/SPI-1, LEE-SPI-2 or SPI-1/SPI-2 double T3SS mutants.

In the A/E pathogens the presence of the LEE confers an extracellular lifestyle, while *Salmonella* SPI-1 and SPI-2 allow an invasive lifestyle through facilitating invasion, and intracellular survival. The presence of all three in *S. salamae* is somewhat contradictory, and especially interesting for a bacterium with contrasting data surrounding its invasiveness. The invasiveness of other *S. enterica* subspecies is also a source of discussion, though the LEE has not been identified in them. Subspecies *salamae* and *diarizonae* isolates from crocodiles were not invasive in an experimental mouse model in comparison to *enterica* subspecies isolates (Madsen et al., 1998). Despite the ability to colonise and spread within chicken organs, *S. salamae* serovar Sofia was not able to induce disease, and *in vitro* could not survive and replicate within J774.A1 macrophages (Gan et al., 2011). Similarly, a study of subspecies *arizonae* and *diarizonae* found that they were significantly deficient in colonisation compared to *S. Typhimurium* in murine models, and only poorly invaded and replicated within J774.A1 macrophages (Katribe et al., 2009). Contrastingly, strains from the *salamae*, *arizonae*, *diarizonae* and *houtenae* subspecies were able to invade Caco2 intestinal epithelial cells, albeit less efficiently than those from the *enterica* subspecies (Bibiloni et al., 1999; Pasmans et al., 2005). The overriding theme here is that although the non-*enterica* subspecies are able invade and replicate within cultured cells, their ability to do so, and their resulting infectivity *in vivo* is attenuated (Lamas et al., 2018). In line with this, the transfer of SPI-2 to *S. bongori* increased

intracellular persistence in cell culture models but did not enable systemic pathogenicity *in vivo* (Hansen-Wester et al., 2004).

In this chapter *S. salamae* strain 3588/07 was able to invade HeLa cells and J774.A1 macrophages, and the absence of *espJ* had no impact upon this process. To further characterise the role of SeoC in *Salmonella* it would be interesting to test the effect of SeoC expression within *S. Typhimurium*. Furthermore, as several reports have documented variation in *S. salamae* invasiveness between different cell lines, it will be necessary to further characterise these strains in a range of lines including primary cells.

Chapter 4 – The *in vivo* impact of EspJ

4 Results – The *in vivo* impact of EspJ

Previously, EspJ has been characterised in the context of cell culture infections of a few hours (Pollard et al., 2016; Young et al., 2014). This contrasts with the physiological interaction between A/E pathogens and intestinal epithelial cells (IECs) *in vivo*, which lasts several days or weeks. It was recently shown that the proteomic responses of IECs could be quantitatively studied after isolation from mice eight days post infection with WT CR, and this demonstrated that CR impacts upon cholesterol homeostasis and cellular bioenergetics (Berger et al., 2017). To investigate the role of *espJ* using this model, a CR mutant strain lacking *espJ* ($\Delta espJ$) was generated, and two further strains by complementation on the genome with WT *espJ* ($\Delta espJ$ c-*espJ*) or *espJ*-R79A (ΔART).

4.1 *In vitro* and *in vivo* *C. rodentium* *espJ* mutant strains characterisation

A resistance cassette-free CR $\Delta espJ$ was generated by triparental conjugation of the suicide vector pSEVA612s (encoding the EspJ flanking regions) into CR allowing integration of this plasmid into the genome by recombination. Subsequently, after its arabinose-induced expression from the pACBSR plasmid, I-SceI causes a double stranded break at the site of integration. Recombination of this break results in reversion to WT or the deletion of *espJ*, which was screened for by PCR. WT and ΔART complemented strains were similarly produced, using CR $\Delta espJ$ as the receiver strain and pSEVA612S-(-)300bp-*espJ*-WT/R79A-(+)300bp as the donor plasmid.

These strains were shown to grow at similar rates to WT CR in Lennox-LB or M9 media (Figure 4.1) indicating that there had not been disruption of CR housekeeping genes in the mutagenesis process. In addition, each mutant strain was able to form actin rich pedestals during on cultured HeLa cells, suggesting that the T3SS was full functional. (Figure 4.2).

As I do not possess a personal licence, *in vivo* work was conducted by Izabela Glegola-Madejska. WT CR, and $\Delta espJ$ and ΔART mutants were used to inoculate C57BL/6 mice (5 mice per group) by oral gavage, and their shedding measured at days 3, 6, 7 and 8 post-gavage by cfu/gram of stool. Both mutant strains displayed an equivalent shedding profile to WT, indicative of similar colonisation dynamics (Figure 4.3). WT CR causes colitis and colonic crypt hyperplasia (CCH), characterised by colonic crypt lengthening and increased cell proliferation. Measurement of crypts after H & E staining of sections from the distal colon of showed that mice infected with $\Delta espJ$ and ΔART CR significantly increased crypt lengths beyond that observed in WT CR-infected mice (Figure 4.4). Ki67 is a marker for cell proliferation. The distance that Ki67 expressing cells spread up the colonic crypts is routinely used to estimate levels of crypt proliferation. This showed that while both $\Delta espJ$ and ΔART CR significantly increased cell proliferation compared to uninfected mice, only $\Delta espJ$ CR significantly increased proliferation in comparison to WT CR-infected mice (Figure 4.4).

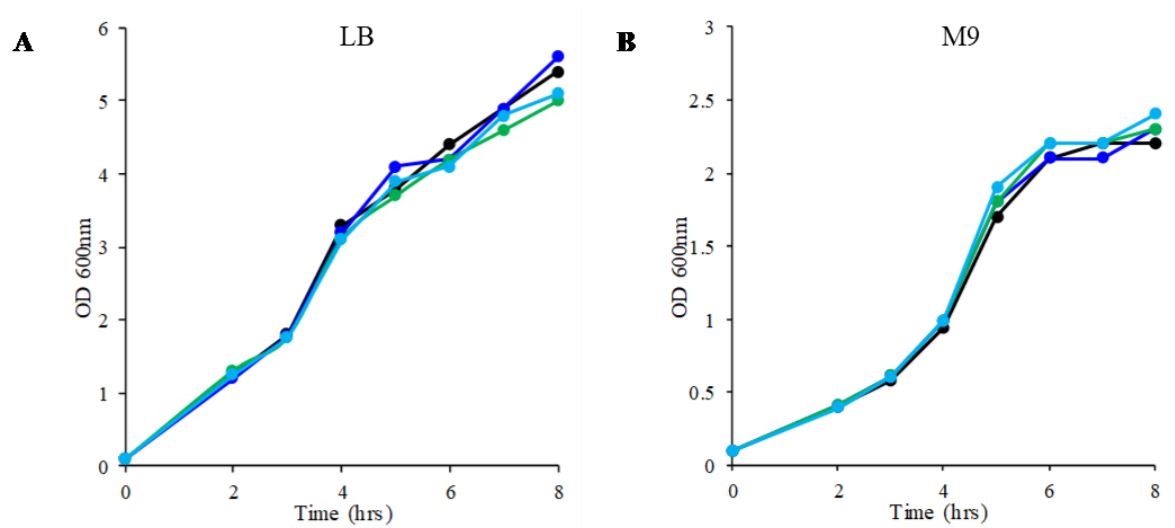


Figure 4.1 - *C. rodentium* *espJ* mutants do not have growth defects in LB or M9 media

WT *C. rodentium* (black) or *C. rodentium* $\Delta espJ$ (dark blue) with or without genomic DNA complementation using WT *espJ* (green) or *espJ*-R79A (light blue) (*c-espJ/c-R79A*), were grown in M9 minimal media or LB-lennox broth and OD-600nm measured every hour from 2 to 8 h.

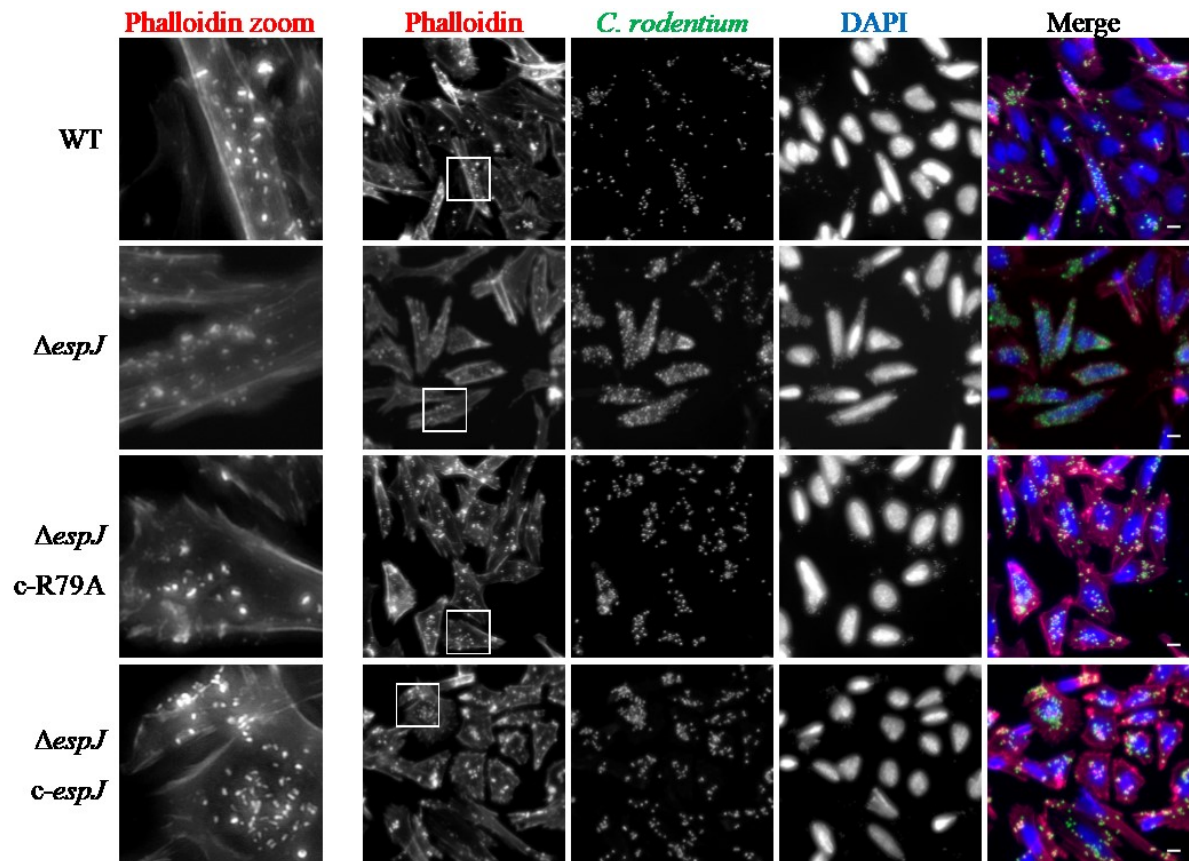


Figure 4.2 - *C. rodentium* *espJ* mutants form pedestals *in vitro*

HeLa cells were infected with WT *C. rodentium* or *C. rodentium* $\Delta espJ$ with or without genomic DNA complementation using WT *espJ* or *espJ*-R79A (*c-espJ/c-R79A*). Immunofluorescence images display phalloidin staining (red), *C. rodentium* (green), DAPI (blue) and a merged image. Zoomed phalloidin regions are shown in white boxes. Scale bars = 10 μ m.

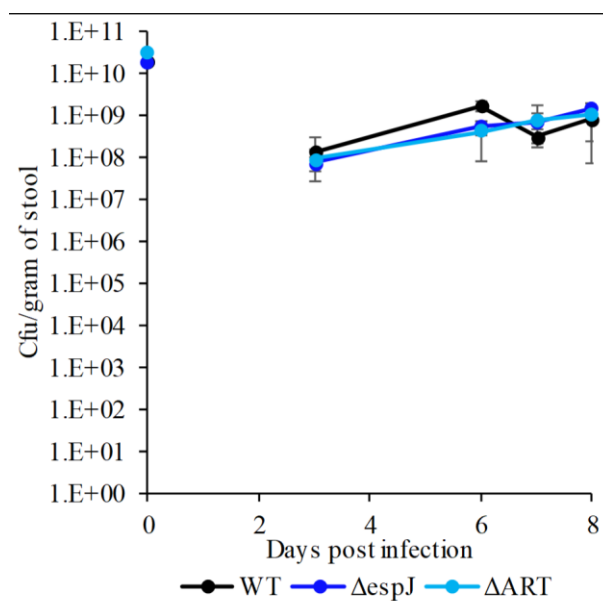


Figure 4.3 - *C. rodentium* *espJ* mutants colonise mice like WT

C57BL/6 mice were inoculated with WT *C. rodentium*, *C. rodentium* *EspJ* catalytic mutant (ΔART) or PBS. Colonisation was assessed by colony forming units per gram of stool at day 3, 6, 7 and 8 post infection. Mean values $\pm$ SEM are displayed from 5 mice per group.

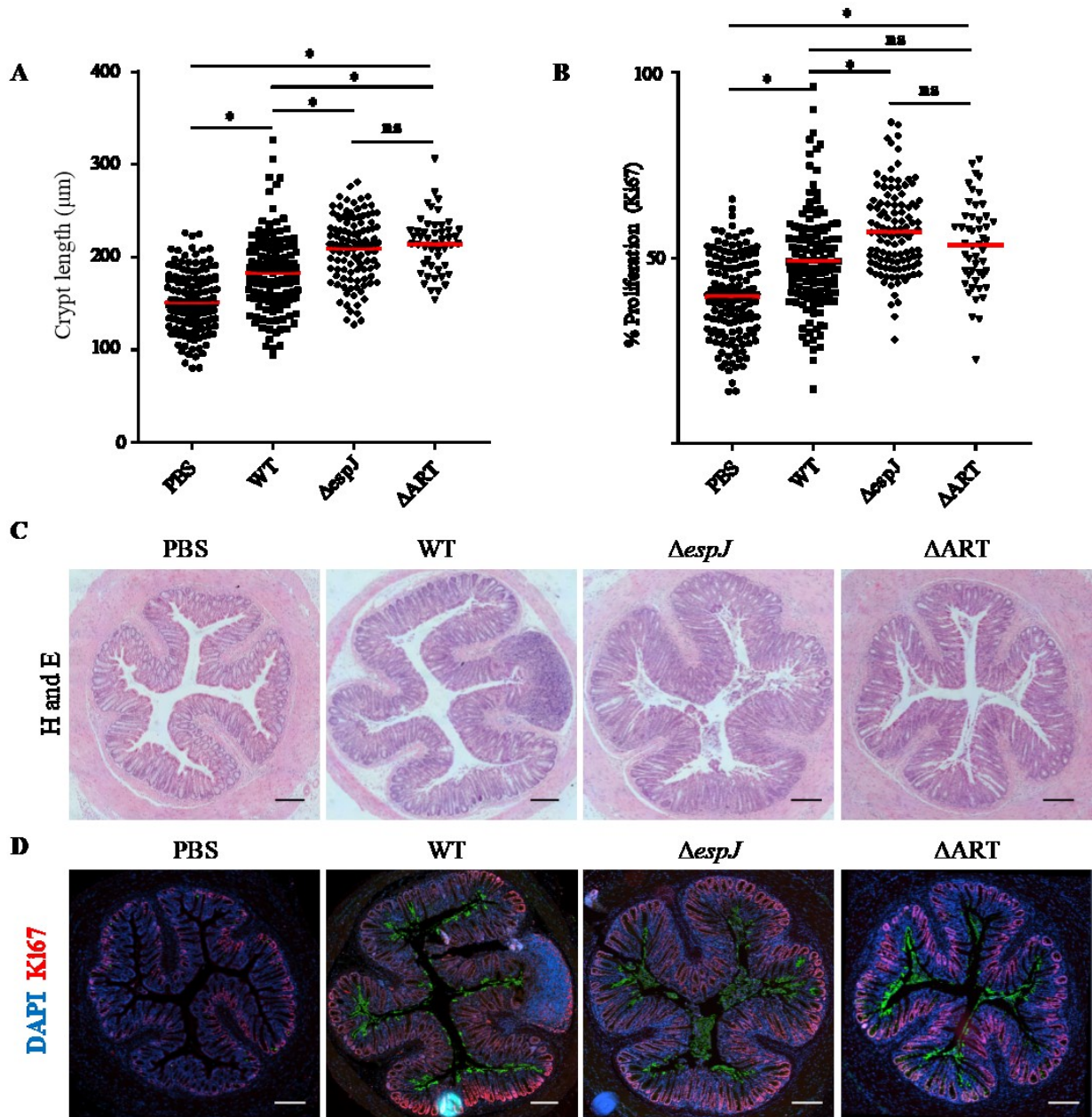


Figure 4.4 - *C. rodentium* *espJ* mutants induce colonic crypt hyperplasia (CCH)

C57BL/6 mice were inoculated with WT *C. rodentium*, *C. rodentium* *EspJ* catalytic mutant (ΔART) or PBS. (A and C) Crypt lengths were measured to assess colonic crypt hyperplasia in H & E sections from the distal colon of PBS or *C. rodentium* (WT/ ΔART) infected mice. (B and D) Proliferation was estimated from the ratio of Ki67 (red) to DAPI (blue) staining by immunofluorescence. The ΔART mutant and WT strains significantly induced lengthening of the crypts, and proliferation to similar levels compared to PBS treated mice. Statistical analysis was performed using a OneWay Anova followed by Bonferroni post-test, * = $p < 0.05$, ns = not significant, red line - mean crypt length, scale bars = 200 μm. Figure from Pollard *et al*, 2018.

4.2 *C. rodentium* WT and Δ ART induce similar global proteomic changes in mice IECs

The function of EspJ catalytic activity was investigated using *in vivo* proteomics analysis of IECs purified from mice eight days after oral gavage with CR WT or Δ ART strains. Tandem mass tag (TMT)-based mass spectrometry was used to quantitatively analyse IEC proteomes. Each sample is labelled with a different isobaric tag which has a unique fragmentation pattern, and samples are mixed prior to analysis. This allows relative quantification of differentially tagged peptides during the same mass spectrometry run and was used to determine protein abundance changes in IECs isolated from mock infected mice or mice infected with WT or Δ ART (4, 5 and 6 mice per group respectively).

7400 mouse proteins were identified and quantified across the three purified IEC samples using this isobaric labelling strategy (FDR <0.01). In comparison to uninfected IECs, the abundance of 3396 and 3166 proteins were changed by ± 1.5 fold after WT CR or Δ ART infection, respectively. 2654 of these protein abundance changes were observed after both infections

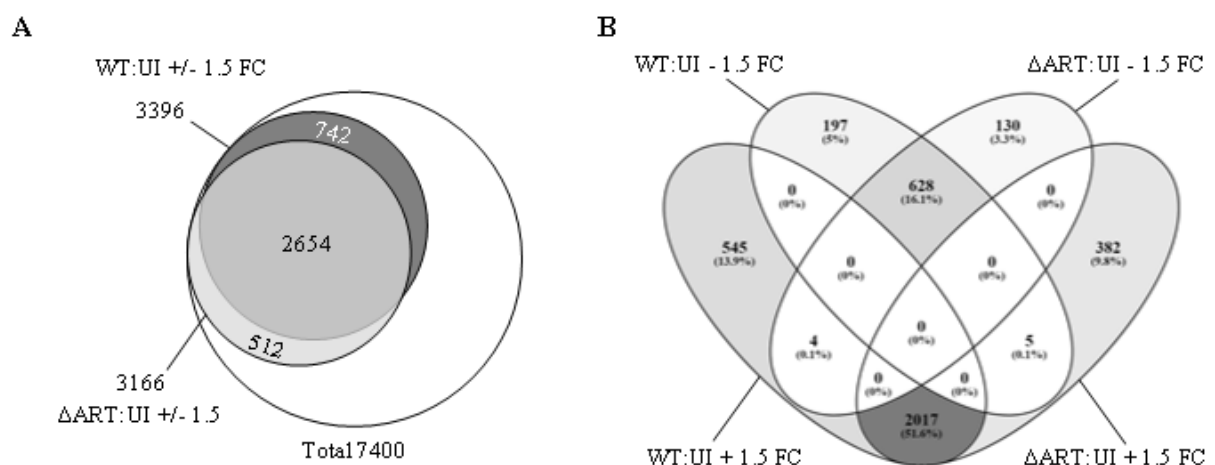


Figure 4.5 – Changes in IEC protein abundances induced by WT and Δ ART *C. rodentium* infections

Venn diagrams to represent IEC proteomics. (A) A total of 7400 proteins were identified from proteomics analysis of IECs. 3396 and 3166 were changed by ± 1.5 -fold compared to infected IECs after infection with WT or Δ ART *C. rodentium*. (B) Four-way Venn diagram shows the proportions of these proteins that are increased or decreased in abundance, coloured according to the percentage of total protein changes (darker = more proteins).

(Figure 4.5). The majority of these shared changes were increased protein abundances, with 2017 proteins increased by >1.5 fold abundance and 628 down regulated by >1.5 fold in both WT and ART samples, compared to uninfected IECs (Figure 4.5).

Berger *et al* analysed the impact of CR on IEC metabolic processes (Berger et al., 2017). KEGG pathway enrichment analysis showed that CR WT and Δ ART strains from this study impacted upon similar metabolic processes to the previous report, with down regulation of proteins involved in the tricarboxylic acid (TCA) cycle, glycolysis, amino acid/fatty acid metabolism and upregulation of proteins involved in DNA replication and repair (Figure 4.6). In conjunction with this, WT and Δ ART infections upregulated proteins linked to ribosome biogenesis, protein processing and the complement system (Figure 4.6).

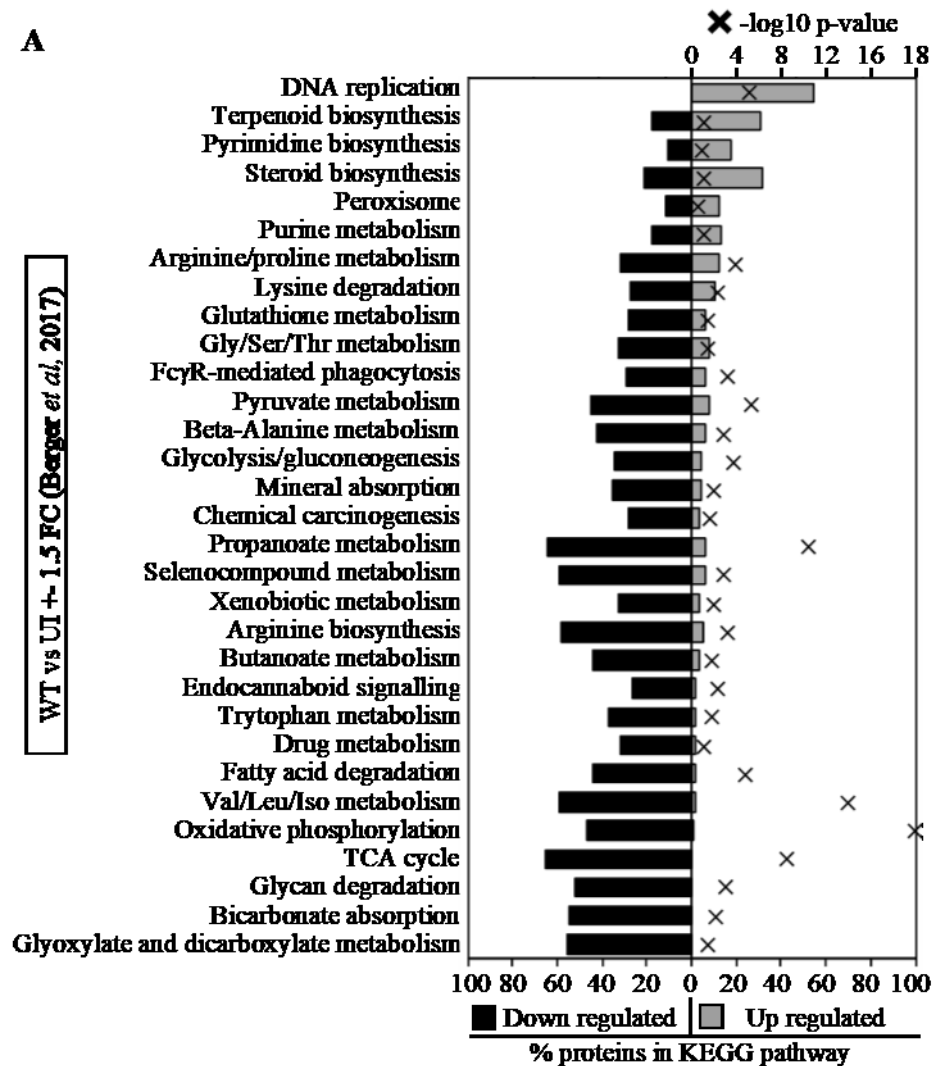
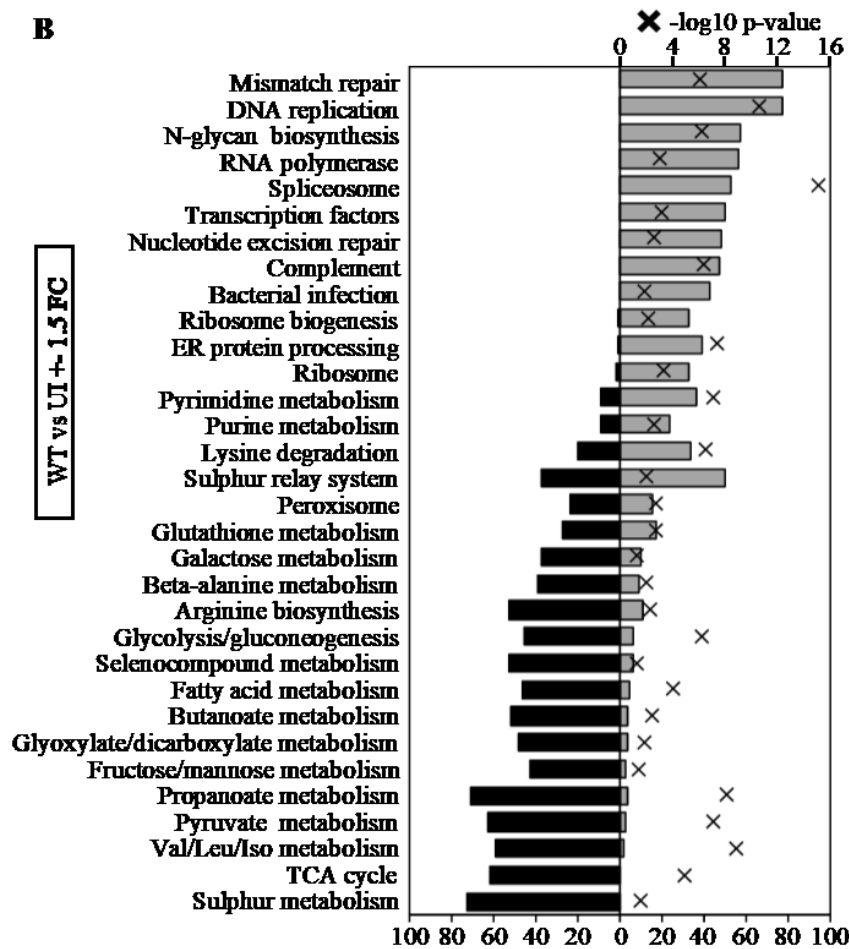


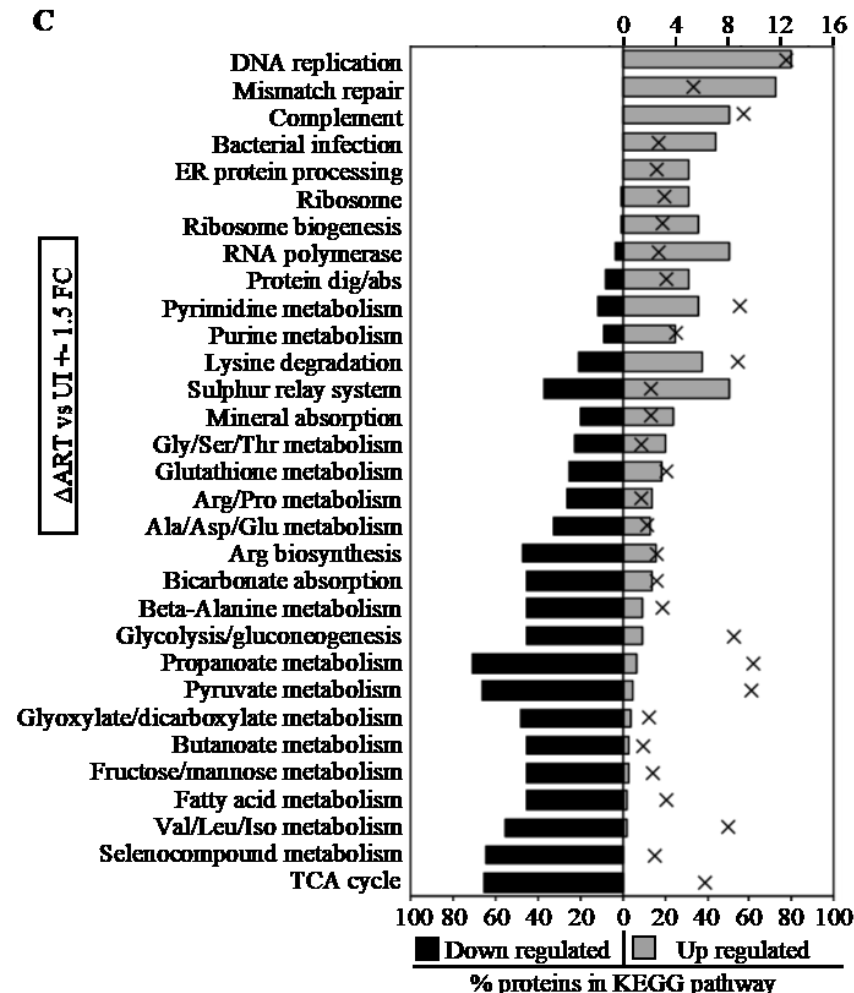
Figure 4.6 - *C. rodentium* Δ ART and WT induce similar global proteomic changes

(A) KEGG pathway analysis of proteins changed by ± 1.5 FC (WT vs UI) from Berger *et al* 2017. This is compared to KEGG analysis of protein abundance changes *versus* uninfected IECs after infection with (B) WT *C. rodentium* or (C) *C. rodentium* EspJ catalytic mutant (Δ ART). Black crosses show the $-\log_{10}$ Bonferroni corrected p-value for each predicted pathway, bars represent the percentage of the pathway present in the group of proteins up (grey) or down (black) regulated by >1.5 fold. Figure adapter from Pollard *et al* 2018.

B



C



4.3 *In vivo* EspJ regulates the IEC immune response to infection

So far, this study has shown that CR *espJ* mutants colonise and induce CCH, and that the Δ ART mutant induces similar global proteomic changes in IECs when compared to WT infections. It is probable that the subtler impacts of EspJ presence/absence are masked by the changes induced by the general infection, as well as the remaining T3SS effectors. To analyse the role of EspJ, the difference in protein abundances between WT and Δ ART infected IECs were directly compared.

From the total 7400 proteins identified, 457 were upregulated in Δ ART vs WT CR-infected IECs, and 159 down regulated by > 1.5 -fold change (Figure 4.7A). ClueGO analysis of the 159 down-regulated genes did not reveal any statistically significant GO-terms. However, the 457 proteins up-regulated by the absence of EspJ activity were linked to the host immune response with GO-terms linked to the defence and inflammatory responses, innate and adaptive immunity, phagocytosis and the actin cytoskeleton (Figure 4.7B). Furthermore, GO-cellular component analysis predicted that these proteins were localised in and around the cell periphery; vesicles/phagosome, cytoskeleton/cortex and cell junctions/adhesions (Figure 4.7C). Further analysis using the ingenuity pathway analysis (IPA) software (Qiagen) was used to infer pathways which may be impacted upon by EspJ and predict the upstream regulators which led to the observed changes in protein abundance. IPA assigns a Z-score to each prediction, suggesting either positive or negative regulation. The top 20 significant pathways suggested that the host immune response is exaggerated in the absence of EspJ activity hinting at an immunosuppressive role for EspJ (Figure 4.8). These pathways include Fc γ - and Fc ϵ -receptor signalling, phagocytosis/micropinocytosis, immune cell maturation and extravasation, cytokine/integrin signalling, reactive oxygen species (ROS) production and thrombin activation (Figure 4.8). Upstream analysis predicted that the absence of EspJ caused an increase in proinflammatory cytokines interleukin IL8, IL6, interferon- γ (IFN γ) and tumor

necrosis factor (TNF), but a decrease in the anti-inflammatory IL10-receptor (Figure 4.8). Interestingly the most likely upstream regulator was bacterial LPS, suggesting that EspJ may be involved in suppressing toll like receptor TLR4 signalling in response to LPS detection.

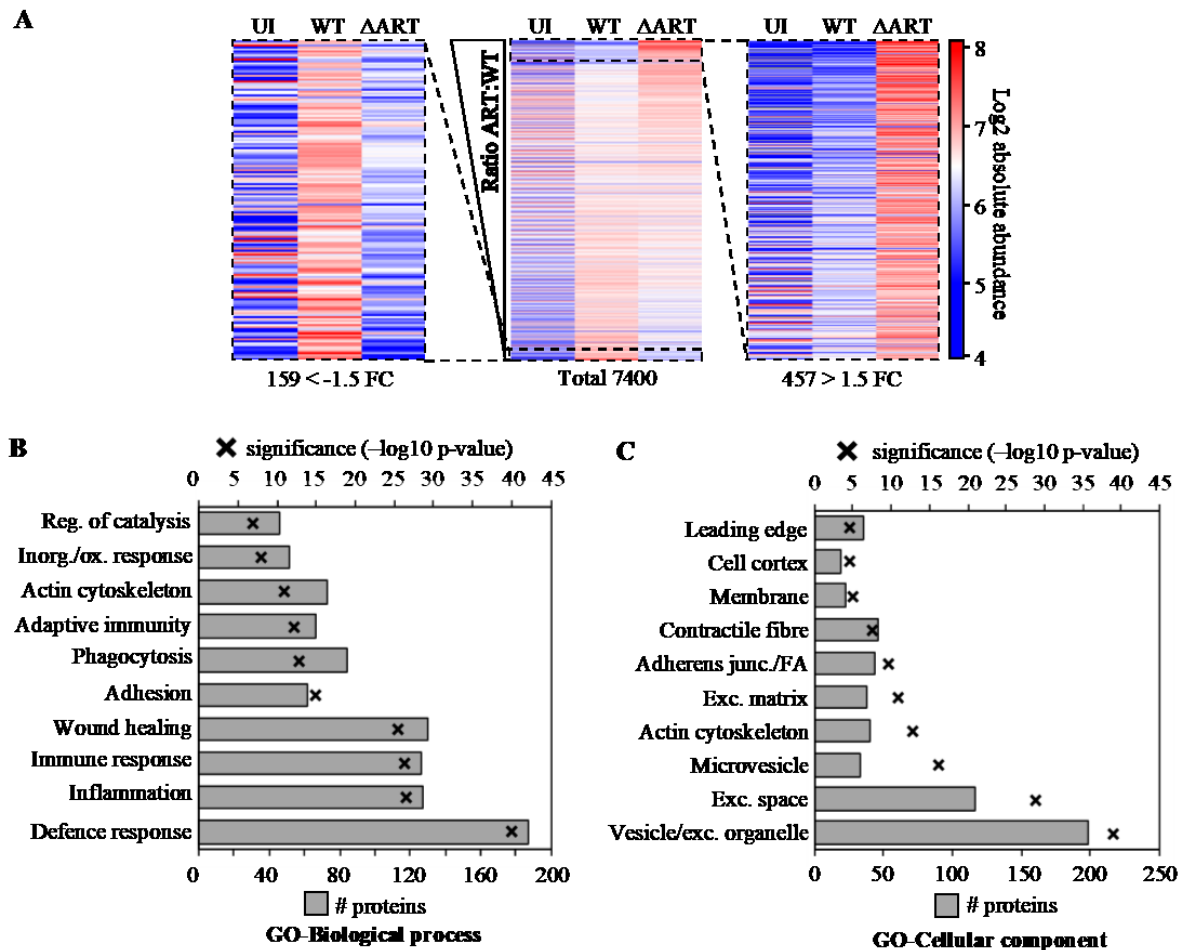


Figure 4.7 - *C. rodentium* espJ is immunomodulatory during *in vivo* infections

(A) A protein abundance ratio from Δ ART to WT *C. rodentium*-infected IECs was made and proteins ranked by the greatest fold change ART versus WT infected IECs (left). The zoomed regions show either 457 proteins with abundance increase of at least 1.5-fold in ART compared to WT infections (right) or 159 with an abundance decrease >1.5-fold (left). Those with increased abundance were assigned to ten GO term groups for biological process (B) and cellular component (C). Analyses are shown with the number of GO-term associated proteins in red bars and the -log p-value in black crosses (with Bonferroni correction). Abbreviations: (-) reg. = negative regulation, inorg./ox. response = response to inorganic substance/oxidative stress, exc. = extracellular, FA = focal adhesion.

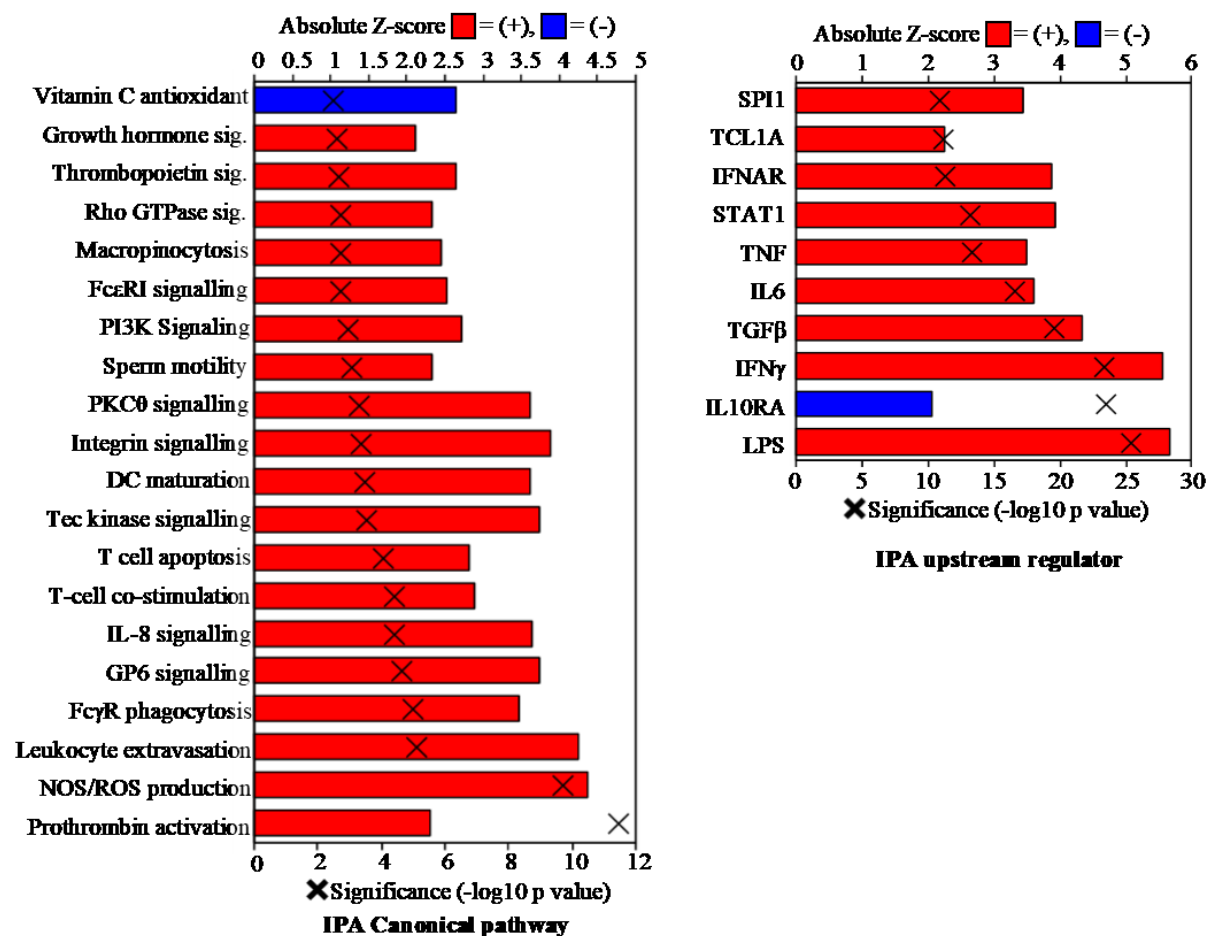


Figure 4.8 - Ingenuity pathway analysis (IPA) of *espJ* induced proteomic changes in IECs

Combined analysis of the IEC protein abundances changed by ± 1.5 fold in Δ ART vs WT-infected IECs, using Qiagen IPA software. (A) Canonical pathways predicted to be regulated by the activity of EspJ and (B) the predicted upstream regulators modulated directly or indirectly by EspJ. Black crosses show the $-\log_{10}$ p value associated with predictions, bars show the absolute Z-score representative of pathway activation (red) or inactivation (blue) in the absence of EspJ catalytic activity. Sig. = signalling.

Due to the prediction of many immune cell processes being regulated by EspJ, it was hypothesised that there may be an increased number of immune cells in the Δ ART-infected IEC samples, either due to recruitment and infiltration or contamination. The proteomics data were searched for immune cell markers commonly used for cell typing (BD biosciences CD marker handbook). CD45, otherwise known as protein tyrosine phosphatase receptor type C (Ptpcr) or leukocyte common antigen (LCA) is found on the surface of T-, B- and NK-cells, macrophages and granulocytes but not platelets, erythrocytes, endothelial or epithelial cells. CD45 was enriched in IECs under both infection conditions by >1.5 FC but was also enriched by > 1.5 FC when comparing Δ ART- to WT-infected IECs suggesting that there is an increased occupancy of immune cells in Δ ART samples (Table 4.1). An increased level of macrophages and endothelial cells in Δ ART samples was also suggested by the presence of CD11b (integrin α_M /CR3) and CD146 (Mcam) respectively. Furthermore, the enrichment of several antimicrobial peptides and proteins supports this theory of increased infiltration; such as lysozyme, myeloperoxidase, lipocalin-2 and granzyme A and B, which are usually associated with immune cells. In contrast, none of the other identified key markers were enriched, and specific T- and B-cell markers were not identified in the IEC proteomes (Table 4.1).

Table 4.1 – Immune cell markers identified in IEC proteomes

The presence of protein markers in numerous cell types is indicated by + (present), - (absent). Log2 protein abundance in uninfected (UI), WT *C. rodentium*-infected (WT) or ΔART-infected (ΔART) IECs are shown and coloured according to intensity (blue – low, white – medium, red- high). Intensity ratios between infection conditions are also shown

														Log2 abundance					
CD	Alias	Cell marker	T cell	B cell	DC	NK cell	Stem cell	Macrophage	Granulocyte	Platelet	Erythrocyte	Endothelial	Epithelial	ΔART:WT	ΔART:UI	WT:UI	UI	WT	ΔART
CD45	Ptprc	General immune	+	+	+	+	+	+	+	-	-	-	-	1.31	2.04	0.73	4.99	5.72	7.02
CD11b	Itgam, CR3	Macrophage			+	+		+						1.09	2.49	1.41	4.36	5.77	6.86
CD146	Mcam	Endothelial	+		+							+		0.79	1.76	0.98	3.73	4.71	5.50
CD9	Tspan29	Platelet	+	+	+		+	+	+	+				0.11	-0.34	-0.46	6.93	6.47	6.59
CD106	Vcam1	Endothelial	-	-	-	-		-	-	-	-	+		0.07	2.05	1.99	4.48	6.47	6.53
Ly6c1		Granulocyte							+					-0.09	3.86	3.95	2.91	6.86	6.77
CD326	Epcam	Epithelial	+		+								+	-0.21	-0.15	0.06	6.56	6.62	6.41
CD34	Mucosialin	Stem	-	-	-	-	+	-	-	-	-	+		-0.35	0.52	0.87	5.20	6.07	5.72
CD3 (δ/ε/γ)	T3	T cell	+			+								N/A	N/A	N/A	N/A	N/A	N/A
CD4	Ly4	T cell	+		+	+	+	-						N/A	N/A	N/A	N/A	N/A	N/A
CD8	Ly2	T cell	+		+									N/A	N/A	N/A	N/A	N/A	N/A
CD19	B4	B cell	-	+	+	-	+	-	-	-	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A
CD11c	Itgax, CR4	DC	+		+	+	+	+	+					N/A	N/A	N/A	N/A	N/A	N/A
CD123	IL3R	DC			+		+	+	+			+		N/A	N/A	N/A	N/A	N/A	N/A
CD335	NKp46	NK				+								N/A	N/A	N/A	N/A	N/A	N/A
CD66b	Cgm6, Cea-3	Granulocyte							+					N/A	N/A	N/A	N/A	N/A	N/A
Ly6g		Granulocyte							+					N/A	N/A	N/A	N/A	N/A	N/A
CD41	Itga2b	Platelet	-	-	-	-	+	-	-	+	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A
CD61	Itgb3	Platelet						+		+		+		N/A	N/A	N/A	N/A	N/A	N/A
CD62p	P-selectin	Platelet								+		+		N/A	N/A	N/A	N/A	N/A	N/A
CD235a	Glycophorin A	Erythrocyte	-	-	-	-	+	-	-		+			N/A	N/A	N/A	N/A	N/A	N/A
CD131	Aic2a	Endothelial	+	+			+							N/A	N/A	N/A	N/A	N/A	N/A

4.4 Selected pathways and proteins modulated by EspJ

Closer analysis revealed that the NOX2 NADPH oxidase complex was enriched in the absence of EspJ catalytic activity during infection, but no alteration of other NOX or DUOX complexes (Table 4.2). This included increased abundance of the constitutively membrane bound gp91-*phox* and p22-*phox* subunits, as well as p47-*phox*, p40-*phox*, p60-*phox* and Rac2 which are recruited to the membrane upon an activating stimulus (Figure 1.7, Figure 4.9) indicating that EspJ may regulate ROS production during the CR infection. This complex is crucial to the phagosomal destruction of pathogenic microbes, a process which appeared enriched in the absence of EspJ. Several key phagocytic receptors were also enriched including the C-type lectin mannose receptor (MR), scavenger receptor CD36 and complement receptors CR1 and CR3 ($\alpha_M\beta_2$ integrin) (Figure 4.9). NOX2 is also crucial to leukocyte extravasation, the process whereby leukocytes are recruited to and infiltrate endothelial or epithelial barriers to enable antigenic sampling. In CR Δ ART-infected IECs key integrins and ERM proteins for leukocyte binding were enriched, as well as matrix metalloproteases (MMP) that act downstream of NOX2 to break epithelial/endothelial barrier contacts allowing extravasation (Figure 4.9, Figure 4.10). Furthermore, proteins involved in the modulation of cytoskeletal and membrane dynamics were also perturbed by the absence of EspJ. These included key kinases the SFK Hck, PI3K, PLC γ and PKC, the GEFs Vav1, DOCK2 and DOCK10, and Rho GTPase Rac2 (Figure 4.10). The complement system was enriched including CR1, CR3 and CR4 as well as complement component (C)3, C4 and C9 which is part of the membrane attack complex (MAC) that forms pores in bacterial membranes causing their lysis. Finally, the abundance multiple zymogens involved in the coagulation cascade were increased including coagulation factors IX/X/XI/XII/XIII, thrombin and fibrinogen (Figure 4.11) along with the cytokine IL36 γ which has been shown to drive mucosal healing during inflammatory bowel disease (Scheibe et al.,

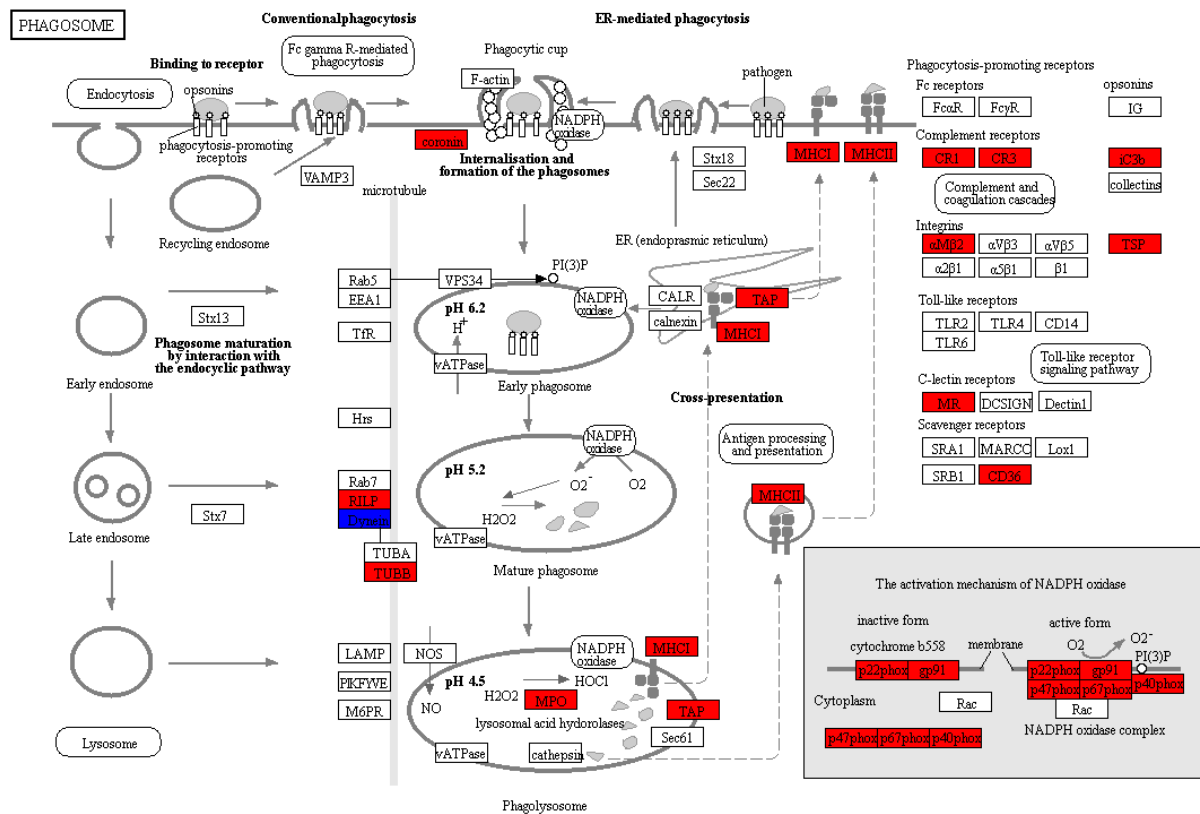
2017). These selected pathways provide a snapshot of the processes that EspJ may directly or indirectly modulate.

These pathways and proteins apparently regulated by EspJ are consistent with the fact that the known EspJ target Src, is membrane tethered, and is important for regulation of the innate immune response and the actin cytoskeleton (Lowell, 2011). However, many kinases are involved in the host immune response and IPA predicted EspJ to regulate Tec kinase, PI3K and PKC θ signalling pathways (Figure 4.8). Because of this and the fact that EspJ ADP-ribosylates a universally conserved kinase domain residue (Src E310) (Young et al., 2014), it was hypothesised that EspJ may modulate multiple kinases.

Table 4.2 – The presence of NADPH oxidase components in IECs from uninfected (UI), WT and Δ ART infected mice

Log2 protein abundances are shown on a blue (low) to white (medium) to red (high) scale.

Complex	Subunit (alias)	Δ ART:WT	Δ ART:UI	WT:UI	UI	WT	Δ ART
Nox2	p47-phox (Ncf4)	1.60	3.24	1.64	4.28	5.92	7.51
Nox2	p40-phox (Ncf2)	1.49	3.27	1.78	4.22	6.00	7.49
Nox1/2/3	Rac2	1.46	1.88	0.41	5.36	5.77	7.23
Nox2	gp91-phox (Cybb)	1.11	2.26	1.16	4.72	5.88	6.98
Nox2	p67-phox (Ncf2)	1.00	1.73	0.73	5.56	6.29	7.29
Nox1/2/3/4	p22-phox (Cyba)	0.88	1.93	1.05	4.99	6.04	6.92
Nox1	Noxo1	0.31	2.97	2.66	4.31	6.97	7.28
Nox1/2/3	Rac1	0.27	0.07	-0.21	6.78	6.57	6.84
Nox1	Noxa1	0.16	1.84	1.67	5.16	6.83	6.99
Nox4	Poldip2	0.05	-0.15	-0.19	6.94	6.75	6.79
Duox2	Duoxa2	0.01	2.19	2.18	4.62	6.80	6.81
Nox1	Nox1	-0.48	2.67	3.15	4.24	7.39	6.91
Nox3	Nox3	N/A	N/A	N/A	N/A	N/A	N/A
Nox4	Nox4	N/A	N/A	N/A	N/A	N/A	N/A
Nox5	Nox5	N/A	N/A	N/A	N/A	N/A	N/A
Duox1	Duox1	N/A	N/A	N/A	N/A	N/A	N/A
Duox1	Duoxa1	N/A	N/A	N/A	N/A	N/A	N/A
Duox2	Duox2	N/A	N/A	N/A	N/A	N/A	N/A



FcγR-MEDIATED PHAGOCYTOSIS

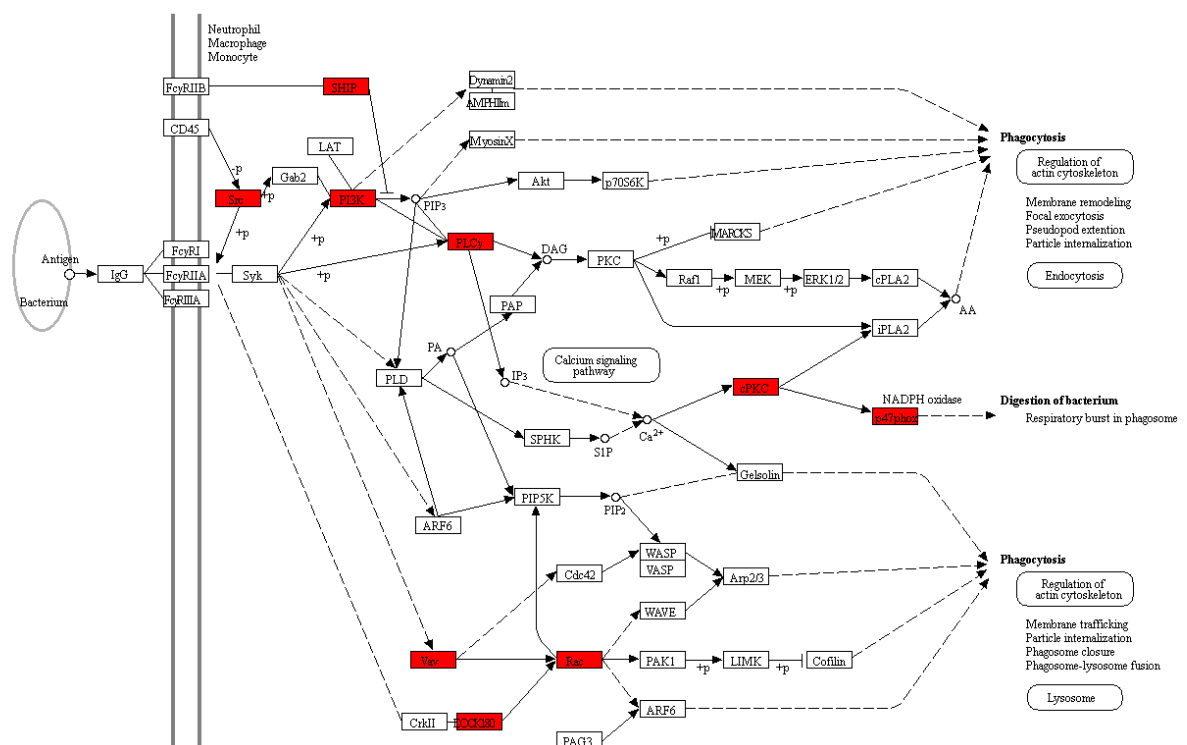


Figure 4.9 – KEGG phagocytosis pathway analysis overlaid with *espJ* induced IEC protein abundance changes

Red = increase > 1.5-fold, blue = decrease > 1.5 fold in ΔART vs WT infected IECs. Images produced using KEGG pathway mapper.

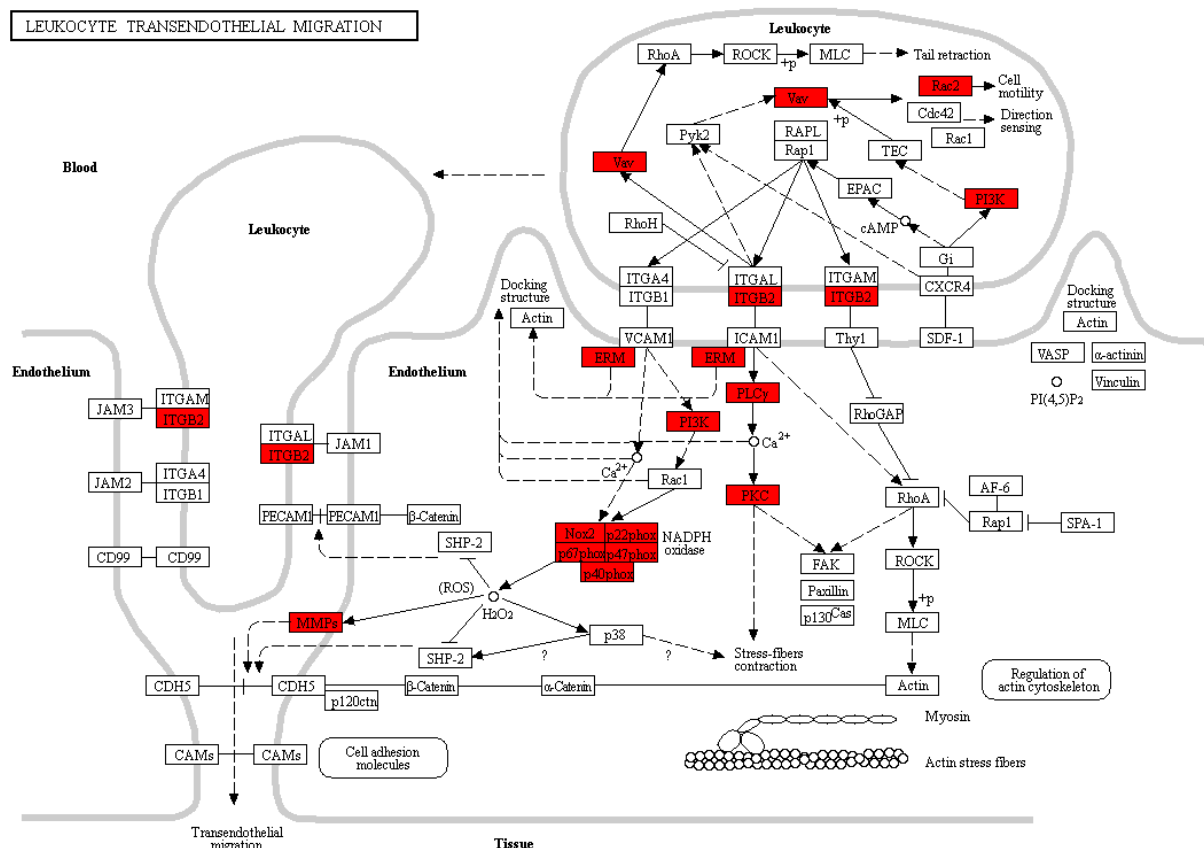


Figure 4.10 – KEGG leukocyte migration pathway overlaid with *espJ* induced IEC protein abundance changes

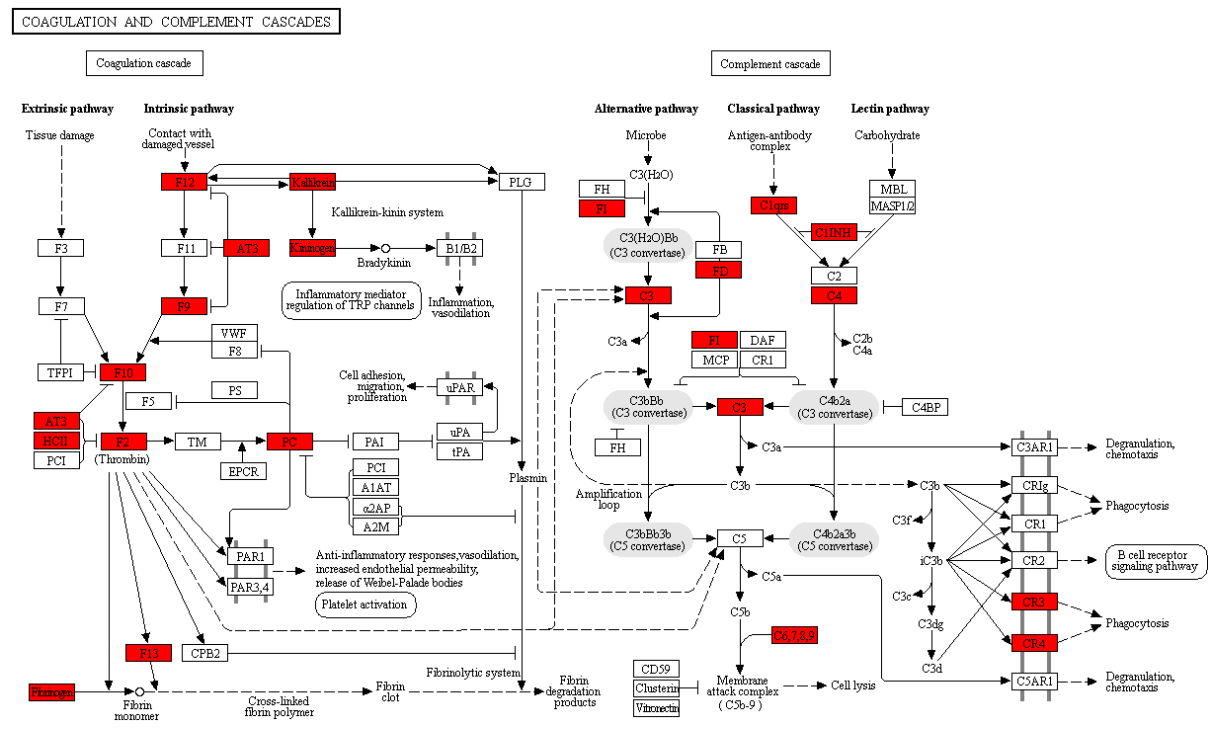


Figure 4.11 -KEGG coagulation and complement cascades overlaid with *espJ* induced IEC protein abundance changes

4.5 EspJ is predicted to modulate multiple tyrosine kinase *in vivo*

To see if the modulation of NRTKs by EspJ is relevant to an *in vivo* setting, analysis of the 457 proteins upregulated in ART versus WT CR infected IECs was performed to predict the upstream kinases regulated by EspJ. We used the kinase expression analysis (KEA) function within expression to kinase (X2K) software to predict upstream regulatory kinases from the group of 457 proteins repressed by EspJ (Chen et al., 2012). KEA uses kinase perturbation studies from multiple protein and gene expression databases, to predict which upstream regulatory kinases are most probably responsible for protein abundance changes. Twelve from the top fifteen most likely kinases regulated by EspJ were tyrosine kinases and ten of these were NRTKs (Figure 4.12). To validate this prediction, ten groups of random 47 proteins, from the total 7400 identified, were also analysed using KEA. None of the top fifteen kinases

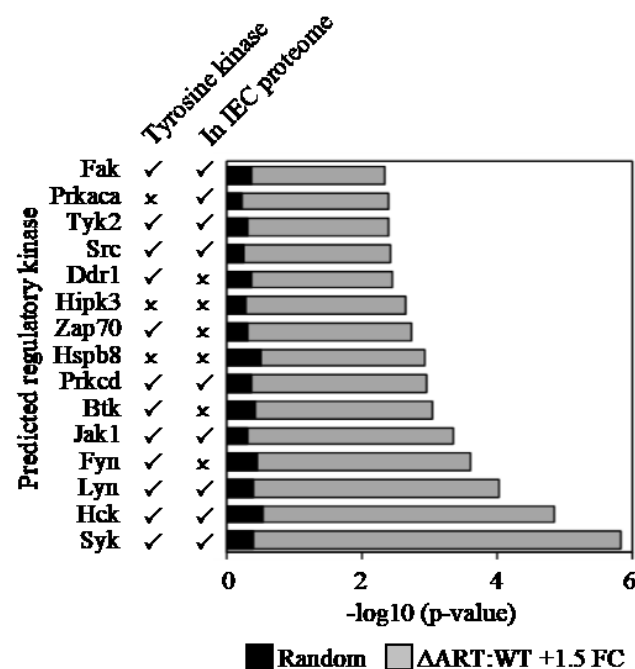


Figure 4.12 - Upstream analysis of 457 proteins upregulated in Δ ART versus WT *C. rodentium* infected IECs.

The fifteen most likely upstream regulatory kinases targeted by EspJ to produce the observed proteomic changes. Grey bars = kinase p-value for the group of EspJ-modulated proteins, black bars = mean p-value of the kinase across X2K runs from ten random lists of 457 proteins from the total 7400. Ticks = tyrosine kinases and whether the kinase was identified in the IEC proteome.

predicted to be regulated by EspJ were also significantly predicted across these ten random datasets. Seven of these predicted kinases were present in the IEC proteomes, but only the abundance of Hck was changed when comparing WT and Δ ART infected IECs, which increased by over two-fold.

4.6 Discussion

In this chapter the role of EspJ was investigated during the CR infection of C57BL/6 mice comparing colonisation dynamics, levels of CCH and proliferation, and IEC quantitative proteomes. Historically the elucidation of T3SS effector roles has relied upon interpretation of cell culture infections, pathological and immunological phenotypes. However, the true role is often eclipsed by the impacts of tens of other effectors *in vivo*. The proteomics methodology in this chapter allowed an unbiased insight into the physiological impacts of the T3SS effector EspJ *in vivo*.

This chapter confirms that EspJ does not play a critical role in the initial colonisation of mice, as CR $\Delta espJ$ (deletion mutant) and Δ ART (deletion mutant complemented with *espJ*-R79A catalytic mutant) from this study colonised normally up to day 8. A previous study of the infection of C57BL/6J mice with a different CR $\Delta espJ$ strain found that during the first 10 days after inoculation colonisation was as expected when compared to WT CR. However, a delayed clearance was displayed by this strain with significantly increased pathogen burdens in the colon at day 14 compared to WT infections. Further, at day 20 when WT CR had cleared fully, the *espJ* deletion mutant was still present in the colon and caecum (Dahan et al., 2005). This prior study used a kanamycin cassette for deletion of *espJ* whereas in this thesis the *espJ* deletion was not replaced with a cassette. However, to further understand the proposed delayed clearance further studies would be required.

Dahan *et al*, observed that CR $\Delta espJ$ caused significant CCH compared to uninfected mice but not significantly different to WT infections. Contrastingly, this thesis suggested that the absence of EspJ, or its catalytic activity, causes increased hyperplasia. It is difficult to compare these experiments as Dahan *et al* did not show the data. It is possible that *in vivo* EspJ repression of Src activities could limit CCH, as Src kinase has pro-proliferative roles (Imada et al., 2016; Thomas and Brugge, 1997), and therefore the removal of EspJ would result in exaggerated CCH. In line with this, Csk deletion *in vivo* has been demonstrated to significantly increase IEC proliferation, and small intestinal/colonic crypt length typical of CCH (Imada et al., 2016).

The CR mouse model of infection has been used to extensively investigate the role of the T3SS and its effectors. Disruption of the T3SS machinery by deletion of the ATPase *escN* removes the CR ability to colonise mice (Deng et al., 2004), as does deletion of the effector Tir or the bacterial outer membrane protein intimin (Deng et al., 2003; Frankel et al., 1996). Several single effector mutants including Δmap , $\Delta nleC$, $\Delta nleF$, $\Delta nleD$, $\Delta espF$, $\Delta espG$ and $\Delta espH$ are outcompeted by WT CR during coinfections *in vivo*, but are not attenuated in single infection studies (Echtenkamp et al., 2008; Ma et al., 2006; Mundy et al., 2004, 2005; Pallett et al., 2017). In contrast $\Delta nleA$ and $\Delta nleB$ deletion mutant strains show significantly impaired colonisation dynamics and did not induce hyperplasia to the same level as WT strains *in vivo* (Kelly et al., 2006; Mundy et al., 2004; Pollock et al., 2017). Like the $\Delta espJ/\Delta ART$ mutants, the absence of *espO* does not impair colonisation dynamics. However, crypt lengths were significantly lower in $\Delta espO$ than WT *C. rodentium* infected colons suggesting that *espO* may have CCH-promoting qualities, opposing the action of *espJ* (PhD thesis (Constantinou, 2013)).

The CCH caused by CR is induced by the loss of epithelial barrier integrity, allowing bacteria to pass into the lamina propria, and the thickening of colonic mucosae arises through excessive epithelial regeneration and repair and is accompanied by intestinal inflammation (Collins et al., 2014). Exaggerated proliferation is thought to be orchestrated by signalling from

WNT- β -catenin (Papapietro et al., 2013), Stat3 (Gibson et al., 2010) and PI3K- β -catenin (Brown et al., 2011) pathways in response to numerous growth factors and cytokines. In addition, pro-inflammatory MEK, ERK and NF κ B pathways are induced by TLR-MyD88 mechanisms in response to epithelial damage (Gibson et al., 2010). While molecular mechanisms underlying CCH are not yet fully understood there is evidence for crosstalk between NF κ B and β -catenin pathways to support CCH manifestation (Chandrakesan et al., 2012, 2013; Wang et al., 2006). In this chapter the quantitative proteomics analysis of WT or Δ ART CR infected IECs suggested that EspJ regulates inflammatory processes, and it could be that in the absence of EspJ increased inflammatory mechanisms contribute to an increase in CCH.

The use of CR Δ ART (*espJ* R79A catalytic mutant) enabled the specific analysis of IEC proteome changes induced by the ART activity of EspJ. Whereas 457 protein abundances were increased by >1.5 FC when comparing Δ ART to WT CR infected IECs only 159 were down regulated. This imbalance and the fact that statistically no significant GO terms were predicted from the down regulated proteins may reflect the inhibitory activity of EspJ. Further analysis suggested that there was an enhanced inflammation, immune responses and wound healing in the absence of EspJ activity, and a prediction of the upstream kinases regulated by EspJ indicated that the observed proteomics changes may arise from modulation of multiple tyrosine kinases.

Upregulation of wound healing and the coagulation cascade was strongly linked to the absence of EspJ activity. CR murine infections are used as models for Crohn's disease and ulcerative colitis, inflammatory bowel diseases to which mucosal damage is a key feature (Rieder et al., 2007). During acute or chronic intestinal inflammation, ROS and extracellular matrix (ECM)-degrading enzymes such as MMPs are released by macrophages and neutrophils inducing damage to the mucosal surface (Rieder et al., 2007). A proteolytic cascade

orchestrated by platelet-born, serine protease coagulation factors is then central to the blood clotting which culminates in the cleavage of fibrinogen to fibrin (Palta et al., 2014). Many of these were more enriched in Δ ART-infected IECs compared to WT CR infections. The fact that leukocyte migration, ROS and NOS production are also predicted to be enriched in the absence of EspJ suggests that the EspJ may regulate the host immune response to protect CR and dampen host tissue damage. It could be hypothesised that in the absence of immunoregulation by EspJ there is exaggerated tissue damage, which ends in the increased abundance of proteins involved in the coagulation cascade.

It is known that EspJ is able to inhibit CR3-mediated and Fc γ R-mediated phagocytosis during cell culture infections (Marchès et al., 2008). The fact that the complement system, and Fc γ R phagocytosis were predicted to be upregulated in IECs in the absence of EspJ catalytic activity suggests that EspJ can also inhibit phagocytosis *in vivo*. Several complement components are constitutively expressed in IECs and levels of C3 are also increased in the dextran sulphate sodium (DSS) model of colitis (Sünderhauf et al., 2017), again suggesting that EspJ may have roles for the protection of host tissue. The abundance of IEC complement components can be increased by LPS stimulation (Sünderhauf et al., 2017) which indicates that EspJ may also modulate signalling downstream of TLR4 recognition of bacterial LPS. In line with this, LPS was predicted as the most likely upstream regulator impacted by EspJ presence/absence.

IEC proteomes suggested that EspJ absence increases the expression of key kinases (PLC γ /PKC/Hck), GEFs (Vav1/DOCK2) and Rho GTPases (Rac2) in the Fc γ R stimulated phagocytosis cascade. In addition, increased levels of MHC-II components as well as iNOS and the entire NOX2 complex indicates that EspJ modulates antigen presentation and ROS/RNS production. However, it is not clear whether these are direct or downstream impacts of the inhibition of kinases at the phagocytic receptor. M-cells sample antigens and whole microorganisms from the intestinal lumen and it is possible that EspJ could interrupt these

processes (Mabbott et al., 2013). Furthermore, IECs express MHC-II molecules and have been shown to function as non-professional antigen presenting cells (Hershberg and Mayer, 2000). The expression of the MHC-II complex is induced by the cytokine IFN γ (Hatano et al., 2013; Matsumoto et al., 1995; Thelemann et al., 2014), the signalling of which was also predicted to be directly or indirectly regulated by EspJ.

IECs induce iNOS expression during WT CR infection, and iNOS deficient mice are delayed in their clearance of the pathogen, suggesting that RNS play a role in the defence against CR (Vallance et al., 2002). NOX2 defects leads to reduced ROS production and susceptibility to recurrent infections in the form of human CGD. It could be hypothesised that the increased ROS/NOS and phagocytic processes observed in this chapter would result in faster clearance of CR Δ ART. However, as previously discussed, a prior study suggests that CR Δ espJ infection persists for longer than WT (Dahan et al., 2005). There is lost of evidence supporting roles of the microbiota in in ROS/RNS homeostasis. A recent study showed that the absence of *gp91-phox* (inactive NOX2) or *p22-phox* (inactive NOX1-4) in germ-free mice, led to worsened CR-induced disease symptoms. Interestingly, H₂O₂ producing bacteria in the microbiome could compensate for NOX absence (Pircalabioru et al., 2016). It is possible that the microbiome can compensate for EspJ inactivation of NOX2 explaining the lack of significant differences in disease symptoms between WT and Δ ART-infections. This could also explain why another study found that while *Rac2*^{-/-} mice had worsened disease symptoms during CR infection, *Nox2*^{-/-} mice did not (Fattouh et al., 2013). It would therefore be interesting to study the impact of CR WT and Δ ART infection on the microbiome or their disease progression in germ free mice.

The reported roles for IECs in regulation of innate and adaptive immunity is growing and data from this chapter may support the view of IECs as pseudo-immune cells. However, it was previously estimated that the IEC purification strategy employed in this study yields about 90%

purity (Crepin et al., 2015). Therefore, IEC sample contamination by immune cells, platelets, and extracellular matrix proteins must be also considered in the interpretation of these data. CR infection causes a host inflammatory response including recruitment of neutrophils, macrophages and dendritic cells (Collins et al., 2014). The further enrichment of Ptpcr and Itga_M as well as immune cell antimicrobial proteins and peptides in Δ ART-infected IECs suggested increased immune cell occupancy in the absence of EspJ. Furthermore, the previously discussed NOX2 complex is regarded as immune cell-specific. However, as key markers for T- and B-cells were either not enriched or not identified, this infiltration may be primarily leukocytes not lymphocytes. The absence of EspJ ART activity was predicted to enrich leukocyte transcytosis which would explain the enriched leukocyte markers.

While this chapter considers IECs as a single entity it is in fact a very diverse cellular population. Interestingly a recent single cell RNAseq survey of mouse small intestine identified a subset of specialised IECs called tuft cells that were Epcam⁺/Ptpcr⁺, showing for the first time Ptpcr in cells of a non-haematopoietic lineage (Haber et al., 2017). Tuft cells have been reported to accumulate and activate T-cells responses during parasitic infections (Gerbe and Jay, 2016), and this Epcam⁺/Ptpcr⁺ subset was enriched during helminth infection of mice (Haber et al., 2017). It is possible that these tuft cells could explain the increased abundance of Ptpcr during Δ ART CR infection, although more in depth studies would be required. As SFKs inhibit differentiation in IECs (Imada et al., 2016), the modulation of Src by EspJ could in theory affect the abundance of highly differentiated cells such as Tuft cells. Haber *et al* demonstrate that it is increasingly difficult to categorise cell types based upon cell markers that were previously considered well established. However, a similar study combining FACS and RNAseq/proteomics study during CR infection would allow the proper characterisation of the impact of EspJ on immune cell recruitment and IEC differentiation. This would also allow the differentiation between EspJ's specific role within IECs and immune cells.

The A/E pathogens are well characterised extracellular bacteria that *in vivo* colonise the apical surfaces of IECs. Here they also come into contact with cells of the innate immune system either indirectly through uptake by microfold cells and presentation to macrophages and DCs in the lamina propria, or directly with infiltrating immune cells protruding through the IEC layer (Etienne-Mesmin et al., 2011). *In vitro* these bacteria can infect immune cell lines and the presence of anti-phagocytosis T3SS effectors such as EspJ, EspH, EspF and EspG (Dong et al., 2010; Humphreys et al., 2016; Marchès et al., 2008) is indirect evidence for the interaction between A/E pathogens and macrophages/neutrophils/DCs *in vivo*. From this proteomics screen it is not clear whether the primary function of EspJ *in vivo* is to inhibit immune cell recruitment through interruption of IEC signalling pathways that otherwise lead to cytokine/chemokine leukocyte attractant secretion, or to directly interrupt immune cell bactericidal activities, or both. The numerous roles of Src kinase, aside from stimulation of ITAM-containing receptors, affords EspJ many opportunities to interrupt innate immune signalling, explaining the diverse observed proteomic changes in this chapter. For example, Src kinase activation is required for TLR4 signalling in response to LPS (Gong et al., 2008; Lee et al., 2012) and Src inhibitors can block signalling in response to the TLR4-induced cytokine IFN γ (Orlicek et al., 1999). Therefore, EspJ may directly inhibit the TLR4 pathway but also dampen the effects of TLR4-induced cytokines downstream. Src kinase is also required for the activation of the NADPH complex, likely through activation of Rho GTPases (Gianni et al., 2008; Lee et al., 2012), but is also activated by ROS (Giannoni et al., 2005). This means that as well as preventing signalling for cytoskeletal remodelling at the phagocyte receptor, EspJ has the potential downstream to inhibit the bactericidal properties of phagosomes, also preventing antigen presentation and leukocyte transepithelial migration.

While a lot has been learned about the role of EspJ *in vivo*, this chapter only describes a proteomics screen of IECs pooled from five mice into one sample, denying the robust statistical

analysis of protein abundance changes. A more comprehensive study with several biological replicates for WT and Δ ART CR-infected mice is needed to enable more in-depth proteomics analysis. There is also the possibility that EspJ may ADP-ribosylate other proteins *in vivo*. Accordingly, upstream analysis of the kinases likely perturbed by EspJ *in vivo* suggested that it targets a range of NRTKs from the Src, Tec, Jak, Syk and Fak families. In addition, many of these NRTKs were identified in the IEC proteomes extracted from CR-infected mice, so are realistic targets. This is plausible given the highly conserved Src kinase glutamic acid ADP-ribosylated targeted by EspJ, and if true would explain how EspJ is able to modulate so many aspects of the immune response. Given this prediction, the combination of IEC a phosphoproteome and ADP-ribosylome could be instrumental in deciphering the targets and specific pathways modulated by EspJ *in vivo*. Defining the target repertoire will be the focus of the next chapter.

Chapter 5 – Biochemistry and substrate repertoire of EspJ

5 Results – Biochemistry and substrate repertoire of EspJ

5.1 EspJ ADP-ribosylation of Src kinase isoforms

It is known that EPEC EspJ is able to modify WT full length Src, the SH1 domain in isolation (residues 250-533) as well as the ‘kinase dead’ K295M mutant form of Src, each being amidated and ADP ribosylated at Src E310 (Young et al., 2014). Co expression of Src and EspJ inhibited Src kinase activity and auto phosphorylation of Y416, suggesting that EspJ can ADP-ribosylate the inactive form of Src preventing auto activation. Defining the repertoire of Src isoforms that EspJ can modify and inhibit *in vitro* is important to fully understand its impacts during infection.

Gel filtration of GST Src₂₅₀₋₅₃₃-K295M revealed two doublets, one corresponding to a GST-Src homodimer and one with a seemingly much higher molecular weight (Figure 5.1A/B). After western blotting it was found that this ‘kinase dead’ mutant was phosphorylated in the homodimeric species (Figure 5.1C). The loss of phosphorylation after Y416A/F mutations (Figure 5.1C) revealed that the ‘kinase dead’ mutant was indeed Y416 phosphorylated. Mass spectrometry has shown that purification of this ‘kinase dead’ Src variant resulted in two populations, one phosphorylated at Y416 and one not, a surprising finding considering that this is the residue for auto phosphorylation (Piserchio et al., 2009, 2012). His-tagged EPEC EspJ₂₈₋₂₁₇ was incubated with each of these species and NAD biotin and ADP ribosylation of Src assessed by western blotting (Figure 5.1D). Src₂₅₀₋₅₃₃K295M was only ADP ribosylated in the homodimeric pY416 isoform of Src. However, gel filtration of Src₂₅₀₋₅₃₃K295M/Y416A/F still eluted as two doublets and EspJ was only able to ADP ribosylate the peak with the same retention volume as the Src-₂₅₀₋₅₃₃K295M single mutant. Nevertheless, this adds to the repertoire of Src isoforms that EspJ is able to ADP-ribosylate; we now know that EspJ can modify Y416 autophosphorylated active Src, the constitutively active Src-Y527F mutant, and the inactive Src-K295M/Y416A double mutant.

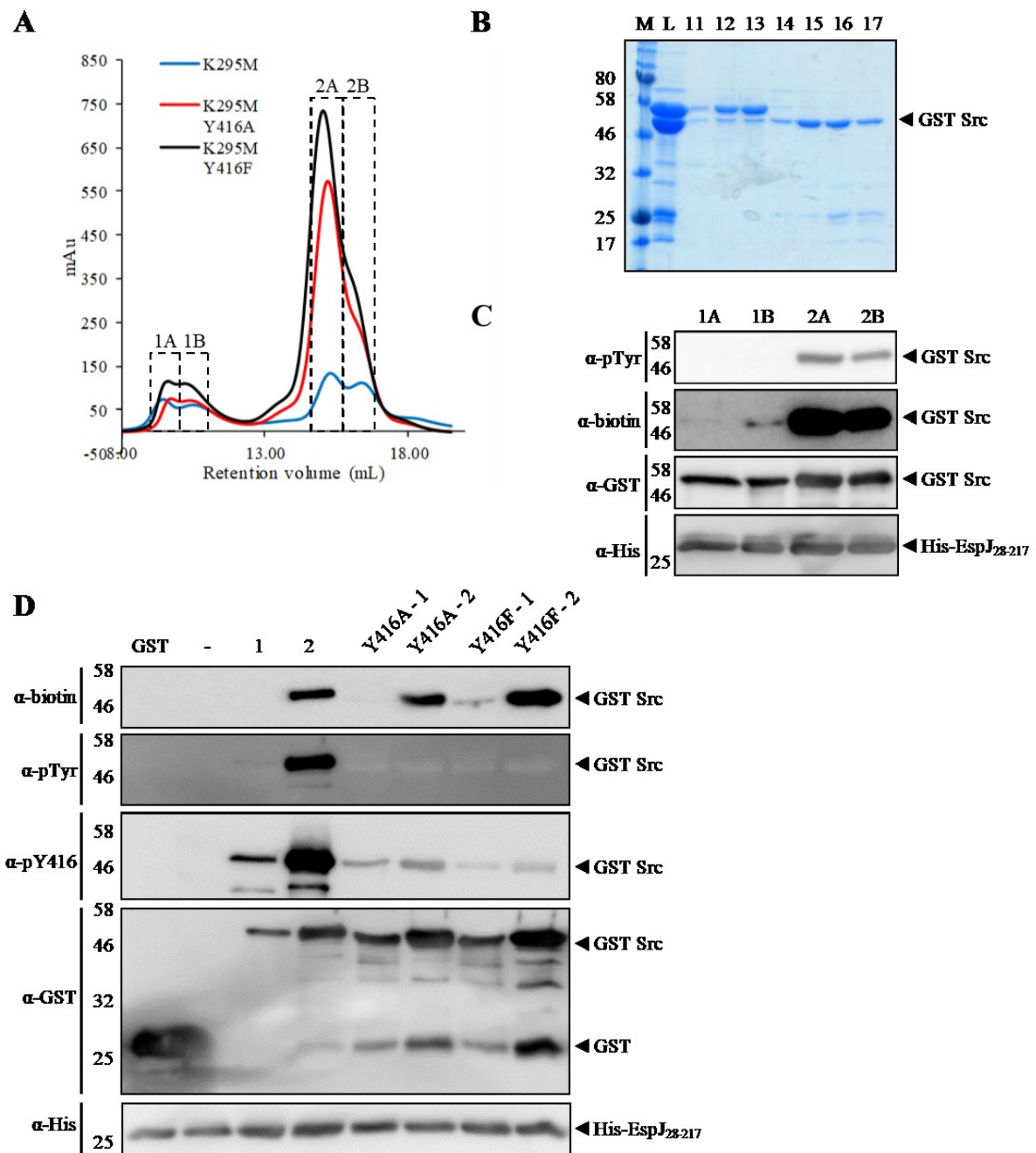


Figure 5.1 - Defining the functional isoforms of Src and EspJ

(A) Gel filtration traces and (B) corresponding SDS-PAGE analysis for GST-Src₂₅₀₋₅₃₃ mutants K295M (blue), K295M/Y416A (red) and K295M/Y416F (black). Elution fractions labelled 1A/1B/2A/2B relate to western blot (C) which shows that EPEC His-EspJ₂₈₋₂₁₇ can ADP-ribosylate Src₂₅₀₋₅₃₃ K295M, peak 2A/B but not 1A/B using NAD-biotin. This also shows that only peak 2 is tyrosine phosphorylated. (D) While Y416A/Y416F mutations to remove this tyrosine phosphorylation, it does not prevent the ADP-ribosylation of peak 2 by EspJ.

5.2 EspJ can ADP-ribosylate Csk

We have shown the ability of EspJ_{EPEC} to ADP-ribosylate multiple Src isoforms. However, as EspJ modifies a highly conserved catalytic residue Src-E310, it was postulated that EspJ may be able to ADP-ribosylate other kinases. As proof of principle for this theory, EspJ was incubated with NAD-biotin and the tyrosine kinase Csk, as well as the EPEC/EHEC T3SS-effector serine/threonine kinase NleH1, alongside Src kinase (Figure 5.2). Western blotting with anti-biotin reagents showed that while EspJ could not modify NleH1, it could ADP-ribosylate Csk. This provided rationale for the in-depth investigation of the EspJ substrate repertoire.

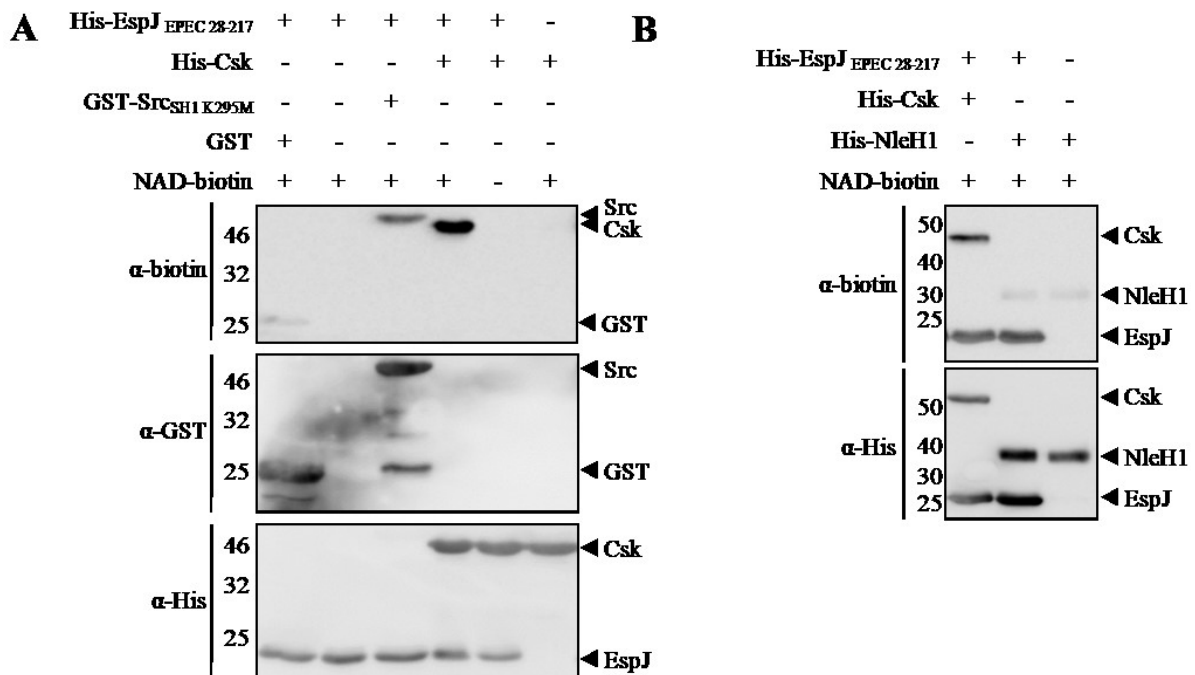


Figure 5.2 - Testing the ability for EspJ to ADP-ribosylate other kinases with NAD-biotin

His-EspJ_{EPEC 28-217} was incubated with His-Csk/GST-Src₂₅₀₋₅₃₃K295M (A) or His NleH1 (B) and NAD-biotin for 1 hour before analysing ADP-ribosylation by western blotting and streptavidin-HRP (α -biotin). (A) EspJ-EPEC can ADP-ribosylate Csk in vitro. (B) EspJ is not able to ADP-ribosylate the serine/threonine kinase NleH1 using the same reaction conditions as for the ADP-ribosylation of Csk.

5.3 EspJ_{EHEC} is more active than EspJ_{EPEC}

Chapter 3 describes initial characterisation of the EspJ homologues purification and enzymatic activities. Multiple *in vitro* ADPr assays suggested that despite being most favourable for purification, recombinant EspJ_{EPEC} was not consistently active for unknown reasons. Despite lower solubility, EspJ_{EHEC} was active in every assay, and optimisation of buffer conditions vastly improved its purification. Here, across a gradient of NAD-biotin concentration EspJ_{EHEC} was consistently more active than EspJ_{EPEC} (Figure 5.3A), and this is further demonstrated by the amount of Src able to be pulled down with Neutravidin resin post ADPr with NAD-biotin (Figure 5.3B). It was hence decided that EspJ_{EHEC} should be used for further characterisation of targets.

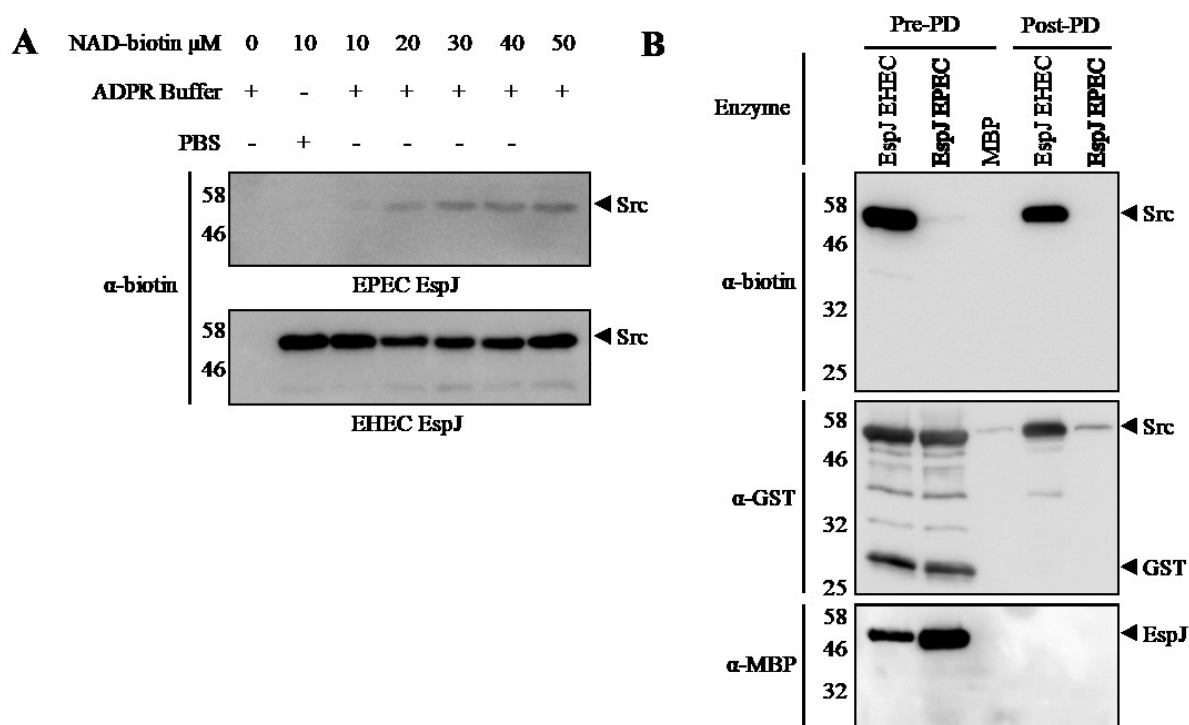


Figure 5.3 – Comparison of EspJ_{EHEC} and EspJ_{EPEC} enzymatic activity

(A) ADP-ribosylation of GST-Src₂₅₀₋₅₃₃K295M/Y416A by MBP-EspJ_{EPEC} (top panel) or MBP-EspJ_{EPEC} (bottom panel) with increasing concentrations of NAD-biotin and in either PBS or a ADP-ribosylation (ADPR) buffer. Analysis by western blotting with anti-biotin reagents show that EspJ_{EHEC} is more efficient (B) The ADP-ribosylation of GST-Src₂₅₀₋₅₃₃K295M/Y416A by MBP-EspJ_{EPEC}, MBP-EspJ_{EPEC} with NAD-biotin (pre-PD) followed by enrichment of ADP-ribosylated Src using neutravidin resin (post-PD).

5.4 Chemical proteomics for the definition of EspJ substrates

In this study 2-ethynyl-adenosine-NAD (eNAD) was used to investigate EspJ targets (Figure 5.4, Figure 5.5). EspJ was able to ADP-ribosylate itself and recombinant Src using eNAD as shown by in-gel fluorescence and anti-biotin western blotting (Figure 5.6A/B) after conjugation of the capture reagent, AzRTB; Az - azide for click chemistry reactivity, R – arginine which can be used to cleave functional groups, T – TAMRA fluorophore, and B – biotin for detection or enrichment. Using Neutravidin resin it was possible to enrich ADP-ribosylated recombinant Src both from the more complex background of A549 lysate (Figure 5.6C/D). By in-gel fluorescence it was not possible to visualise the ADPr of proteins by EspJ in A549 cell lysate (Figure 5.6D). This indicated that mass spectrometry may be required to detect the ADPr of EspJ substrates in cell lysate.

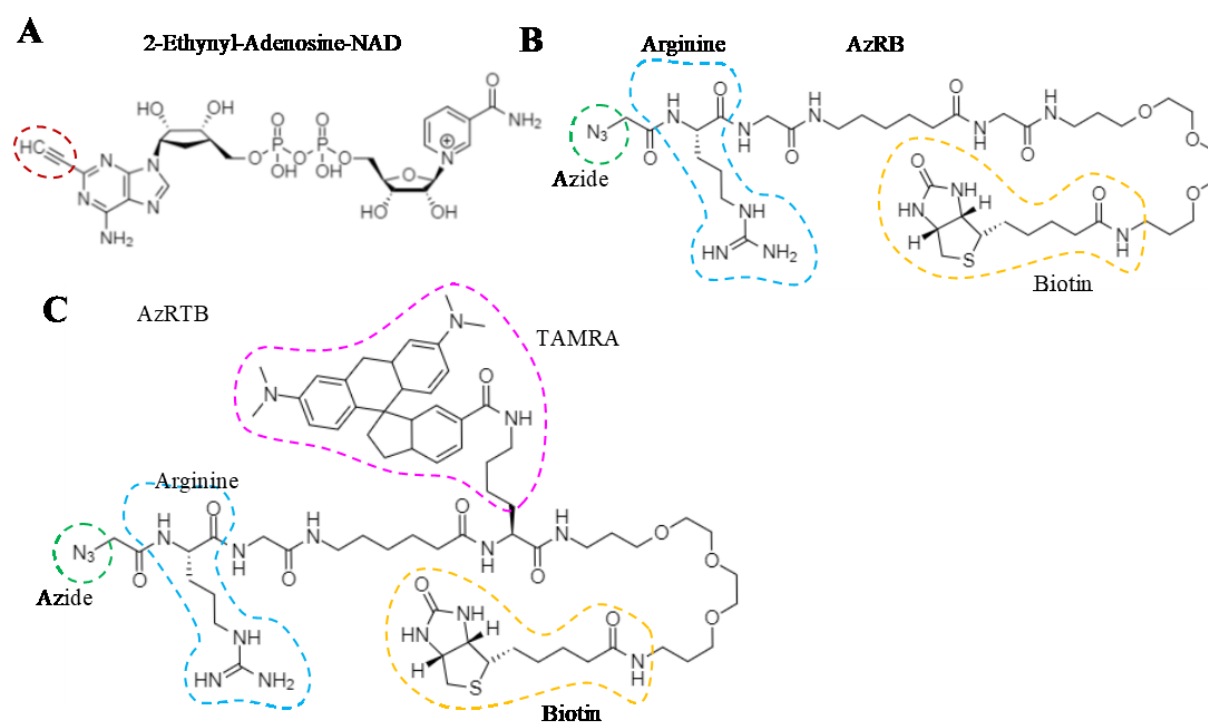


Figure 5.4 - Chemical reagents used for click-chemistry in this study

Skeletal structures for (A) 2 ethynyl adenosine NAD, (B) AzRB and (C) AzRTB with key groups circled. Red – alkyne, green – azide, blue – arginine for trypsin cleavage, yellow – biotin and magenta – TAMRA.

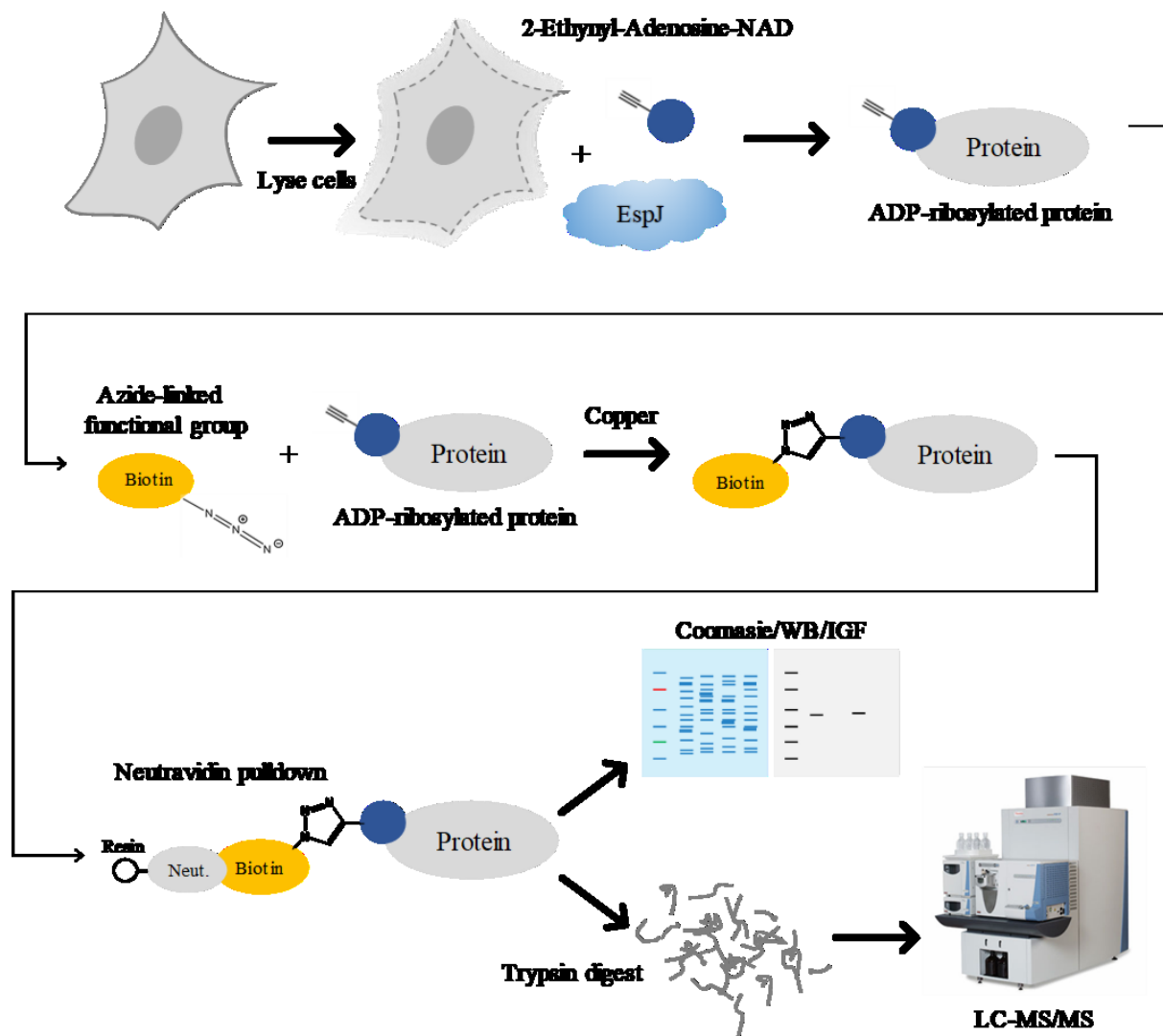


Figure 5.5 – Workflow for analysis of EspJ ADP-riboyslation by click-chemistry

EspJ is incubated with eNAD and either cell lysate or a recombinant protein substrate in an ADP-ribosylation reaction. ADP-ribosylated proteins are then labelled with a biotin payload through the click chemistry reaction between azide-biotin and the alkyne of ADP-ribosylated proteins. Biotin-labelled proteins are then enriched via a Neutravidin pulldown and are analysed by SDS-PAGE, western blotting. Alternatively, proteins are digested with trypsin before LC-MS/MS analysis.

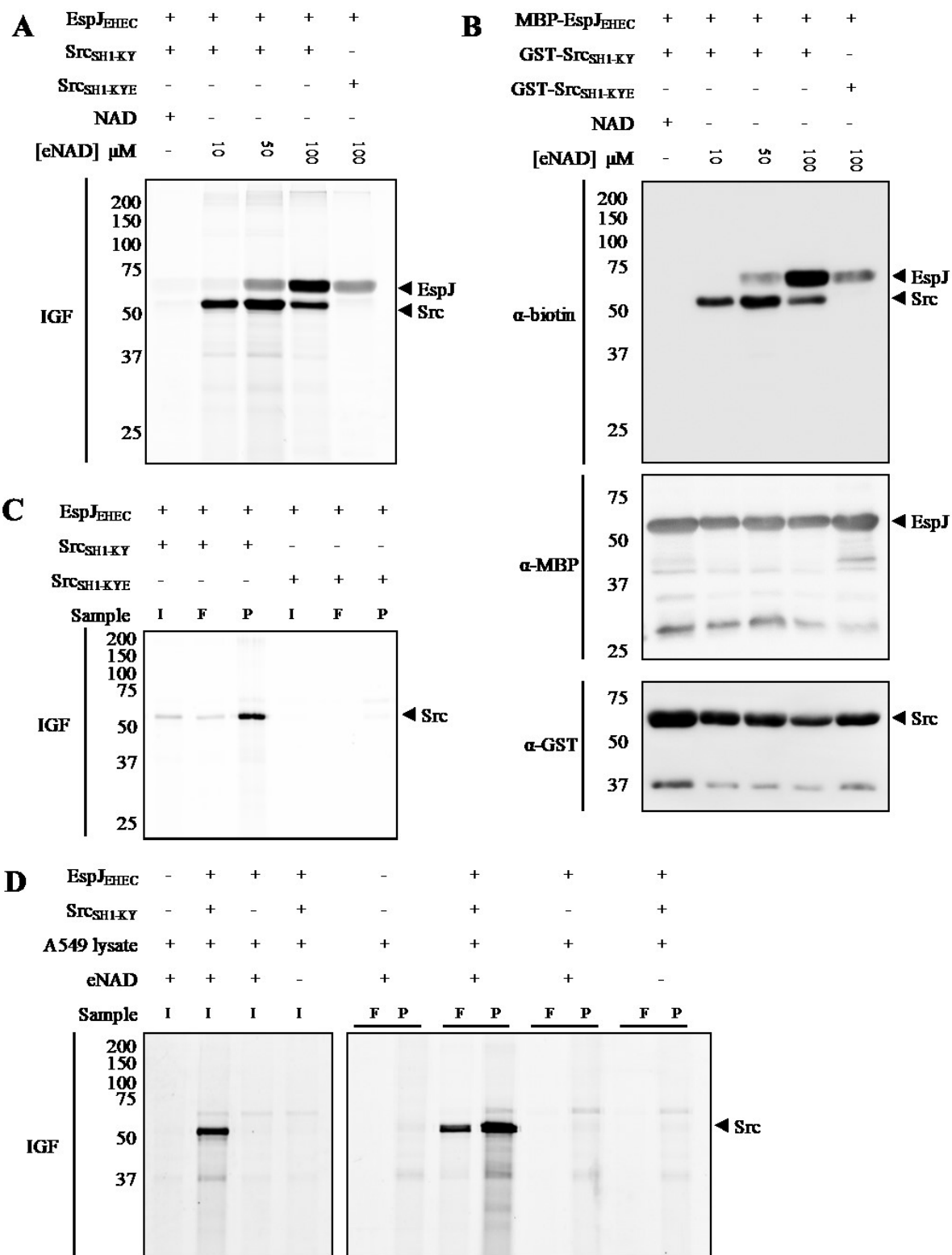


Figure 5.6 – Src can be enriched after ADP-ribosylation by EspJ using eNAD

EspJ can ADP-ribosylate Src₂₅₀₋₅₃₃-K295M/Y416A (Src₂₅₀₋₅₃₃-KY) but not Src₂₅₀₋₅₃₃-K295M/Y416A/E310A (Src₂₅₀₋₅₃₃-KYE) using eNAD as shown by (A) in gel fluorescence and (B) anti-biotin western blotting after reaction with AzRTB. (C) and (D) Resulting ADP-ribosylated recombinant Src can be enriched by Neutravidin pulldown. I – input, F – flow through, P - pulldown

5.5 EspJ ADP-ribosylates a suite of NRTKs in cell lysate

After incubation of eNAD with mammalian cell culture lysates, biotin tagging and Neutravidin enrichment of ADP-ribosylated substrates, label free LC-MS/MS was used to identify ADP-ribosylated proteins. This showed that ADP-ribosylated proteins such as ARTD1, which strongly auto-ADP-ribosylates, could be enriched using this methodology (Figure 5.7). Accordingly, MBP-EspJ_{HEC} and eNAD were spiked into various cell lysates before tagging with AzRB, enriching with Neutravidin resin and quantifying by label free mass spectrometry. Statistical analysis of protein abundances from samples with or without EspJ indicated that in HeLa (human cervical cancer cells) and A549 (human alveolar epithelial cells) lysate EspJ could ADP-ribosylate Src and Csk. In polarised Caco2 (human epithelial colorectal cells) lysate Yes1 kinase was significantly enriched in addition to Src and Csk. In differentiated Thp1 (human monocytes) lysate EspJ modified a range of NRTKs; Csk, the SFKs Lyn and Hck, the spleen tyrosine kinase Syk, and the Tec family kinases Tec and Btk (Figure 5.8). Substrates of EspJ were identified using a two-sided T-test with an FDR-corrected p value < 0.01 and a fudge value (s0) of 1. Non-statistical analysis showed that Lyn (Caco2 lysate) and Abl kinase (from HeLa and Caco2 lysates) were enriched in EspJ-containing samples but after imputation of missing values are below the significance threshold (Figure 5.9, Figure 5.10).

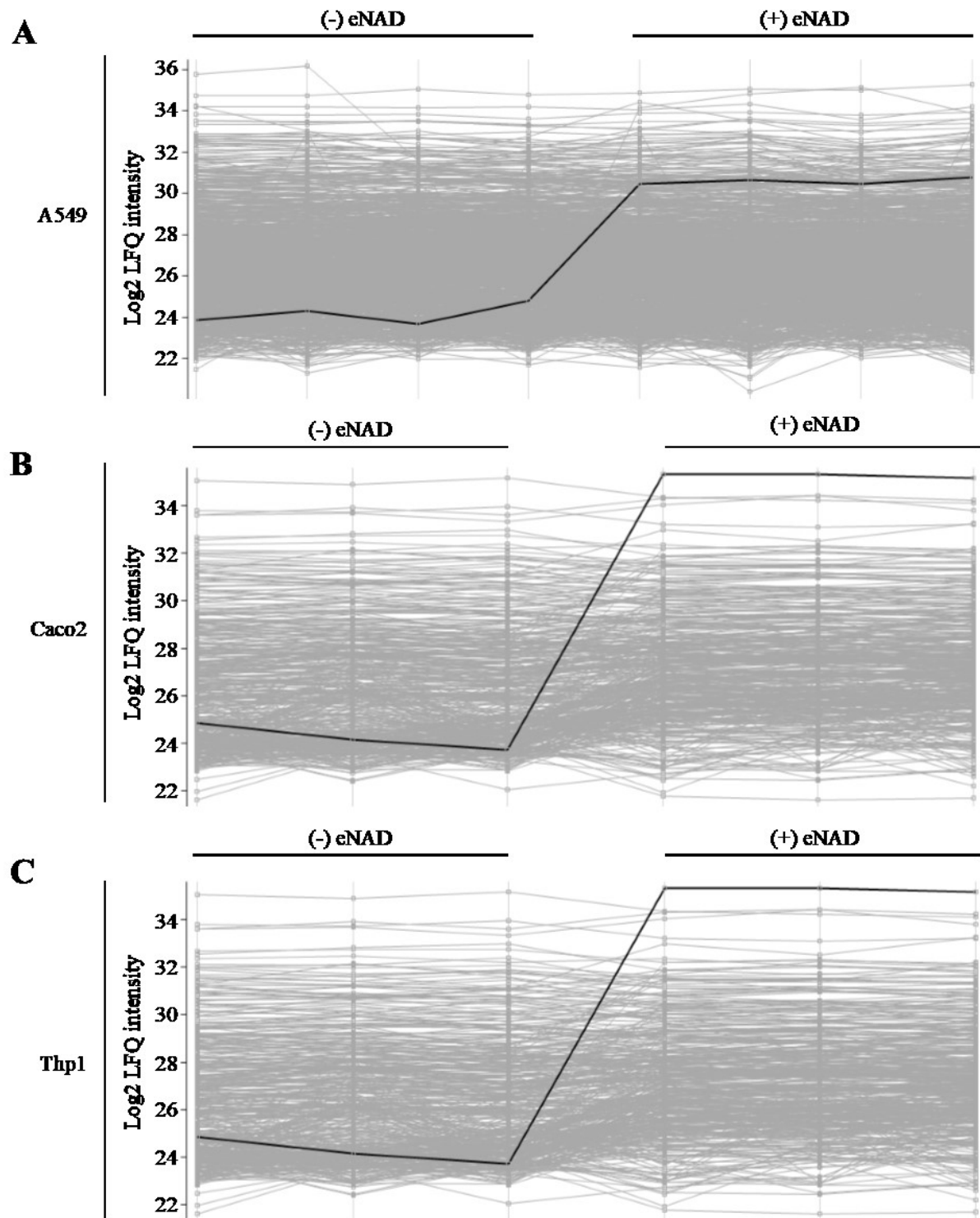


Figure 5.7 - ARTD1 can be enriched from cell lysates after ADP-ribosylation by eNAD

A549 (A), Caco2 (B) and Thp1 (C) cell lysates were incubated with (+) or without (-) 2-ethynyl-adenosine-NAD (eNAD), tagged with biotin by CLICK chemistry and enriched with Neutravidin resin before analysis by label free (LFQ) mass spectrometry. Profile plots highlight ARTD1 (black line) which is strongly enriched in the presence of eNAD, owing to its auto-ADP-ribosylation activity.

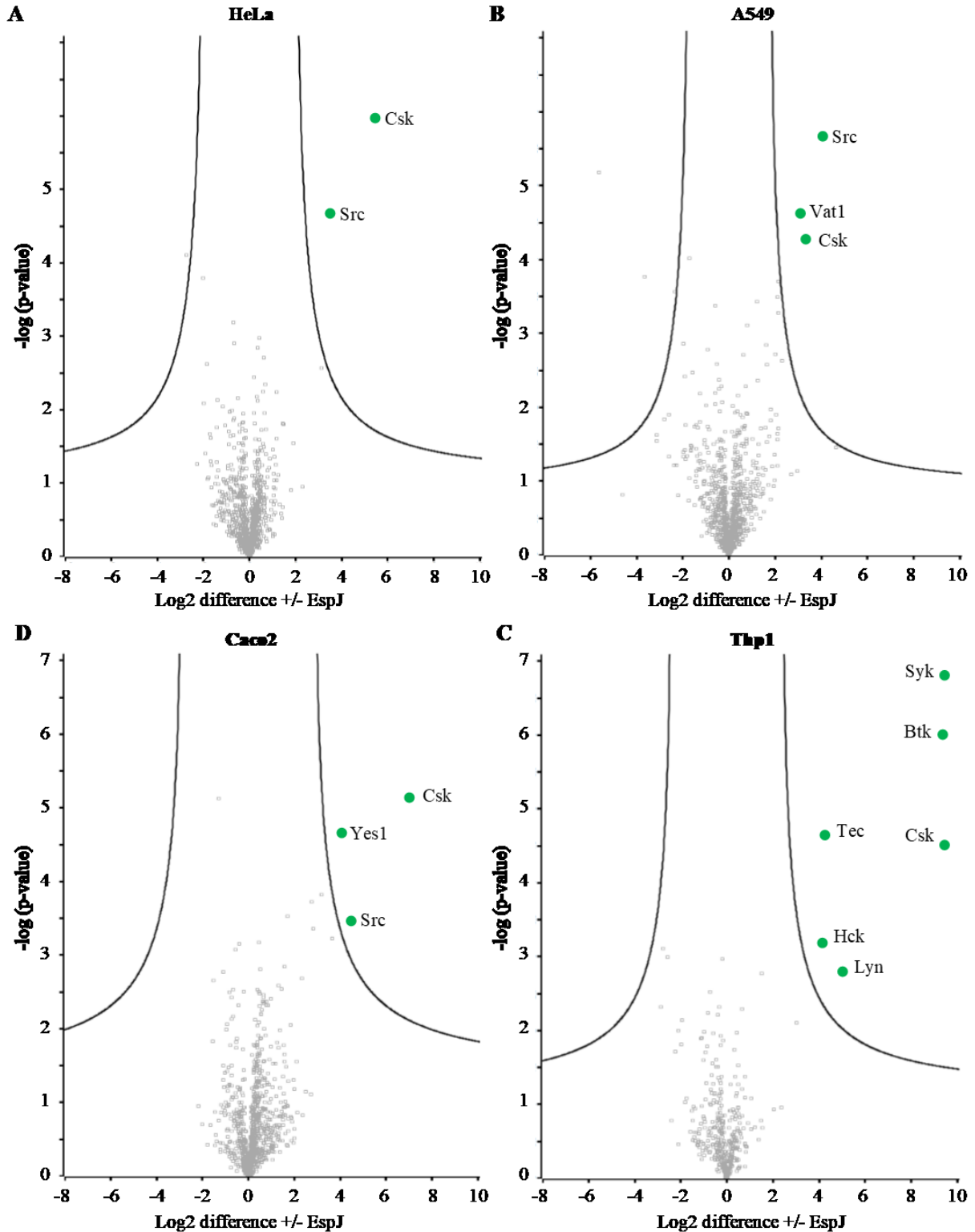


Figure 5.8 - *in vitro* EspJ_{HEC} ADP-ribosylates non-receptor tyrosine kinases

Lysate from (A) HeLa, (B) A549, (C) polarised Caco2 and (D) differentiated Thp1 cells were incubated with eNAD and EspJ_{HEC}. After tagging with biotin ADP-ribosylated proteins were enriched with Neutravidin resin and subsequently analysed by label-free quantification (LFQ) mass spectrometry. Green dots mark significantly enriched proteins in the presence versus absence of EspJ. Black lines display the significance cut-off with an FDR-corrected p-value of 0.01 and s0 value of 1 after a two-sided T-test. Figure adapted from Pollard *et al* 2018.

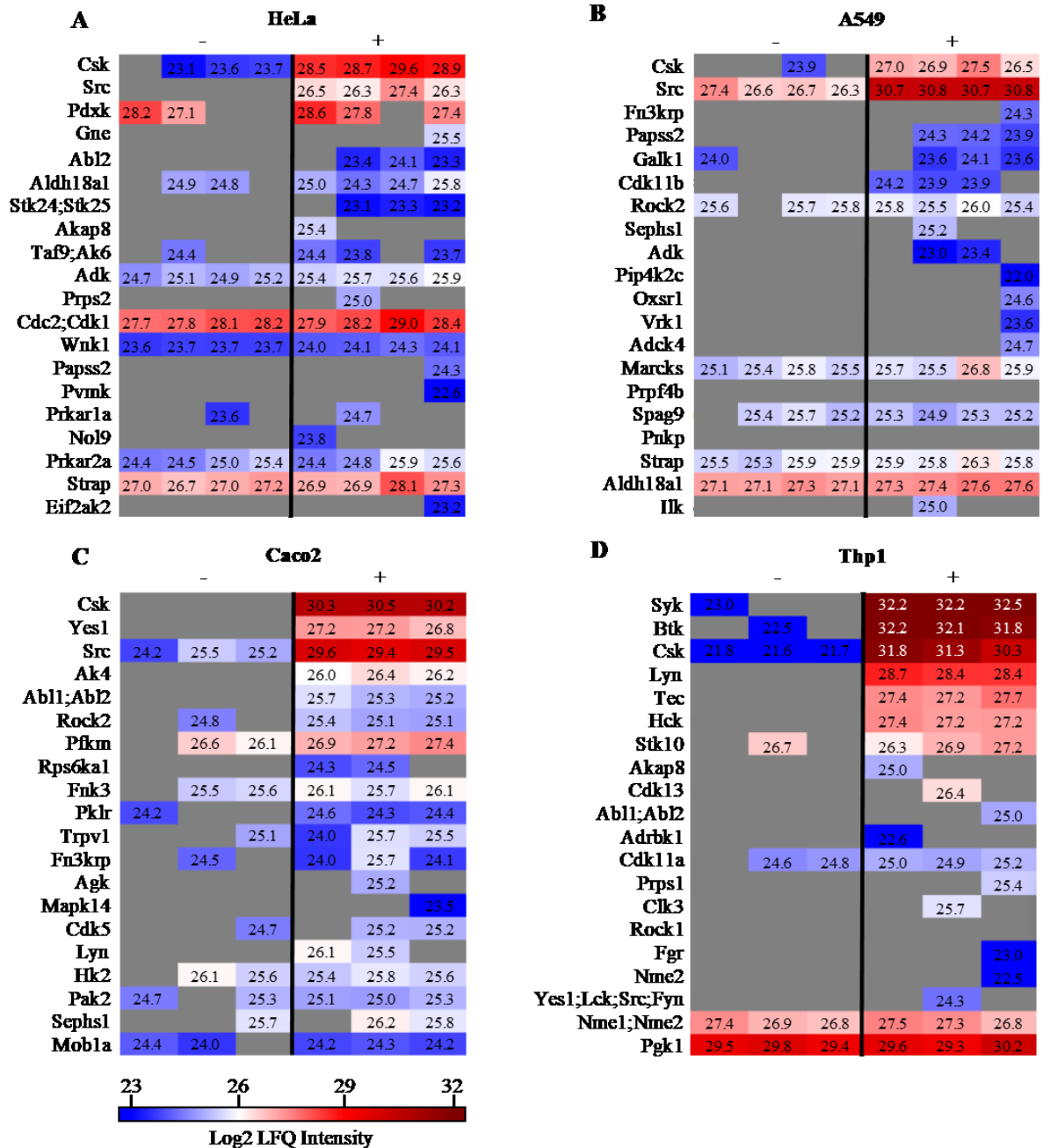


Figure 5.9 - Kinases targets of EspJ

Analysis of the kinases enriched after EspJ mediated ADP-ribosylation of cell lysate proteins from (A) HeLa, (B) A549, (C) Caco2 and (D) Thp1 cells. A text filter was used to select kinases. Missing values were replaced by imputing values from a normal distribution, and kinases were then ranked by the difference in Log2 LFQ intensity between EspJ presence (+) and absence (-). Imputed values were removed after ranking. The top 20 kinases are displayed for each cell lysate. The blue (low) to red (high) colour scale represent Log2 LFQ intensities of kinases in each sample, with enumeration overlayed. From Pollard *et al.*, 2018.

5.6 Modification of NRTKs depends upon a functional EspJ catalytic domain

Either MBP-EspJ_{EHEC}-R79A or MBP-EspJ_{EHEC}-D187A were incubated with A549/Caco2/Thp1 cell lysates alongside WT MBP-EspJ. Statistical analysis revealed that when comparing the presence of EspJ_{WT} to EspJ-D187A, Csk, Lyn, Hck, Tec, Btk and Syk were all significantly enriched, showing that their enrichment is dependent upon a functional EspJ ART domain. Similarly, Csk was significantly enriched from HeLa Caco2 lysate when comparing EspJ_{WT} to EspJ-R79A containing samples. Interestingly, while EspJ presence significantly enriched Src (HeLa lysate) and Yes1 (HeLa and Caco2 lysate) in comparison to EspJ absence, there was no significant difference between abundance levels in EspJ_{WT} and EspJ-R79A containing samples (Figure 5.10). *In vitro* ADPr of Src with NAD-biotin revealed that MBP-EspJ_{EHEC}-R79A can still modify Src, albeit to a lesser extent than WT, while D187A or R79A/D187A double mutant displayed almost no catalytic activity (Figure 5.11). This explains why Src and Yes1 were still enriched after incubation with eNAD and EspJ_{EHEC}-R79A but does not explain why Csk is not. Further, this is surprising when considering that MBP-EspJ_{EPEC}-R79A is not able to ADP-ribosylate Src, and that EspJ_{EPEC/EHEC}-R79A and *S. salamae* SeoC-R79A do not inhibit phagocytosis (Pollard et al., 2016; Young et al., 2014).

Together these data suggest that EspJ can ADP-ribosylate NRTKs from the Src, Abl Csk, Tec, and Syk families. The identification of numerous serine/threonine kinases and RTKs within the cell lysate proteomics datasets which are not enriched by the presence of EspJ suggest that despite targeting a universally conserved residue in the Src kinase domain, EspJ manages to retain substrate specificity. Src, Abl, Csk, Tec and Syk kinases all possess SH2 domains that bind to phosphotyrosine residues, but this domain is not found in other important NRTKs FAK and JAK. To test the importance of the SH2 domain for EspJ substrate recognition, GFP-tagged Src_{WT} and Src_{R175K} (SH2 mutant unable to bind phosphotyrosines) were immunoprecipitated from HeLa cells prior to incubation with NAD-biotin and MBP-EspJ_{EHEC} (Figure 5.12A). As

EspJ could ADP-ribosylate GFP-Src^{R175K} and GFP-Src^{WT} at similar efficiency it was deduced that EspJ may draw its specificity via mechanisms aside from a functional SH2 domain.

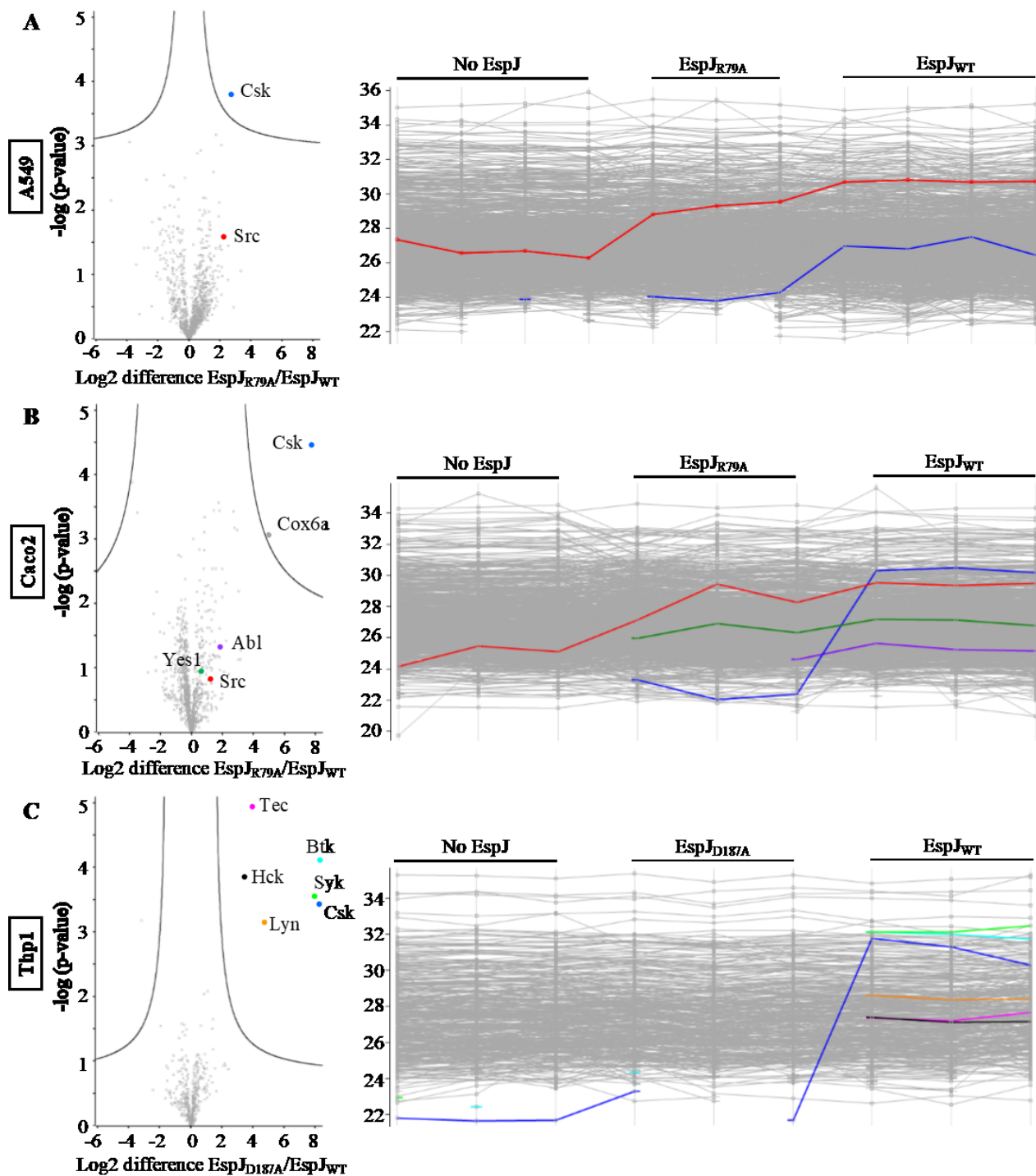


Figure 5.10 – EspJ WT and catalytic mutant ADP-ribosylation of NRTKs

See next page for legend

Figure 5.10 - EspJ WT and catalytic mutant ADP-ribosylation of NRTKs (previous page)

(A) A549, (B) polarised Caco2 and (C) differentiated Thp1 lysates were incubated with eNAD and EspJ_{EHEC} (WT or R79A or D187A) catalytic mutants. ADP-ribosylated proteins were enriched by Neutravidin pulldown after click-chemistry addition of a biotin group and analysed by label-free quantification (LFQ) mass spectrometry. Volcano plots (left) highlight significantly enriched proteins outside the curve, when comparing WT EspJ to ART mutants (R79A or D187A). Non-significant proteins of interest are also highlighted inside the curves. Black lines display the significance cut-off with an FDR-corrected p-value of 0.01 and s0 value of 1 after a two-sided T-test. Right panel - profile plots of sample LFQ-intensities. Dark blue - Csk, red - Src, dark green - Yes1, purple - Abl, light blue - btk, magenta - Tec, black - Hck, orange - lyn, light green – Syk.

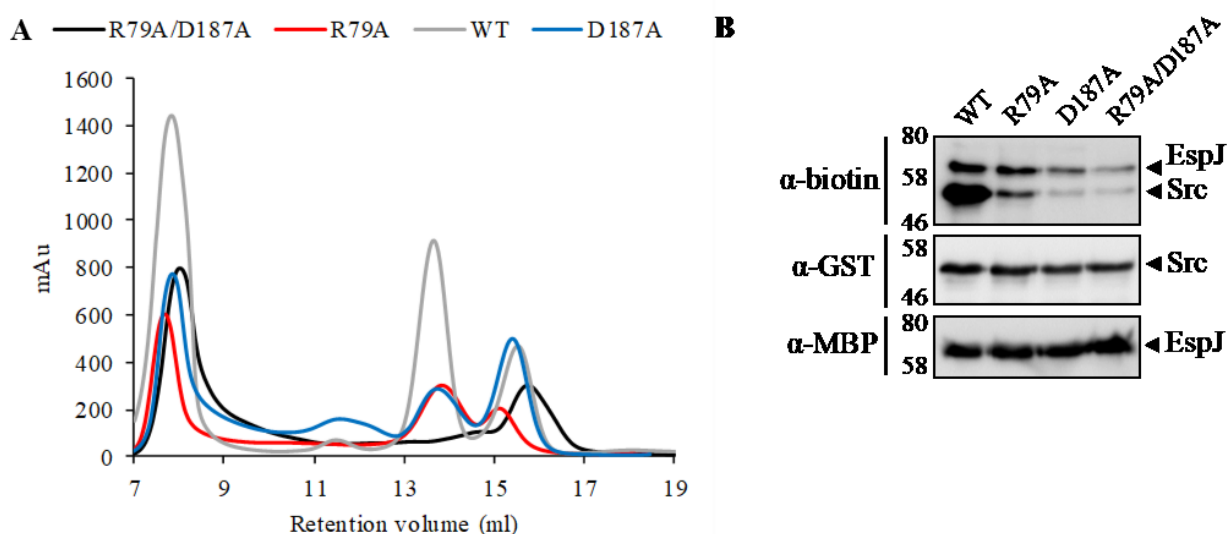


Figure 5.11 - Gel filtration and catalytic activities of EspJ_{EHEC} WT and ART mutants

(A) Gel filtration chromatography for MBP-EspJ_{EHEC} WT (grey), R79A (red), D187A (blue), R79A/D187A (black). Peaks at ~8 ml represent soluble aggregates, ~12 ml MBP-EspJ dimer/trimers, ~14 ml MBP-EspJ monomers and ~16 ml free MBP. (B) ADP-ribosylation of GST-Src₂₅₀₋₅₃₃ K295M/Y416A by EspJ WT and ART mutants bound to amylose resin.

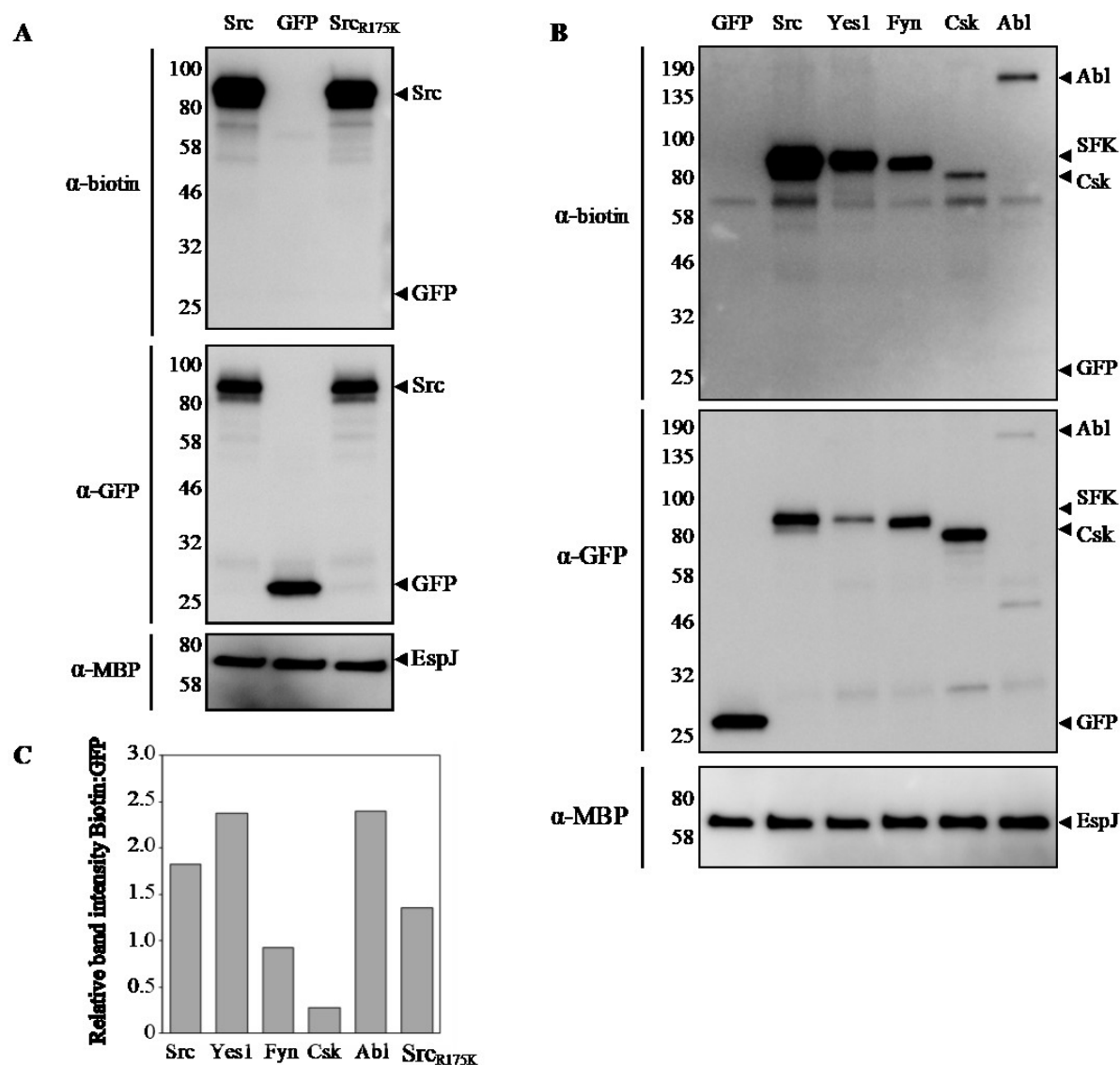


Figure 5.12 - EspJ ADP-ribosylation of non-receptor tyrosine kinases displays specificity

HeLa cells were transfected with GFP, GFP-tagged Src or Src_{R175K} (SrcRK - SH2 domain mutant) (A) and Src, Yes1, Fyn, Csk and Abl (B). NRTKs were immunoprecipitated using anti-GFP antibodies, before incubation with MBP-EspJ_{EEHC} and NAD-biotin. Western blotting and anti-biotin reagent were used to detect ADP-ribosylated proteins. (C) The relative band intensity for α -biotin (ADP-ribosylation) and α -GFP (total protein kinase) was assessed by densitometry as an estimation of ADP-ribosylation efficiency. The mean ratio of between 3 and 5 repeats is shown for each transfected NRTK, indicating that EspJ preferentially ADP-ribosylates Src, Yes1 and Abl over Fyn and Csk. Figure from Pollard *et al* 2018.

5.7 EspJ displays specificity within its NRTK substrates

The activity of EspJ in cell lysates suggested that of the three ubiquitously expressed SFKs, EspJ could modify Src and Yes1 but not Fyn. To assess this apparent specificity, the ADPr of several immunoprecipitated GFP-tagged tyrosine kinases was tested, after incubation with NAD-biotin and EspJ. All the tested kinases (Src, Yes1, Fyn, Abl, and Csk) were ADP-ribosylated by EspJ. Semi-quantitative densitometry of western blots was used to estimate ADPr efficiency based upon the ratio of ADP-ribosylated (anti-biotin) to total (anti-GFP) protein. Across several experiments the Fyn was consistently modified at less than half the efficiency of its family members Src and Yes1, and the non-SFK Abl kinase (Figure 5.12B/C). Interestingly, Csk was the weakest GFP-tagged substrate in this assay, despite being the only kinase suggested to be ADP-ribosylated by EspJ in all four cell lysates.

5.8 Csk and ADP-ribosylation localises to pedestals independently of EspJ

To investigate the relevance of this modification during the infection process, Csk localisation was monitored during EHEC infection of HeLa cells. Infection of HeLa cells ectopically expressing Myc-Csk demonstrated that Csk localises to the sites of bacterial attachment, independently of *espJ* presence (Figure 5.13). However, as the ectopic expression of Csk caused excessive cell rounding and detachment, further analysis was not possible. Immunofluorescence revealed that endogenous Csk in uninfected cells diffusely occupied the cytoplasm, at 2.5 and 5 h post infection Csk colocalised with EHEC actin-rich pedestals (Figure 5.14). At 2.5 but not 5 h post-infection, Csk staining was also concentrated at cellular leading edges/protrusions. Both the localisation to pedestals and cell edges were independent of *espJ*. Interestingly, staining for poly-ADPr showed similar dynamics to Csk localisation with early (2.5 h) accumulation at cell edges, followed by clear colocalisation with EHEC pedestals

(Figure 5.15). However, this was apparently also EspJ independent and is difficult to interpret due to the use of an anti-poly-ADP-ribose antibody to study mono-ADPr by EspJ.

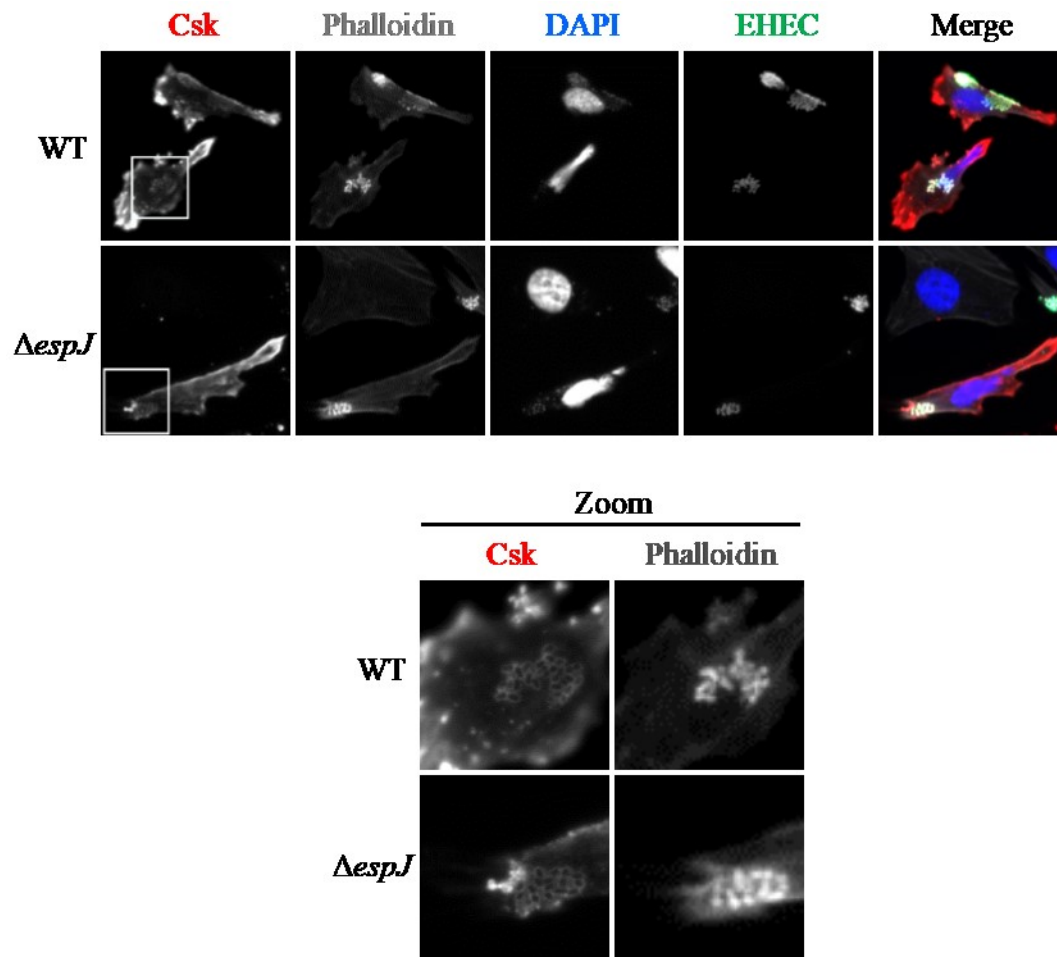


Figure 5.13 – Overexpressed Csk localises to sites of bacterial attachment

HeLa cells were transfected with pRK5-Myc-Csk before infecting with EHEC WT or $\Delta espJ$. Immunofluorescence staining reveals that Csk localises around the site of bacterial attachment and pedestal formation independently of the presence of EspJ. Red – Csk, grey -actin, blue -DAPI, green -EHEC. White boxes show the zoomed areas.

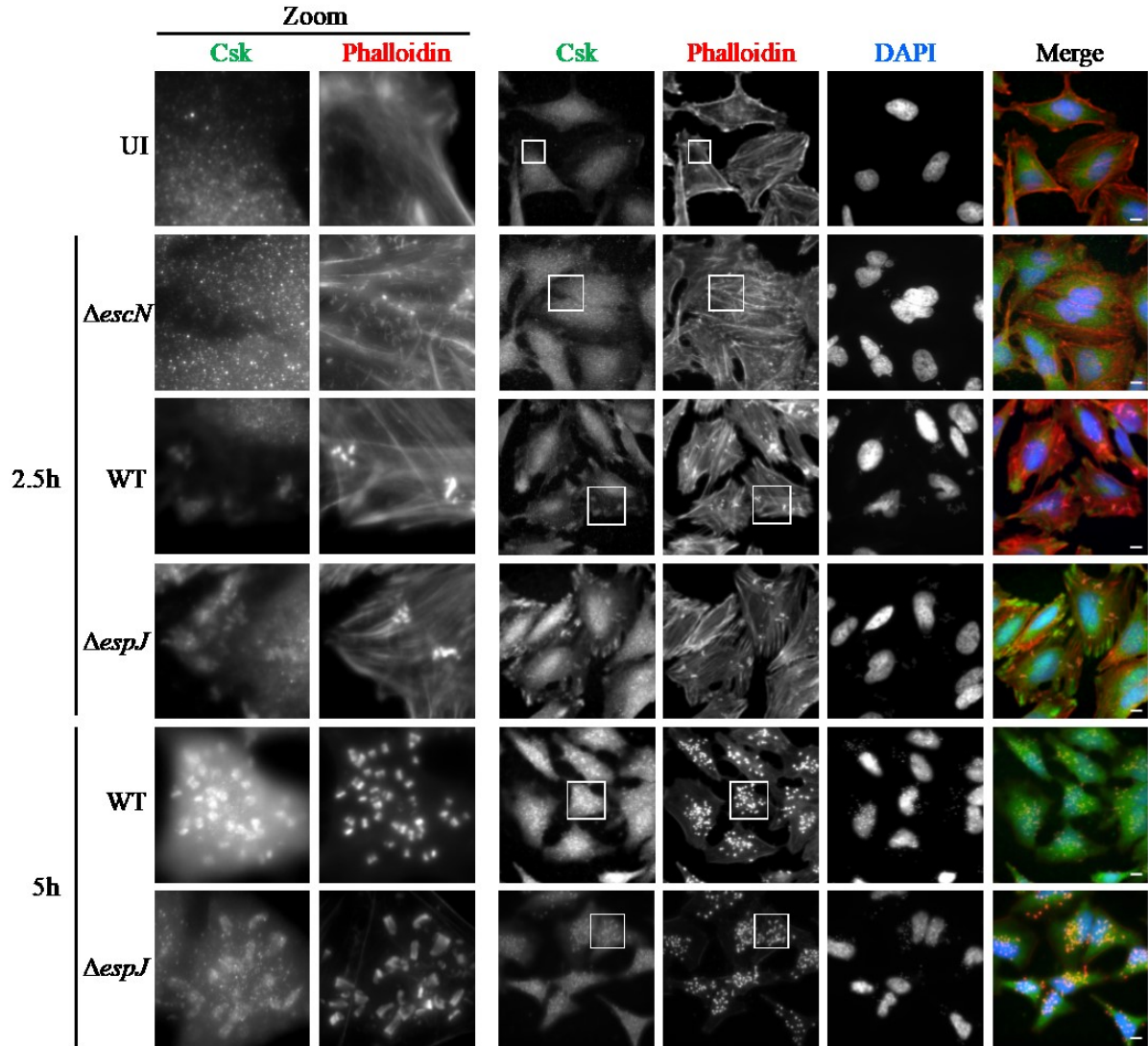


Figure 5.14 – Endogenous Csk localises to cell protrusions and pedestals

HeLa cells were infected with EHEC WT, *ΔespJ* or *ΔescN* for 2.5 or 5 h. Cells were then stained for Csk (green), and with phalloidin (red) and DAPI (blue). At 2.5 h Csk was more strongly localised at cell edges/protrusions compared to staining in uninfected cells. At 5 h Csk was colocalised with actin rich pedestals underneath the sites of bacterial attachment. Both observed phenotypes were independent of *espJ*. White boxes show zoomed area.

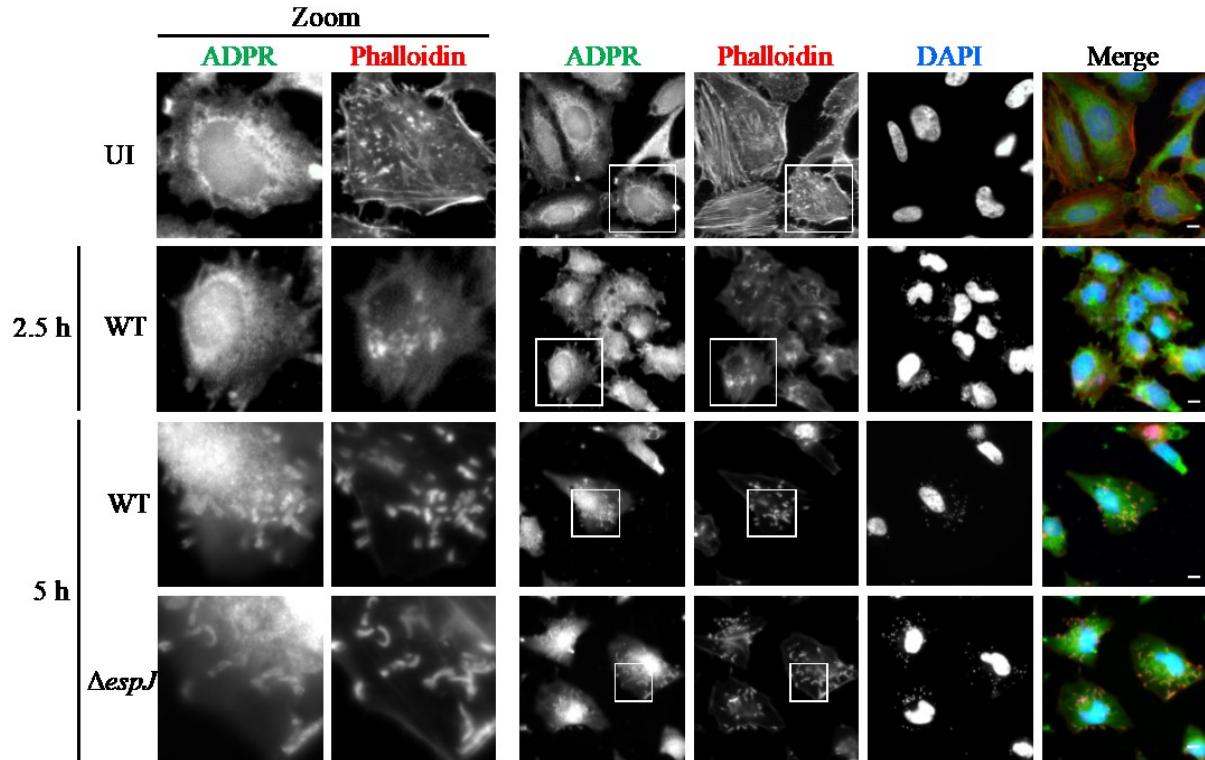


Figure 5.15 - ADP-ribosylation localises to pedestals during EHEC infection

HeLa cells were infected with EHEC WT, or $\Delta espJ$ for 2.5 or 5 h. Cells were then stained for PARylation (ADPR) (green), and with phalloidin (red) and DAPI (blue). At 2.5 h ADPR was more strongly localised at cell edges/protrusions compared to staining in uninfected cells. At 5 h ADPR was colocalised with actin rich pedestals underneath the sites of bacterial attachment. Both observed phenotypes were independent of *espJ*. White boxes show zoomed area.

5.9 EspJ inhibits Csk by ADP-ribosylation of E236

This chapter suggest that EspJ can inhibit the SFKs and but ADP-ribosylate their inhibitor Csk. This was further investigated to fully understand this intriguing EspJ-Src-Csk relationship. This thesis showed that the *Salmonella* EspJ homologues SeoC and SboC can ADP-ribosylate Src, while *Yersinia* YspJ cannot, and this trend is conserved in relation to the ADP-ribosylation of Csk (Figure 5.16A). While the EspJ_{EHEC}-D187A catalytic mutant could not ADP-ribosylate Csk, EspJ_{EHEC}-WT modified Csk in a time-dependent manner (Figure 5.16B), and mutation of Csk E236, analogous to Src E310 prevented ADPr suggesting that EspJ ADP-ribosylates the same conserved residue in Csk and Src (Figure 5.16C).

Because Csk activation or inhibition could have drastic differential effects on the regulation of Src kinase, the result of Csk ADPr by EspJ was investigated. To assess this Csk was first ADPr by EspJ for 2 h using native NAD, before spiking in buffer and a substrate; either the peptide tyrosine kinase substrate poly(glu₄, tyr) or Src₂₅₀₋₅₃₃K295M/Y416A/E310A (K295M – catalytic mutant, Y416A - non-autophosphorylated, E310A – not ADP-ribosylated by EspJ). Pre-incubation with WT EspJ_{EHEC} fully abolished Csk mediated phosphorylation of poly(glu₄, tyr) and Src, as shown by phospho-tyrosine western blotting. Contrastingly, after pre-incubation with D187A EspJ_{EHEC} or TssF1 (uncharacterised *Pseudomonas aeruginosa* protein used as a control), Csk was still fully active, indicating that its inhibition is dependent upon a functional ART domain (Figure 5.17). Therefore, by ADP-ribosylation EspJ can inhibit both the SFKs and their inhibitor Csk.

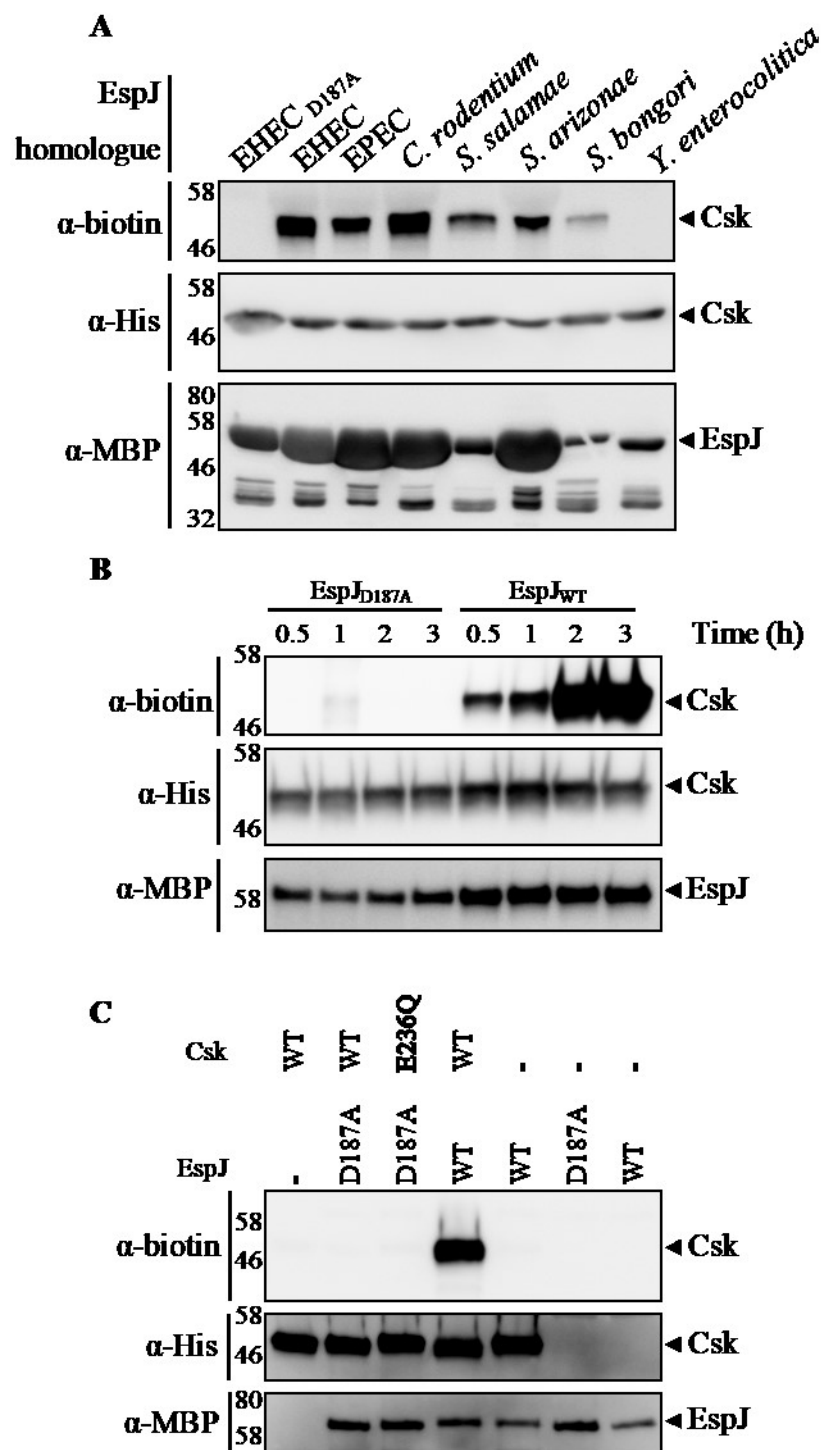


Figure 5.16 - EspJ ADP ribosylates Csk E236

MBP-EspJ homologues and or MBP-EspJ-D187A catalytic mutant were incubated with His-Csk (WT or E236Q) and NAD biotin. Western blotting and anti-biotin reagents were used to detect ADP ribosylated Csk. (A) MBP-EspJ_{EHEC} WT but not D187A (catalytic mutant) ADP ribosylates Csk in a time dependent manner. (B) Apart from *Yersinia* YspJ, the EspJ homologues are able to ADP ribosylate Csk; (C) the E236Q mutation of Csk prevents ADP ribosylation by EspJ. Adapted from Pollard *et al* 2018.

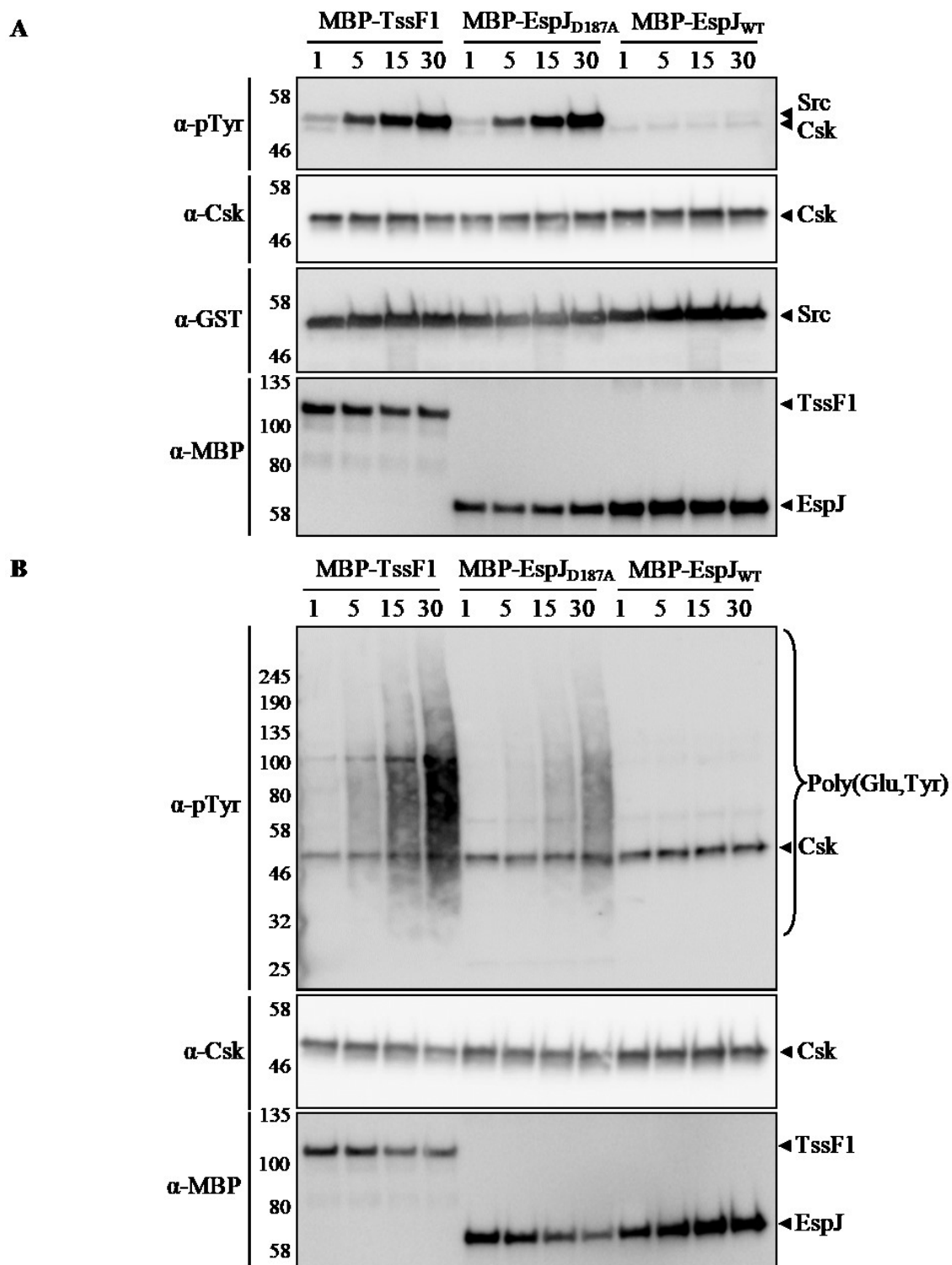


Figure 5.17 - EspJ inhibits Csk kinase activity

Csk was incubated with native NAD and MBP-EspJ-WT, the catalytic mutant MBP-EspJ-D187A or the negative control MBP-TssF1 for 2 h. Subsequently (A) GST-Src₂₅₀₋₅₃₃ K295M/Y416A/E310A or (B) poly(Glu4, Tyr) substrates were added for a 30 min time course kinase assay. Substrate phosphorylation was assessed by western blotting and an anti phosphotyrosine (pTyr) antibody. Csk was able to phosphorylate Src and poly(Glu4, Tyr) after pre incubation with MBP-EspJ-D187A or MBP-TssF1, but its ADP ribosylation by MBP-EspJ-WT inhibited Csk catalytic activity. Adapted from Pollard *et al* 2018.

5.10 Proteomics for the identification of EspJ targets during infection

Due to the apparently broad range of EspJ targets in cell lysate, protocols were next developed to study the ART activity of EspJ during tissue culture infections. ADPr during EHEC infections of HeLa cells and polarised Caco2 cells were first characterised using western blotting. HeLa cells were infected for 2.5 h and 5 h before lysing in denaturing conditions. Western blotting for PARylation revealed interesting ADPr dynamics with several infection induced bands and one band at about 60 kDa only observed in WT EHEC-infected cells. This ADP-ribosylated band was proposed to be EspJ-specific and was only present at 2.5h (Figure 5.18A). An EHEC time-course infection of polarised Caco2 cells also revealed a potentially EspJ specific band at around 60 kDa, but only at 5 h post infection despite testing 2, 4, 5, 6 and 8 h (Figure 5.18B). Analysis of the soluble and insoluble fractions after lysis of HeLa and Caco2 cell EHEC infections showed that the prominent infection specific ADPr events were present in the insoluble fraction (Figure 5.18)

I. Alkyne-tagged adenosine-based enrichment

Recently several methodologies have been developed to enrich ADP-ribosylated proteins and peptides for the purpose of defining the ADPr proteome (ADP-ribosylome). To circumvent the non-cell permeable properties of NAD, alkyne tagged adenosine analogues have been used, which are up taken into mammalian cells where they are incorporated into NAD. Subsequently, ADP-ribosylated proteins can then be enriched using the same click-chemistry based approach as used in this chapter to discover the targets of EspJ in cell lysate. This approach has been used to investigate the ADP-ribosylome in HeLa cells in response to oxidative stress (Westcott et al., 2017). This method avoids issues with the solubility of the insoluble nature of the infection specific and EspJ specific ADPr events (Figure 5.18C/D), by enrichment of proteins under denaturing conditions.

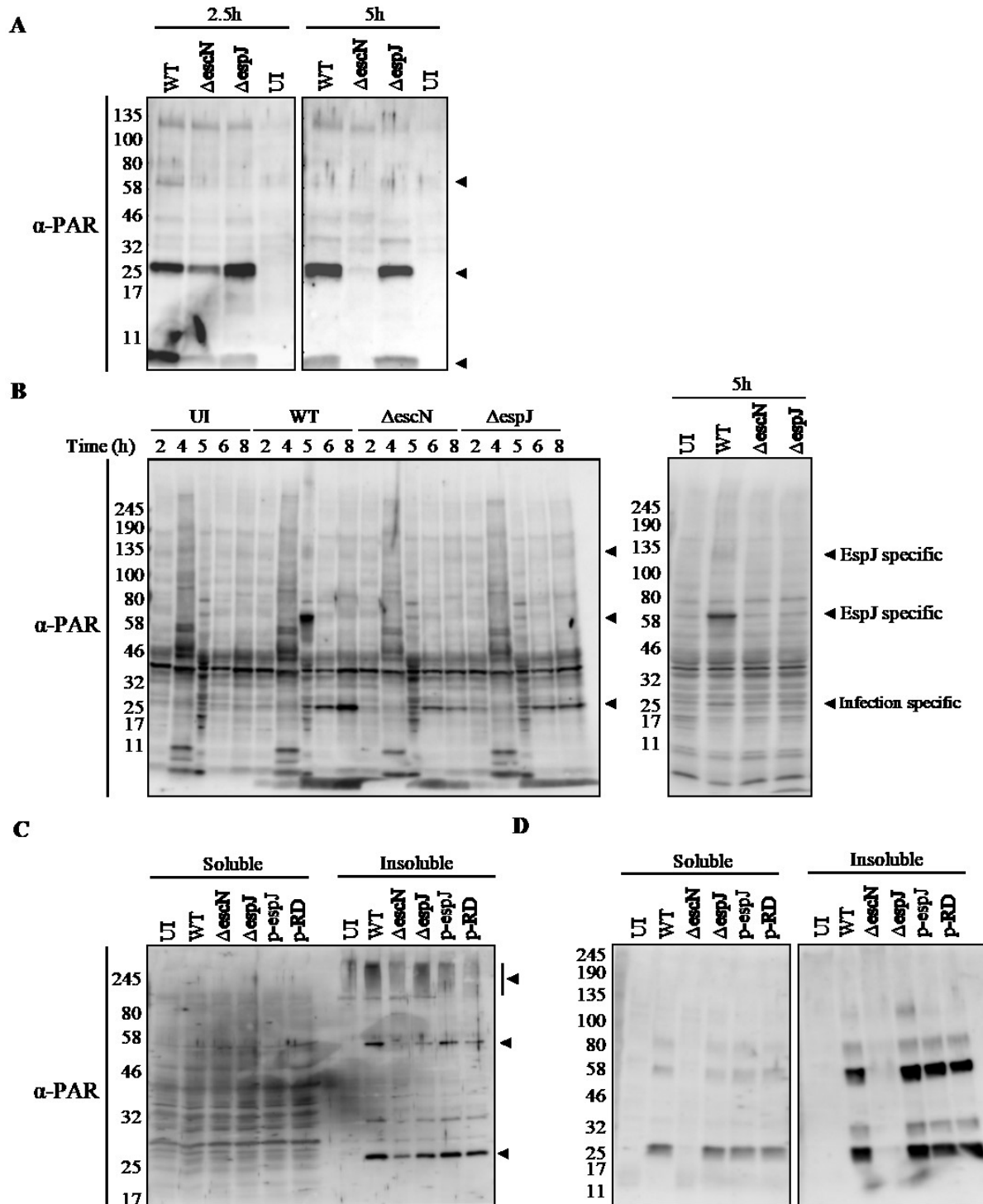


Figure 5.18 – EHEC-induced infection- and EspJ-specific ADP-ribosylation in tissue culture

EHEC WT, Δ escN, Δ espJ or Δ espJ complemented with WT *espJ* (p-espJ) or *espJ*-R79A/D187A (p-RD) were used to infect HeLa (A and D) or polarised Caco2 (B and C) cells. Infection lysates were analysed by western blotting with an anti-PARylation antibody. (A and B) Cells were infected over time 5 and 8 h time courses respectively, lysing in a 4% SDS buffer. (C) and (D) were infected for 5 and 2.5 h respectively before lysis in a RIPA buffer and analysis of the resulting soluble and insoluble fractions. Arrows represent ADP-ribosylation bands of interest. (A) was performed by Carmen Pee, ICL.

The uptake of alkyne tagged adenosine (2YnAd, provided by Pete DiMaggio, Imperial College London) by HeLa or Caco2 cells from cell culture medium was tested at 20 μ M, 50 μ M and 200 μ M for 2.5 h and 5 h. 2YnAd was incorporated into HeLa/Caco2 cells in a time and concentration dependent manner as previously reported (Westcott et al., 2017), as showed by anti-biotin western blotting (Figure 5.19). Using a Neutravidin pulldown, biotinylated proteins were increasingly enriched with increasing concentrations of 2YnAd incubated with Caco2 cells. ADP-ribosylated proteins were also able to be enriched, but this did not display a dependency on the concentration of 2YnAd (Figure 5.19). In addition, after infection of Caco2 cells with EHEC WT, $\Delta escN$, $\Delta espJ$ at increasing concentrations of 2YnAd, it was not possible to enrich infection specific ADP-ribosylated proteins. Unfortunately, neither anti-Src and anti-Csk revealed enrichment of either EspJ targets (data not shown).

II. *Afl521 macro-domain based enrichment*

An alternative methodology utilises the ADP-ribose binding domain (macro domain), of *Archaeoglobus fulgidus* Afl521, to enrich ADP-ribosylated proteins. GST-tagged WT Afl521 and G42E Afl521 (non-binding mutant) were expressed and purified from BL21 STAR *E. coli* before chemically crosslinking it to sepharose resin using a dimethyl pimelimidate (DMP) methodology. Published protocols using this methodology enrich ADP-ribosylated proteins in a non-denaturing RIPA buffer (Martello et al., 2016). In an effort to solubilise and enrich interesting ADP-ribosylated proteins from the insoluble fractions, infected/uninfected Caco2 cells were lysed in 10 x RIPA buffer, 8M urea- or 6M GuHCl-based buffers before a 20x dilution prior to Afl521 pulldowns. ADP-ribosylated proteins were most successfully enriched in a WT-Afl521 dependent manner in RIPA based buffer showing that even low concentrations of urea or GuHCl disrupted the pulldown (Figure 5.20). Using a similar protocol, proteins were enriched from uninfected Caco2 cells or Caco2 cells infected with WT EHEC, $\Delta escN$ EHEC (T3SS mutant) or $\Delta espJ$ mutants in an effort to investigate infection-dependent,

T3SS-dependent and *espJ*-dependent ADP-ribosylation. Western blotting showed that ARTD1 could be enriched in a WT-Afl521 dependent manner. Furthermore, anti-PARylation reagent showed that this method can enrich infection- and T3SS-dependent ADP-ribosylated proteins but there was no evidence of EspJ-specific ADPr, and Src kinase was not enriched (Figure 5.20).

Label free LC-MS/MS experiments were performed using both methodologies with uninfected or WT/ Δ *escN*/ Δ *espJ* EHEC-infected Caco2 cells (data not shown). Unfortunately, neither experiment revealed significantly enriched infection-, T3SS- or *espJ*-dependent ADPr events and both protocols will require further optimisation to study EspJ targets during infection. Interestingly, targeted inspection of MS data revealed that Src, Csk and Yes1 were identified in numerous samples suggesting that they maybe endogenously ADP-ribosylated. In line with to this, ADPr of SFKs has been regularly observed by similar proteomics methods in other laboratories (personal communications Professor Michael Hottiger, Zurich University).

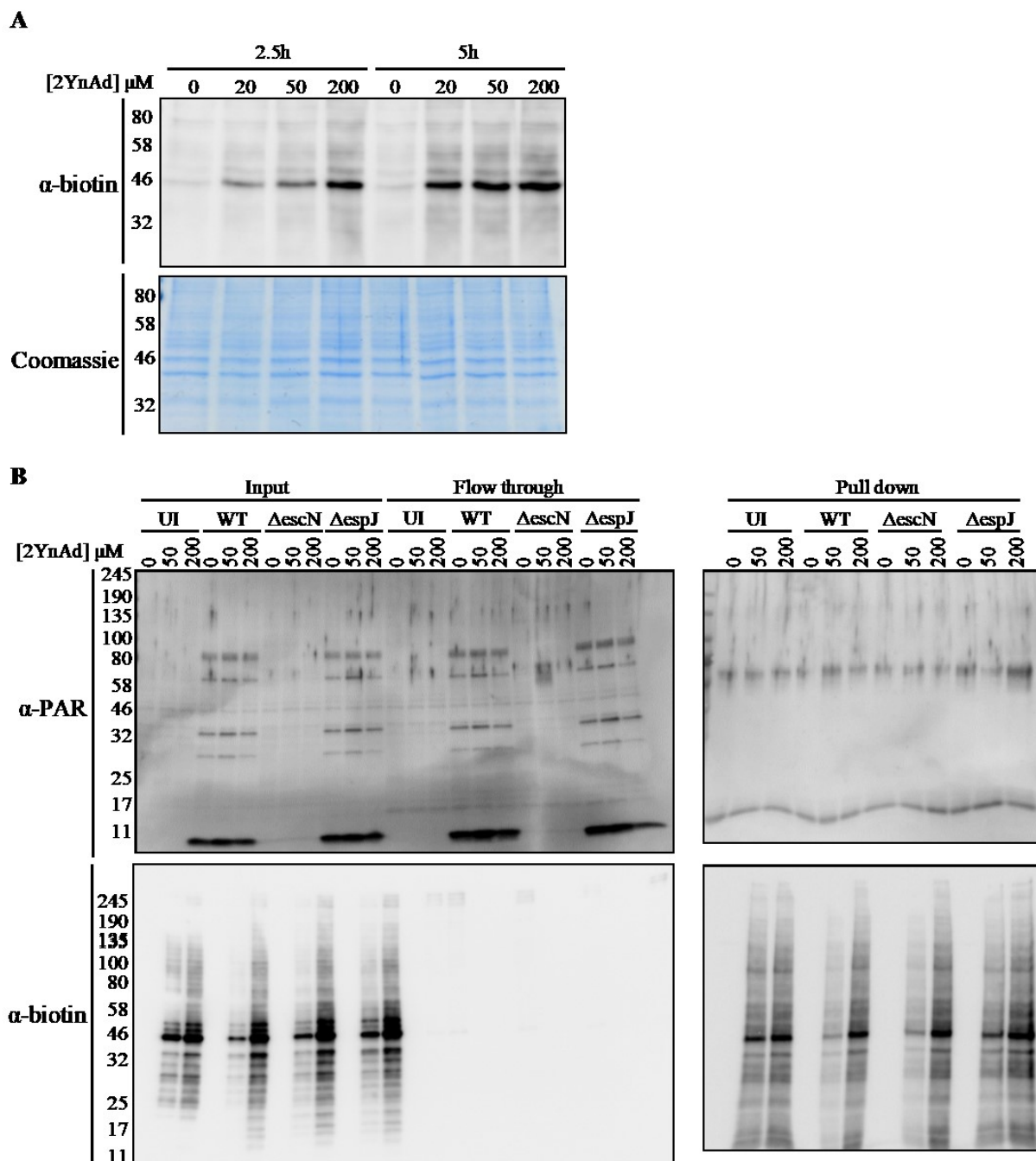
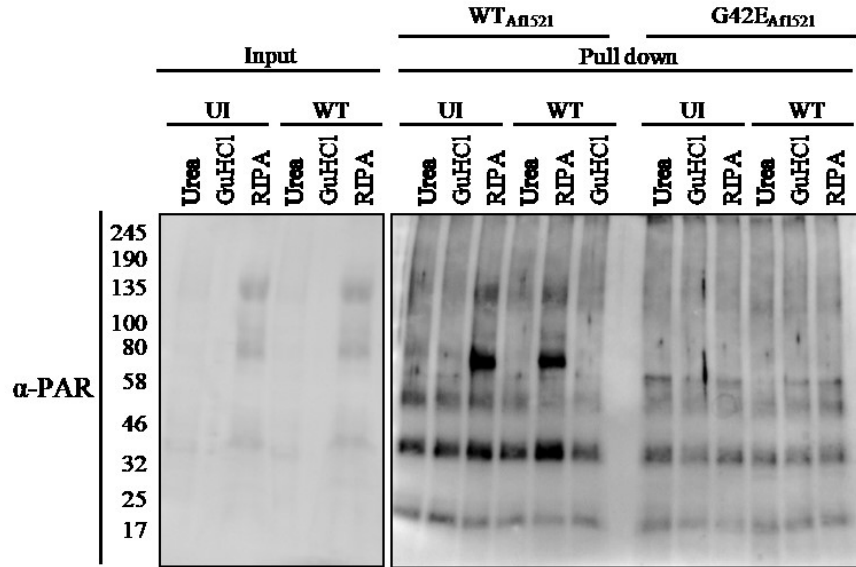


Figure 5.19 – Uptake of 2YnAd and enrichment of ADP-ribosylated proteins

(A) HeLa cells were incubated with 0 μ M, 20 μ M, 50 μ M and 200 μ M of the alkyne tagged adenosine analogue, 2YnAd for 2.5 h and 5 h before lysis in 4% SDS buffer. After click chemistry-mediated addition of a biotin tag to ADP-ribosylated proteins, anti-biotin western blotting shows that 2YnAd is up taken and incorporated into proteins in a time and concentration dependent manner. (B) EHEC WT, Δ escN or Δ espJ were used to infect polarised Caco2 cells for 5 h. Using a similar workflow ADP-ribosylated proteins were enriched and analysed by anti-biotin western blotting.

A



B

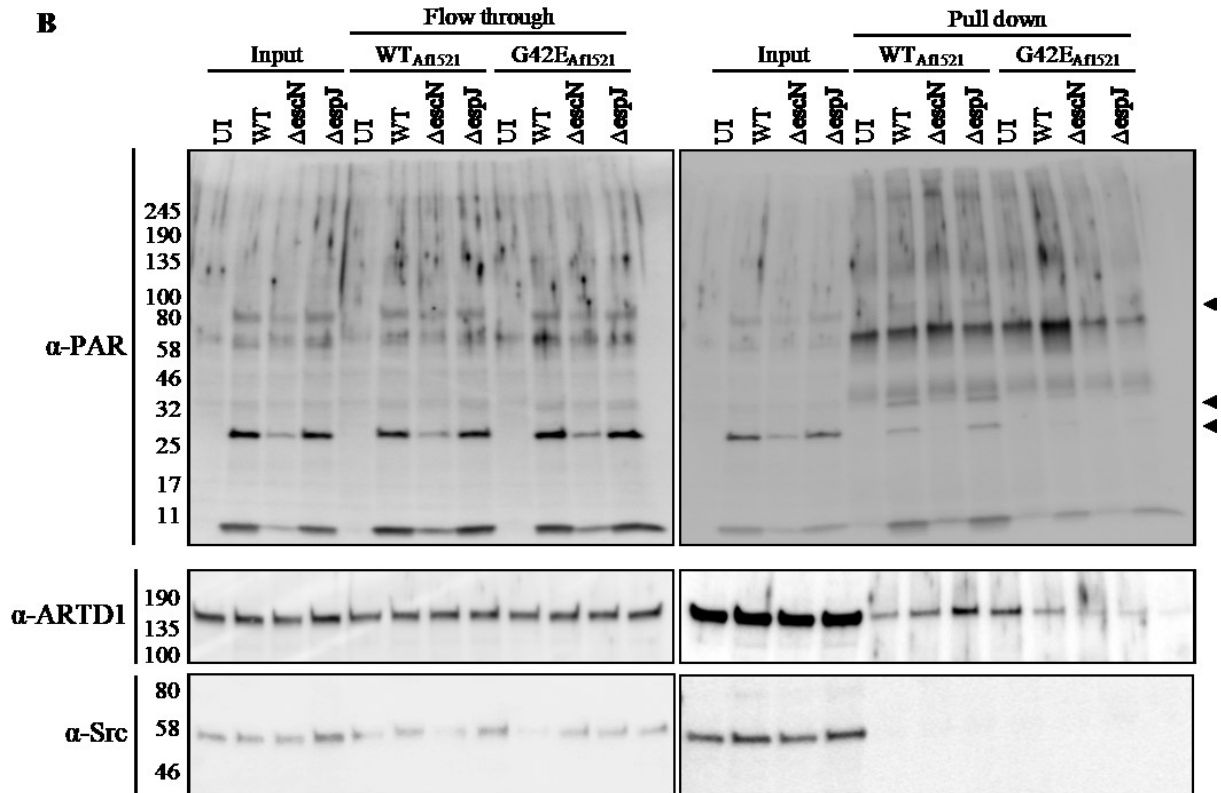


Figure 5.20 – Enrichment of ADP-ribosylated proteins using Af1521 macro domain

Caco2 cells were infected with EHEC WT, EHEC WT, $\Delta escN$ or $\Delta espJ$ before lysis and enrichment using sepharose-crosslinked GST-Af1521 (WT or G42E non-binding mutant). (A) 5 h EHEC infections or uninfected Caco2 cells were lysed in 10 x RIPA buffer, 6M GuHCl-based buffer or 4 M urea-based buffer before dilution and enrichment using Af1521 pulldowns. This showed that lysis in RIPA buffer was superior to GuHCl or urea for enrichment of ADP-ribosylated proteins – as displayed by anti-PARylation antibodies. (B) Caco2 infections lysed in 10 x RIPA buffer were enriched with Af1521 WT or G42E showing that infection specific bands (arrows) could be enriched in Af1521 WT but not G42E pulldowns.

5.11 Discussion

This chapter made inroads to defining the substrate repertoire of the bacterial ART EspJ showing that Src kinase is not its only target, and that it can modify other SFK family members, as well as NRTKs from the Abl, Tec, Syk and Csk families.

Predictions from the previous chapter suggested that EspJ may also modulate kinases from the Jak and Fak families, but these proteins were not ADP-ribosylated in cell lysate. However, this could also be due to low abundances of these kinases in the cell lysate. Fak is expressed in most tissues and cell lines due to its critical role in focal adhesion regulation whereas Ptk2 has a more restricted expression pattern (Parsons, 2003). Similarly, Jak1, Jak2 and Tyk2 are thought to be ubiquitously expressed while Jak3 is only found in haematopoietic cells (Yamaoka et al., 2004). Therefore, it is probable that Fak and Jak family members were present in HeLa/A549/Caco2/Thp1 cell lysates. The ubiquitous SFK Fyn was also not enriched from cell lysate in the presence of EspJ but was later shown to be targeted by EspJ in isolation, albeit less efficiently than Src and Yes1. Global proteomics analysis of the HeLa, A459, Caco2 and Thp1 lysates used in these assays should be performed to investigate the abundance of Fak/Jak/Fyn, and the *in vitro* ADPr of recombinantly purified Fak/Jak is necessary to make confident conclusions. Nonetheless, the ADPr of proteins in cell lysate represents a non-biased approach to identify targets and while not an exhaustive list, this chapter has greatly advanced the known repertoire of EspJ substrates.

Reasons for specificity within the NRTKs was proposed to be due to the presence of SH2 domains in all the EspJ targets but not in Fak/Jak kinases. Originally characterised for binding for phosphotyrosine residues, SH2 domains have recently been shown to also bind PIP₂/PIP₃ membrane lipids, further regulating the activity of SH2 domain-containing proteins (Park et al., 2016). EspJ was still able to ADP-ribosylate a non-functional SH2 mutant of Src. This may not be surprising given that EspJ can also modify the Src kinase domain in isolation. However,

the reaction could be forced by high protein concentrations, at physiological concentrations or in cells the SH2 domain may still enable EspJ selectivity. The *P. syringae* T3SS effector HopF2 is possibly the closest homologue to EspJ aside from SeoC/SboC/YspJ. It shares around 15% sequence identity to the EspJ family and interestingly also ADP-ribosylates a kinase with immunomodulatory implications. However, as HopF2 modifies MKK5 R313, just N-terminal to the kinase domain it doesn't offer insight into the EspJ target specificity (Wang et al., 2010).

This chapter also suggested several EspJ specificities within the SFKs. Src and Yes1 were both significantly enriched after ADPr by EspJ in cell lysate, but Fyn was not. Furthermore, EspJ ADPr of GFP-tagged NRTKs suggested that Fyn was a less favourable target than Src and Yes1. The latter observation could be an artefact from the use of GFP-fusion proteins as targets, or possibly different proteoforms of the SFKs were immunoprecipitated from the HeLa cell transfection. In addition to this, while the major haematopoietic SFKs Hck and Lyn appear to be EspJ targets, Lck was not identified. However, Lck is regarded to be T-cell specific so may not have been present in cell lysates.

Multiple mouse knockout studies clearly demonstrate variable roles of the SFKs (Lowell and Soriano, 1996). Single knockout of Hck or Fgr result in subtle deficiencies in myeloid cells (Lowell and Soriano, 1996), whereas a Src knockout causes issues with bone remodelling (Soriano et al., 1991), Lyn or Lck knockout impairs B- and T-cell function, respectively, and Yes or Blk knockout has no apparent implications (Stein et al., 1994; Texido et al., 2000). More severe problems arise with double or triple knockouts which may explain why EspJ targets multiple SFKs. The majority of Src/Yes, Src/Fyn or Hck/Src knockouts are fatal and Hck/Fgr mutants have severely compromised immune systems (reviewed in (Lowell and Soriano, 1996)).

The SFKs vary the most in their unique N-terminal SH4 domain (Arbesú et al., 2017) which contains lipidation sites and also modulates intramolecular interactions. All family members have N-terminal myristoylation and palmitoylation sites except Src and Blk which lack a palmitoylation site and this has been proposed to cause differential spatial regulation of Src, Fyn and Yes (Sandilands et al., 2007). Despite the highly conserved nature of SH2/SH3 domains, by making SH2/SH3 chimera SFKs it was possible to show that they too orchestrate specific interactions that differentiate SFK functions (Summy et al., 2000). These structural differences may allow EspJ to preferentially target certain SFKs, but specificity is also likely to come from spatiotemporal regulation of SFKs and EspJ.

Src, Yes1 and Fyn are the most closely related SFK family members, and for a long while researchers struggled to develop small molecule inhibitors that displayed selectivity within the Src family. More recent studies showed that selectivity is possible with one compound displaying an 8 fold selectivity for Src over Yes1 and >40 fold selectivity of Fyn which is interesting considering the apparent preferences of EspJ found in this chapter (Brandvold et al., 2012). Src, Fyn and Yes regulate an overlapping set of cell processes and in some instances are able to compensate for the loss of the other ubiquitous SFKs *in vivo* (Stein et al., 1994). Interestingly, despite reports on the redundancy of tyrosine kinases for EPEC pedestal formation (Bommarius et al., 2007; Swimm et al., 2004a), it has been shown that deletion of Fyn alone was sufficient to prevent Tir phosphorylation and pedestal formation (Hayward et al., 2009; Phillips et al., 2004). It is therefore possible that a preference for the inhibition of Src/Yes1 over Fyn means that EspJ does not inhibit EPEC pedestals under physiological infection conditions, but its ectopic expression forces Fyn inhibition to prevent pedestal formation. Similarly, the inferior catalytic activity of EPEC EspJ compared to EHEC EspJ shown in this chapter may also be a mechanism to allow Tir phosphorylation, whereas because

EHEC pedestal formation does not rely on tyrosine kinases, EHEC EspJ can have superior activity.

Interestingly, the *in vitro* proteomics suggested that EspJ-R79A could ADP-ribosylate Src but not Csk in cell lysate. This may indicate that Csk is a weaker substrate for EspJ due to the apparent inability of the EspJ-R79A mutant to ADP-ribosylate it. Contrastingly, in Thp1 lysate EspJ-D187A showed no sign of modifying any substrates. The ADPr of recombinant Src showed similar dynamics with only partially attenuated modification by EspJ-R79A but full inhibition by EspJ-D187A. Similar experiments must be performed with Csk to confirm this observation. Based upon studies of EspJ, HopF2 and DT, EspJ R79 is known to be essential for NAD binding, and D187 is involved cleavage of NAD to release nicotinamide (Wang et al., 2010; Young et al., 2014). In cell culture the R79A and D187A mutations have previously been proven, independently or in combination, to prevent the ability of EspJ to inhibit phagocytosis, or pedestal formation (PhD thesis Young, 2013; Young et al., 2014). Perhaps it is only at non-physiological concentrations of EspJ, substrate and NAD⁺, that R79A is able to catalyse ADPr.

EspJ/SboC/SeoC were all able to ADPr Csk, and it was shown that EspJ_{EHEC} inhibits Csk by modifying the same conserved glutamic acid residue as previously shown for Src. However, immunoprecipitated GFP-Csk was a much weaker target than GFP-Src. Regardless, if EspJ, Src and Csk colocalise during infection, EspJ has the potential to dynamically regulate SFK signalling at different locations or time points by inhibiting either Src or Csk (Okada, 2012). In combination with previous studies, this thesis has shown that EspJ can ADP-ribosylate both kinase dead Src (K295M), the Src autophosphorylation mutant (Y416A), and constitutively active Src (Y527F). Whether EspJ can ADP-ribosylate Src that has been phosphorylated by Csk (Src pY527) will be pivotal to understanding this counter-intuitive relationship; if EspJ cannot modify Src-pY527 then it provides rationale for inhibiting Csk to give EspJ full control

over Src activity. Furthermore, it will be interesting to see if EspJ can ADP-ribosylate Cbp-bound Csk.

Csk is cytoplasmically localised in the resting state. SFK activation results in phosphorylation of the membrane-bound Cbp which recruits Csk in a negative feedback loop. It has been shown that after an initial period of Fyn activity Csk is recruited to EPEC pedestals where it phosphorylates and inhibits Fyn, suggesting that Csk is not inhibited by EspJ (Hayward et al., 2009). Immunofluorescence in this chapter also showed the recruitment of Csk to pedestals along with general ADPr, although neither was EspJ dependent. Hayward *et al* showed that after depletion of Csk, phosphorylation of Fyn Y417 (Src 416), which is indicative of Fyn activity, was still reduced after the initial strong phosphorylation. This inactivation of Fyn could be a result of the ADPr by EspJ. EspJ may also regulate signalling from the anti-inflammatory Tir ITIM domain, the tyrosine phosphorylation of which recruits phosphatases SHP1/SHP2 for the inhibition of TRAF6 inflammatory signalling (Yan et al., 2012, 2013). This scenario would be in line with immunomodulatory roles of EspJ suggested in the previous chapter and can be applied in general to other ITIM containing receptors. Src, Syk, and Tec family kinases, can phosphorylate ITIM domains to enable this anti-inflammatory signalling and can each be inhibited by Csk meaning that EspJ can positively and negatively regulate this process. Previous studies have shown that while EspJ does localise to pedestals during infection, it also spreads around the cell (PhD thesis (Young, 2013)). Therefore, EspJ has the potential to modulate the Src/Csk signalling axis elsewhere and even modulate Src-independent functions of Csk. For example, Csk can stimulate the degradation of the AP-1 transcription factor subunit c-Jun, thereby inhibiting AP-1 stimulated gene expression (Zhu et al., 2006), is recruited to G-proteins where it links GPCR activation to cytoskeletal rearrangements (Lowry et al., 2002), and can phosphorylate and activate phosphatases Ptpn6 and SHP-1 (Autero et al., 1994).

The roles of NRTKs in the host immune response and cytoskeletal signalling is extensive, which provides a strong link between the target repertoire of EspJ *in vitro* and the observed modulation of inflammation and immunity during *in vivo* CR infection of mice. Aside from EspJ, many bacterial effector proteins modulate host NRTKs to their advantage. The activation of several EPEC Tir-based signalling pathways has been discussed in detail in this thesis and the phosphorylation of similar motifs in *Helicobacter pylori* CagA by Src and Abl enables the regulation of cell elongation and motility (Krisch et al., 2016). Src and Abl are also recruited by *Shigella* to orchestrate the cytoskeletal rearrangements necessary for invasion (Mounier et al., 2009) while Btk is key to the formation of *Shigella* actin comet tail formation which enables cell-to-cell spreading (Dragoi et al., 2013). In contrast, bacterial effector protein phosphatases can inactivate NRTK signalling. *Salmonella* SptP can dephosphorylate Syk, and *Yersinia* YopH regulates bacterial internalisation through dephosphorylation of host NRTKs and their downstream targets within integrin and immunoreceptor signalling pathways (Choi et al., 2013; Rolán et al., 2013).

This chapter sought to investigate the relevance of Src/Csk ADPr, along with other suggested NRTK targets during infection, through using emerging proteomics technologies to study physiological ADPr. While data suggested that EspJ-specific and infection-specific ADPr could be detected and enriched from infections, initial proteomics studies were not able to uncover the identity of the modified proteins. It seems that at least the SFKs and perhaps other EspJ targets are ADP-ribosylated independently of EspJ (personal communication, Professor Michael Hottiger, University of Zurich), so the assessment of ADPr enrichment at a protein level is unlikely to uncover the physiological EspJ target repertoire. To maximise PTM identification, pioneering ADP-ribosylome studies are instead performing peptide enrichment with macro-domain pulldowns post-protein digestion which would greatly enhance the chances of identifying EspJ targets (Martello et al., 2016). It is likely that the protocols used in this

study are biased towards the enrichment of PARylated proteins due to the additive effect of the Af1521-ADP-ribose or Neutravidin-ADP-ribose interaction with PAR chains over MARYlation. To circumvent this, current studies are digesting PARylated proteins using the hydrolase PARG, before enrichment (Martello et al., 2016). Nevertheless, the study of MARYlation is still hindered by the absence of methods for specific MAR detection and enrichment. In another methodology, snake venom phosphodiesterase I enzyme is used to digest MAR and PAR down to a single phospho-ribose at the site of modification. This phospho-ribose moiety can then be enriched using IMAC protocols similar to the enrichment of phosphopeptides, and analysed by mass spectrometry (Daniels et al., 2014, 2017). Like the Af1521 protocol, this is superior to chemical proteomics methodologies as it can be applied to *in vivo* studies. However, the co-enrichment of phosphopeptides by IMAC greatly complicates the the resulting mass spectrometry analysis in comparison to the macro-domain enrichment.

Unfortunately, such protocols were beyond the scope of this study, but the next few years will be a very exciting time in the ADPr field. To elucidate the physiological repertoire of EspJ, *in vitro* ADPr followed by enrichment of EspJ targets should first be performed in primary IECs. In addition to this, the latest Af1521-based protocols should be used to enrich of EspJ targets from cell culture infection, and from IECs purified from CR infected mice.

Chapter 6 – General discussion and conclusions

6 Final discussion

6.1 Overview

This study focussed on furthering the understanding of the T3SS ARTs EspJ, SeoC, SboC and YspJ, from their *in vitro* biochemical activities to their role during *in vivo* infection. Previously EspJ had been characterised as an A/E pathogen T3SS effector protein, able to inhibit Src kinase by ADPr, disrupt host opsono-phagocytosis and regulate EPEC-induced actin rich pedestal formation. However, the specificity of EspJ ART activity, the functions of its homologues from *Salmonella* and *Yersinia* and its implications *in vivo* were unknown. Data from this thesis demonstrate that EspJ_{EHEC} can ADP-ribosylate a range of NRTKs from the Src, Abl, Csk, Syk and Tec families, providing a mechanism for the efficient inhibition of phagocytosis. It was shown that the EspJ homologues *S. salamae*/*S. arizonae* SeoC, *S. bongori* SboC and *Y. enterocolitica* YspJ also inhibit opsono-phagocytosis, and except for YspJ, all homologues could ADP-ribosylate Src and Csk. SeoC was proven to be a substrate of the *Salmonella* SPI-1 T3SS and could inhibit *trans*-opsono-phagocytosis during infection. However, like EspJ_{EPEC}, SeoC had no impact on the *cis*-phagocytosis of bacteria by macrophages and played no role in *S. salamae* invasion of epithelial cells. The absence of EspJ catalysis during CR infection of mice resulted in an increased level of hyperplasia, without altered colonisation dynamics. Quantitative proteomics of CR-infected mouse IECs strongly linked EspJ to the modulation of a broad range of host inflammation and immune system processes. Taking *in vitro* and *in vivo* data together, this suggests that EspJ could modulate multiple aspects of proliferative and immunoregulatory pathways in multiple cell types during infection.

6.2 Concluding remarks

Though direct evidence for the ADPr of host kinases by EspJ during infection is yet to be displayed, its ability to ADP-ribosylate a range of host kinases *in vitro* is an indication of how EspJ may broadly regulate the host immune response. However, several of these new EspJ targets must be further studied to prove their modification and inhibition. EspJ was predicted to modulate Fak and Jak but *in vitro* studies did not highlight them as targets. It is important to confirm this specificity, as modulation of Jaks would have large implications on the host immune system through disruption of IFN-stimulated Jak-Stat signalling and gene expression (O'Shea et al., 2015). Furthermore, the inhibition or absence of FAK has been shown to block leukocyte transmigration, a process also predicted to be affected by the activity of EspJ (Parsons et al., 2012). Src, Hck, Syk and Btk have each also been linked to leukocyte recruitment during inflammation (Volmering et al., 2016). Therefore, even without Fak and Jak inhibition, it is feasible that EspJ could modulate immune cell migration, and this also holds true to many processes predicted to be regulated by EspJ during CR mouse infection. Increased IEC expression of NOX2 subunits in the absence of EspJ ART activity links EspJ to the inhibition of microbicidal ROS production. The *P. aeruginosa* T3SS ARTs ExoS and ExoT have recently been shown to independently block neutrophil ROS production through the perturbation of PI3K signalling (Vareechon et al., 2017). Like EspJ, ExoS and ExoT can inhibit phagocytosis via their ART activity, and ExoS ADPr of Ras also prevents the Ras-PI3K interaction necessary for NOX2 assembly. Several NRTKs including Src, Syk and Tec family kinases are involved in the activation of PI3K signalling. It is therefore possible that EspJ regulates similar pathways to ExoS/ExoT, but by inhibiting NRTKs upstream of the Ras/PI3K complex.

EspJ has the potential to regulate signalling at many points downstream of multiple stimuli, for example ITAM/ITIM-containing receptors. Through the inhibition of SFKs kinases EspJ will

directly prevent phosphorylation of the receptor cytoplasmic tyrosines. Along with other adapter proteins Syk is recruited to phosphorylated ITIM/ITAMs for further signal transduction. Syk has also been reported to perform this same phosphorylation in the absence of SFKs (Turner et al., 2000) so to fully ablate these pathways EspJ must also inhibit Syk, as suggested by this study. SFKs and Syk are responsible for the downstream activation of Tec kinases. This may be through a direct relationship, or indirectly through their activation of PI3K which generates membrane PIP₃ which in turn binds and activates Tec kinases. Therefore, EspJ has the potential to directly or indirectly inhibit Tec family kinases, preventing its activation of PLC γ . The A/E pathogens have been shown to interrupt pathways such as pro-inflammatory and cell death cascades at multiple checkpoints through the utilisation of several effector proteins (Pearson et al., 2016; Raymond et al., 2013). However, the A/E pathogens may also disrupt numerous nodes of ITAM receptor signalling through the use of a single T3SS effector EspJ. This also applies to signalling downstream of PRRs such as TLR4, to which multiple NRTKs have been linked (Chattopadhyay and Sen, 2014; Chen et al., 2003; Gong et al., 2008; Lin et al., 2013). SFK inhibitors PP1 and PP2 are able to block LPS-induced TNF production (Smolinska et al., 2008, 2011). Therefore, SFKs are crucial in TLR4 signalling and are thought to activate Tec and Syk kinases to mediate pro-inflammatory signalling from numerous TLRs (Page et al., 2009). Interestingly, EspJ may be able to reverse these pathway blockages through the inhibition of Csk, though the mechanism of selectively ADP-ribosylating Src or Csk remains to be uncovered.

Given the apparent broad regulation of NRTKs by EspJ, and their critical roles in the immune response, it may have been predicted that Δ ART CR would be cleared faster and could have more severe inflammation than WT. Indeed, assessment of hyperplasia suggested greater CCH in Δ ART CR infections, but colonisation was not altered, despite the fact that the absence of EspJ has previously been reported to delay the clearance of CR (Dahan et al., 2005). It is

possible that EspJ interactions with host or bacterial proteins causes a spatiotemporal regulation to limit the ability for EspJ to dramatically disrupt the host immune response. Previously a yeast-2-hybrid approach suggested that EspJ interacts with the GEF RIC8A, the intraflagellar transport system component IFT20 and centromere protein CENPH (Blasche et al., 2014). Contrastingly, the LC-MS/MS analysis of proteins pulled down from HEK293 lysate by GST-EspJ suggested WD repeat-containing protein (WDR)-23 as a binding partner. RIC8A is a GEF for G-proteins that can be activated by of Src family kinases, so could be a realistic EspJ-binding partner (Yan et al., 2015). IFT20, aside from its role in cilium formation, is required for T-cell activation through the recruitment of Lat to the membrane, a process also required for FcγR-mediated phagocytosis (Vivar et al., 2016). WDR23 is not well characterised, but in *C. elegans* functions to target the transcription factor SKN-1 for degradation, preventing the expression of genes involved in the response to xenobiotic and pathogenic stresses (Staab et al., 2013). The links between these proteins and EspJ are tenuous, and studies in the Frankel lab have failed to demonstrate any stable EspJ interaction partners (PhD thesis (Young, 2013)). It may be that infection models are needed to further investigate EspJ interaction partners. For example, tagging EspJ on the CR chromosome and subsequent co-immunoprecipitation of the EspJ fusion protein from CR-infected mouse IECs may allow the isolation of EspJ physiological protein complexes, helping to further define its specific roles.

This study expanded the known protein targets for the ART EspJ_{EHEC} *in vitro*. Although its homologues were also shown to ADPr Src and Csk it will be interesting to perform similar screens to determine the substrate repertoires SeoC, SboC and YspJ. This is especially important when considering that YspJ inhibited macrophage phagocytosis in cell culture despite showing no evidence of ART activity. Like *P. aeruginosa* ExoS and ExoT which inhibit phagocytosis through ADPr of a different sets of proteins (Barbieri and Sun, 2004;

Maresso et al., 2007), YspJ may have different targets to EspJ/SeoC/SboC. The use of emerging proteomics methods for studying “ADP-ribosylomes” will allow non-biased analysis of the EspJ homologue targets. To further investigate the differences between these homologues it may also be useful to test the ability of *seoC/sboC/yspJ* to complement CR $\Delta espJ$, by comparing CR-infected mouse IEC proteomes, although this may be affected by differences in host tropism.

To date the study of T3SS effectors has mostly depended upon *in vitro* studies of recombinant proteins, overexpression of effectors in cell culture, or their deletion during cell culture infections. Despite a multitude of evidence of how, for example, the A/E pathogens subvert inflammatory signalling, it is increasingly important to study the physiological roles of T3SS effectors *in vivo*. Improving techniques are enabling increasingly deep global proteomics analysis and the study of an expanding range of PTMs. This thesis is an example of how such methods can now be used to investigate the more-subtle impacts of effectors *in vivo*. This project should now combine the use of such proteomics techniques after further purification IECs, leukocytes and lymphocytes to differentiate the roles and substrate specificities of EspJ in different cell types.

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