Cellular Subversion: Towards a Complete Repertoire of Type-III Secretion System Effectors in Enterohaemorrhagic Escherichia coli O157:H7

By

SOPHIE ANNE MATTHEWS

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School of Immunity and Infection
College of Medical and Dental Sciences
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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a formidable pathogen that uses a type-III secretion system to inject bacterial 'effector' proteins directly into host cells. Most effectors that are encoded within the locus of enterocyte effacement (LEE) have been studied extensively. This study aimed to characterise a selection of recently discovered non-LEE-encoded effectors using a variety of model systems. Firstly, a β-lactamase translocation assay was used to demonstrate translocation of novel effectors into host cells. The localisation of selected effectors was then investigated using mammalian cells and a yeast cell model. The effector EspM2 was shown to induce the formation of actin stress fibres in transfected HeLa cells and caused growth retardation when expressed in yeast. A number of NleG effectors also caused growth retardation and morphological changes when expressed in yeast. Growth retardation caused by the effector NleG8-2 was shown to be dependent on three conserved cysteine, aspartic acid and histidine residues. Transcriptomics and a high copy yeast gene suppression screen revealed that NleG8-2 may disrupt yeast physiology by affecting the secretory pathway. This study confirms that the effector repertoire of EHEC O157:H7 is much larger than previously imagined and provides insight into the function of selected novel effectors.

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Author Contribution

All the work presented here is that of the author except where indicated in the appropriate chapter. In summary, work presented in chapter 3 formed part of a collaborative effort between the Pallen group (University of Birmingham), the Frankel group (Imperial College, London) and the Hayashi group (Osaka University, Japan). Bioinformatics work was performed by Dr Scott Beatson, secretome, Flag and CyaA assays were performed by Toru Tobe, Hisaaki Taniguchi and Hiroyuki Abe at Osaka University. β-lactamase assays were prepared by the author, Scott Beatson, Rasha Younis, Amanda Fivian and Chris Bailey. β-lactamase assays were performed by the author, Chris Bailey and Scott Beatson under the supervision of Olivier Marches. In chapters 4-6 Gateway cloning of effector genes into entry and destination vectors was performed by the author and Rasha Younis equally. Transfections of HeLa cells as described in chapter 4 were performed by the author and Rob Shaw equally. Site-directed mutagenesis of NleG8-2 as described in chapter 5 was performed by Rasha Younis. In chapter 6, preparation of nucleic acids from yeast for hybridisation to a microarray, data collection and statistical analysis of microarray data were performed by Bharat Rash and Andrew Hayes at the Consortium for Functional Genomics of Microbial Eukaryotes (COGEME) facility at the University of Manchester.

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CHAPTER ONE

Introduction

1. Introduction

1.1. *Escherichia coli*

Escherichia coli is a Gram-negative bacterium that lives as a commensal in the lower-gut of humans and animals [4]. The bacterium is a useful model organism and is often used as a workhorse for molecular biology. However, *E. coli* is very diverse and numerous strains have the ability to cause disease. Diseases such as neonatal meningitis and urinary tract infections are caused by extra-intestinal pathogenic *E. coli* (ExPEC) whereas enteric disease is caused by diarrhoeagenic *E. coli* [5]. Infections with pathogenic *E. coli* costs millions of pounds in lost work time and hospital visits every year [6]; research into these pathogens is therefore important.

Diarrhoeagenic *E. coli* strains are grouped into six distinct pathogenic variants (pathovars) based on clinical presentation, virulence factors and epidemiology [7]. The pathovars include enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [8]. The characteristics of each are summarised in table 1.1. *Shigella* has been shown to be taxonomically indistinguishable from EIEC at the species level. However, the distinction between *E. coli* and *Shigella* is maintained due to the clinical significance of *Shigella* [8].

Pathovar	Characteristics
DAEC	Possesses fimbrial adhesins that cause the formation of cellular extensions which wrap around the bacteria [9]. Causes diarrhoea in children >12 months old [10].
EAEC	Possesses enterotoxin and cytotoxin. Forms biofilms and autoaggregates in a distinctive 'stacked brick' pattern [5]. Causes outbreaks of secretory diarrhoea [11].
EHEC	Possesses one or more Shiga toxins. Intimately adheres to and effaces enterocytes. Affects children and elderly people in developed countries. Causes severe bloody diarrhoea [5].
EIEC	Invades host cells [12]. Causes watery diarrhoea. Closely related to *Shigella*.
EPEC	Intimately adheres to and effaces enterocytes [13]. Causes diarrhoea in children in developing countries [14].
ETEC	Possesses heat stable (ST) and heat labile (LT) toxins [15]. Causes watery diarrhoea in children and travellers in developing countries [5].

Table 1.1: Diarrhoeagenic pathovars of *E. coli*

1.2. EHEC

Enterohaemorrhagic *E. coli* strains form a subgroup of Shiga-toxin-encoding *E. coli* (STEC) [16]. EHEC O157:H7 was first recognised as a cause of human disease in 1982 when it was identified as the cause of two outbreaks of haemorrhagic colitis in the USA [17, 18]. Shiga toxin and/or Shiga-toxin-producing *E. coli* in the stools of affected patients were also linked to sporadic cases of haemolytic-uraemic syndrome (HUS) [19]. EHEC is characterised by the ability to cause HUS and haemorrhagic colitis [5]. HUS is defined by three features: acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia [20]. HUS is especially dangerous in young children where it causes death in 5% of cases [20]. Haemorrhagic colitis is characterised by severe abdominal cramps, bloody stools, lack of fever, colonic mucosal oedema and haemorrhage.

EHEC O157:H7 is a formidable pathogen. The infectious dose may be <100 organisms and treatment of infection with antibiotics may exacerbate disease [21]. There is currently no vaccine to prevent infection with EHEC O157:H7. Outbreaks of EHEC O157:H7 are often associated with consumption of contaminated meat products, particularly undercooked beef products [22]. The largest-ever outbreak occurred in Sakai City, Japan in 1996; it caused >6000 cases and three deaths [23] [24]. The EHEC strain isolated from the Japanese outbreak (RIMD 0509952) is known as EHEC O157:H7 Sakai – the genome of this strain was sequenced in 2001 [25]. Most outbreaks of EHEC in the USA, UK and Japan are caused by the EHEC O157:H7 serotype whereas non-O157 serogroups such as O26 and O111 are important in other countries [5] [26].

Cattle and other ruminants are the main reservoir of EHEC O157:H7 [27] [28] [29]. EHEC O157:H7 colonises the terminal rectum of cattle [30]. EHEC O157:H7 is released into the external environment in bovine faeces [31] and can persist in the farm environment for many months in water/feed troughs, making it difficult to prevent infection of other animals [32] [33] [34]. A major cause of EHEC infection is direct or indirect contact with ruminant faeces [35]. A number of infections, mainly in children, have been attributed to farm visits [36].

Infection of the gastrointestinal tract of adult cattle and weaned calves with EHEC O157:H7 serotype is asymptomatic [37] although intimate adherence to cattle intestinal epithelia and induction of a mild inflammatory response has been documented in some cases [38]. This is in contrast to infection with other serotypes such as EHEC O26 which cause significant morbidity and mortality in cattle [39]. In fact, one could argue that EHEC O157:H7 should no longer be considered a commensal of cattle as originally proposed [40]. Infection of susceptible calves with EHEC O157:H7 produces diarrhoea, epithelial damage and neutrophil infiltration of the lamina propria and intestinal lumen [41]. The relationship between EHEC O157:H7 and cattle is therefore complex with the outcome (i.e. disease/asymptomatic carriage) depending on many factors including the age of the animal [41]. Recent publication of the genome sequence of cattle may help researchers understand the relationship between EHEC O157:H7 and cattle [42].

An increasing number of EHEC O157:H7 outbreaks have been linked to vegetable and salad products [43]; for example, radish sprouts were implicated in the Sakai outbreak [24] and

ready-to-eat spinach was identified as the cause of a recent outbreak in the US [44]. Recent research has shown that EHEC O157:H7 and other pathogens such as *Salmonella* are able to survive on and colonise plant surfaces using type III secretion systems [45-47]. This has implications for food safety as techniques such as washing that are currently used to decontaminate plant material may need to be more stringent.

1.3. EHEC virulence factors

A wide variety of virulence determinants are used by EHEC O157:H7 to cause disease including Shiga toxin and the Esc-Esp type III secretion system (T3SS) encoded by the locus for enterocyte effacement (discussed in later sections). Examples of other EHEC O157:H7 virulence factors are shown in table 1.2. Space does not permit a full discussion of each – the reader is directed to a review of EHEC virulence factors by Nataro *et al* [5].

EHEC strains produce one or more types of Shiga toxin [48]. The toxin belongs to the AB_5 group of toxins and is thought to be the main factor in the development of HUS in infected patients. The toxin binds to globotriaosylceramide-3 (Gb3) receptors on host cells using the B subunits [49]. The toxin is then endocytosed by the cell [50] and the catalytic A domain acts as a glycosidase to depurinate ribosomes. This causes irreversible inhibition of protein synthesis and death of the cell [51] [52]. Interestingly, Shiga toxin has been shown to significantly enhance the survival of EHEC O157:H7 and *E. coli* K-12 in the presence of predatory bacterivorous protozoa [53]. Therefore, it is possible that the Shiga toxin gene is maintained in the EHEC genome to protect the bacterium against predators in the host gut.

EHEC O157:H7 virulence factor	Example	Characteristics
Endotoxin	O157 LPS	Induces cytokine expression through TLR4. O-antigen-specific antibodies are elicited in calves infected with EHEC O157:H7 but this does not result in clearance of the bacteria [37]. Vaccines based on the O157 LPS elicit specific antibody responses in humans and mice but mice were not protected against intestinal colonisation by EHEC O157:H7 [54] [55] [56].
Capsule	Group 4 capsule	Temporal expression of a group 4 capsule (co-ordinated with T3SS expression) is required for colonisation of the host intestine [57].
Adhesins	Intimin	Binds T3SS effector Tir to mediate intimate adherence to host enterocytes [58].
	ToxB	Encoded on pO157, influences expression of T3SS and promotes adherence to host cells but is not required for colonisation of young calves [59] [60].
	Fimbriae	EHEC O157:H7 encodes 14 fimbrial operons [61]. Fimbriae are involved in initial attachment of bacteria to host cells but may interfere with T3SS mediated intimate attachment [62]. Type I fimbriae are not expressed in EHEC O157:H7 due to a deletion in the fimA promoter [63]. Long polar fimbriae (LPF1) may contribute to colonisation *in vivo* [64] [65].
Pili	Type 4 pili	Long bundles of T4P form haemorrhagic coli pili (HCP) that mediate interbacterial, bacterial-epithelial cell and bacterial-extracellular matrix interactions. This contributes to colonisation of the host [66].
Toxins	Stx	Shiga toxin depurinates rRNA leading to inhibition of protein synthesis and induction of apoptosis [51] [52].
	EhxA	Enterohaemolysin causes cell lysis of erythrocytes and leukocytes [67].
Flagellar T3SS	Flagella	Used for bacterial motility/chemotaxis [68]. Also increases bacterial adhesion to epithelial cells *in vivo* [69] [70]. Flagellin binds TLR5 which activates IL-8 expression and an inflammatory response [71].
Proteases	EspP	Type V secreted serine protease encoded on pO157 that cleaves human coagulation factor V [72]. Required for adherence to and colonisation of bovine intestine [73].
	StcE	Metalloprotease, C1-esterase inhibitor, disrupts complement cascade [74].

Table 1.2: EHEC O157:H7 virulence factors (excluding nf-T3SS)

1.4. Type III secretion

Secretion of proteins is important for bacterial pathogens as it allows them to transport a variety of virulence determinants from their cytosol to the external milieu and host cells. The Gram-negative cell envelope consists of an inner membrane, a thin peptidoglycan layer (contained within the periplasm) and an outer membrane [75]. Gram-negative bacteria including pathogenic *E. coli* are able to move proteins across the cell envelope using at least six different secretion systems. Each system uses its own dedicated structural machinery and auxiliary proteins, such as chaperones, to support the different modes of secretion. Secretion may occur in a single step as in types I, III and IV or in multiple stages as in types II and V. Table 1.3 summarises each secretion system.

There are two main classes of type III secretion system (T3SS), the flagellar and the non-flagellar. The subsystems are homologous and monophyletic i.e. they have evolved from a common ancestor [76-78]. Each system is comprised of over 20 proteins and they form some of the most complex bacterial structures characterised to date. The flagellar T3SS is involved in flagellar biosynthesis [79]. Flagella are used by bacteria for swimming and swarming. The non-flagellar T3SS (nf-T3SS) is used by both commensal and pathogenic bacteria to deliver bacterial 'effector' proteins directly into eukaryotic cells through a 'molecular syringe' [80]. This may enable bacteria to colonise, disseminate and survive within the mammalian/plant/insect host. T3SSs are versatile – they are not limited to a particular bacterial species, host environment or disease spectrum. The work presented in this thesis concentrates on the nf-T3SS; subsequent reference to T3SS refers to the nf-T3SS unless otherwise stated.

System	Example	Mechanism
Type I (T1SS)	α-haemolysin secretion by uropathogenic *E. coli* [81]	One-step Sec-independent secretion. Substrates are secreted across the cell envelope using an ABC transporter, a membrane fusion protein and a pore-forming outer membrane protein to cross the inner membrane, periplasm and outer membrane respectively [82]. Substrates possess a C-terminal signal peptide that is not cleaved during secretion.
Type II (T2SS)	Pullulanase secretion by *Klebsiella oxytoca* [83].	Two-step Sec-dependent secretion of proteins. Proteins cross through the inner membrane using a multi-subunit translocon (general secretory pathway). Proteins then cross the outer membrane through a ring-shaped secreton complex [84]. Substrates have an N-terminal signal peptide that is cleaved during secretion.
Type III (T3SS)	Yop secretion by *Yersinia pestis* [85]	One-step Sec-independent secretion of proteins through a central channel that spans the cell envelope. A hollow filament extends from the outer membrane. The filament is either used to deliver bacterial proteins into eukaryotic cells or for locomotion depending on the type of system (flagellar/non-flagellar) [86].
Type IV (T4SS)	Pertussis toxin secretion by *Bordetella pertussis* [87]	One-step Sec-independent secretion mechanism. The apparatus is similar to T3SS in structure and function i.e. substrates are secreted into host cells through a pilus. However, substrates can include DNA as well as proteins [88].
Type V (T5SS)	IgA protease secretion by *Neisseria gonorrhoeae* [89]	Two-step Sec-dependent secretion. Proteins are secreted across the inner membrane by the general secretory pathway as in T2SS. The C-terminal β-barrel domain of the substrate (Type Va) or its partner (Type Vb) then forms a pore in the outer membrane allowing the substrate to pass through the outer membrane [90]. Substrates have an N-terminal signal peptide that is cleaved during secretion.
Type VI (T6SS)	Haemolysin-coregulated protein (HCP) secretion by *Vibrio cholerae* [91]	A newly identified system. The structure, function and localisation of many components of the system, are poorly understood although there is evidence that proteins are translocated directly into host cells as in T3SS and T4SS [92]. Substrates have no N-terminal secretion signal.

Table 1.3: Secretion systems used by Gram-negative bacteria.

The T3SS requires several types of protein:

- Structural proteins – form an injectisome. The injectisome comprises two rings (one in each bacterial membrane) joined by a short rod (periplasmic) and a needle that extends from the outer membrane ring. Bacterial proteins pass through the injectisome from the bacterial cytosol to the eukaryotic cytoplasm in one step. The structure of the Esc-Esp T3SS is shown in figure 1.1.
- Translocator proteins – located at the tip of the needle; allow passage of effector proteins into the host cell.
- Effector proteins – secreted into eukaryotic cells by the T3SS; subvert normal host cell processes to the benefit of the bacteria.
- Chaperones – assist in the unfolding and secretion of effector proteins.
- Regulatory proteins – control expression of the T3SS and secretion of translocators and effectors.

Phylogenetic analysis of the ATPases of injectisomes reveals that there are seven families of T3SS [76] [86]. The families of T3SS with examples of the organisms that possess them are shown in table 1.4. Individual species of bacteria can contain more than one type of T3SS. For example, *Burkholderia pseudomallei* contains two plant pathogen-like T3SS (TTS1 and TTS2) [93] and one animal pathogen-like T3SS (Bsa) [94]. *Salmonella* possesses two T3SSs; T3SS-1 and T3SS-2 which are used for initial invasion of host cells [95] and for survival and replication within host cells respectively [96].

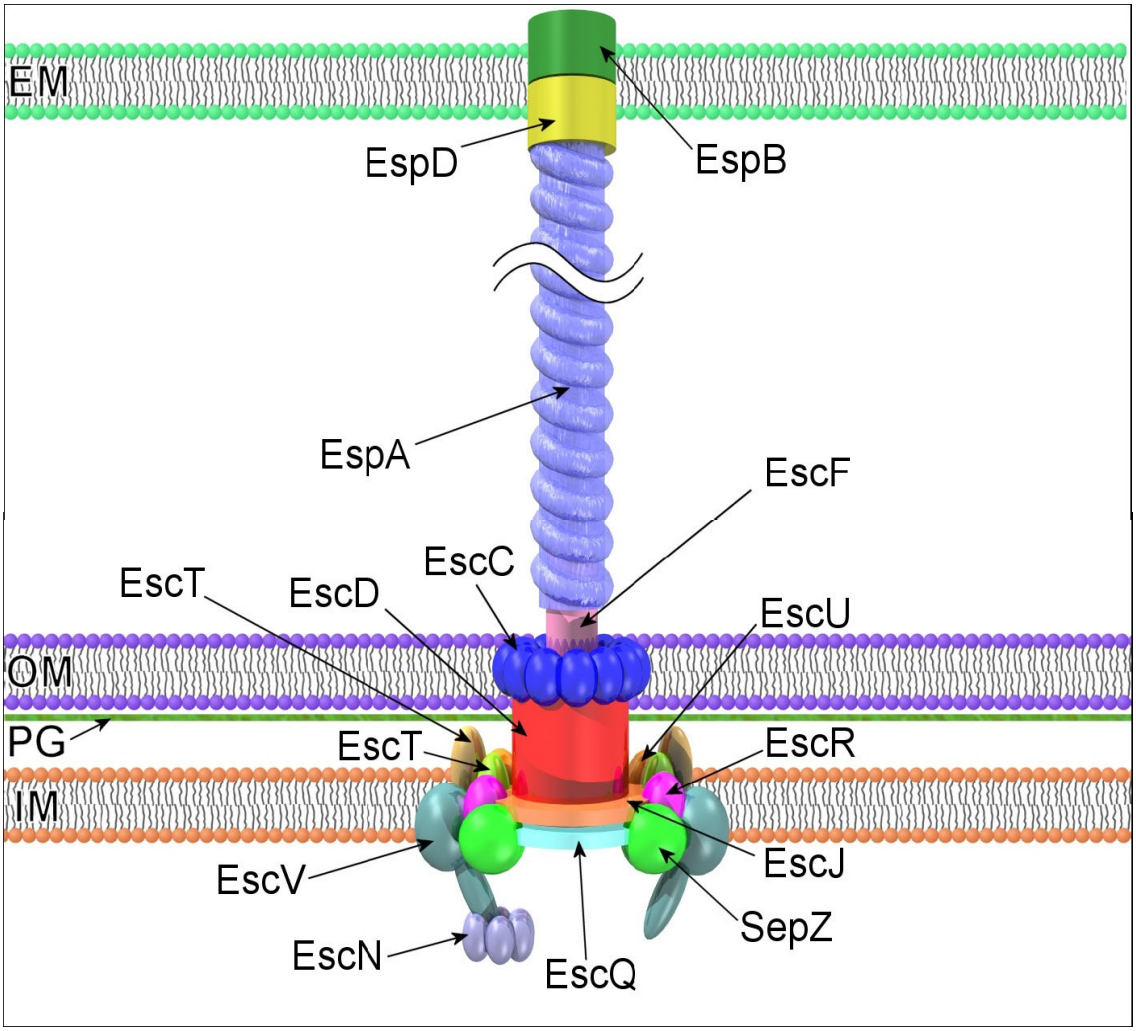

Figure 1.1: Graphical representation of the Esc-Esp T3SS. EM; eukaryotic membrane, OM; outer membrane, PG; peptidoglycan layer, IM; inner membrane. Image used with permission from Pallen *et al*., 2005 [1].

T3SS Family	Organisms	Examples
Ysc	*Yersinia* *Pseudomonas aeruginosa* *Aeromonas salmonicida*	Includes the Ysc T3SS found in *Yersinia* spp (where it is necessary for survival within macrophages [97, 98] [99]) the Psc T3SS of *Pseudomonas aeruginosa* and the Asc T3SS of *Aeromonas* sp.
Inv-Mxi-Spa	*Shigella* *Salmonella*	Includes the Inv-Mxi T3SS found in *Shigella,* the Inv-Spa T3SS of *Salmonella* and the Bsa system in *Burkholderia pseudomallei*. These T3SSs are involved in uptake of the bacteria by host cells to promote intercellular dissemination within the host [100] [101] [102].
Ssa-Esc	*E. coli* *Salmonella*	EPEC and EHEC use the Esc-Esp T3SS to intimately attach to and efface host cells [103]. *Salmonella* uses the SPI-2 encoded Ssa T3SS to interfere with phagosome maturation in macrophages to allow bacteria to multiply within host cells [104].
Rhizobiales	Symbiotic bacteria of plants such as the nitrogen-fixing *Rhizobiaceae* family	This system is necessary for the establishment of a beneficial symbiosis between the bacterium and plant host e.g. by inducing root hair deformation and nodule formation [105] [106] [107].
Chlamydiales	*Chlamydia*	T3SS used to modulate host cell cytokine release and to support intracellular survival and growth [108].
Hrc-Hrp1 and Hrc-Hrp2	Plant pathogens including *Pseudomonas syringae, Xanthomonas campestris*	T3SS play a significant role in colonisation of the plant surface and in the formation of lesions [109-111]. T3SS enables bacteria to cause disease on susceptible hosts [112] but induces the hypersensitive response in non-susceptible hosts [113].

Table 1.4: Families of T3SS

Both flagellar and nf-T3SSs play an important role in bacterial virulence and symbiosis (the flagellar system also has an important role in motility) [114] [115]. Our understanding of these processes therefore depends upon understanding both types of T3SS and the functions of nf-T3SS effector proteins. The study of T3SSs is also important as the T3SS provides an attractive target for novel vaccines/drugs [116]. For example, EspA-based vaccines have been designed to reduce colonisation of cattle by EHEC O157:H7 as the EspA filament is exposed to host immune cells during infection [117]. Also, screening of small molecule inhibitors in *Yersinia* [118], *Chlamydia* [119, 120], EPEC [121] and *Salmonella* [122] has identified several compounds capable of inhibiting the T3SS without affecting bacterial replication [122]. The T3SS may also provide an attractive drug/vaccine delivery tool – biologically active cytokines and antigens have been delivered into eukaryotic cells using T3SSs of attenuated *Shigella* [123] and *Salmonella* [124].

1.5. T3SS effectors

Effector proteins are bacterial T3SS proteins that function primarily inside the host cell [125]. It is predicted that effectors are secreted in a denatured/partially denatured state although it is unclear as to how (or whether) effectors depend upon host cell molecules to fold into a functional state after secretion. Studies of BopE (*B. pseudomallei* effector) suggest that effectors may be capable of spontaneously folding into a functional state after translocation into the host cell cytoplasm [126].

Translocation of effectors into the host cell leads to subversion of the host cell [127]. Effectors may cause local (e.g. cytoskeletal) or systemic (e.g. immune response) changes in the host. Therefore, subversion can have a variety of outcomes for example, invasion of the host cell [128], evasion of the host immune system [85] or development of a beneficial symbiosis between the host and bacterium [107]. Examples of T3S effectors from various T3S organisms are shown in table 1.5.

Local disruption of the cytoskeleton allows invasive bacteria to both survive/multiply within host cells and also to move intra/intercellularly to disseminate within the host and avoid host immune surveillance [129]. For example, many *Shigella* and *Burkholderia pseudomallei* T3SS effectors are involved in subversion of the actin and microtubule cytoskeleton of host cells, pathogen escape from endocytic vesicles and intercellular spread [128] [102]. T3SS effectors and actin-based motility are central to the pathogenicity of these pathogens [130] [131] [132].

Systemic changes are caused by numerous effectors – many modulate the host immune response e.g. effectors may affect IL-8 synthesis to cause or prevent attraction of neutrophils [133] [134]. *P. aeruginosa* induces IL-8 production using T3SS effector ExoU. ExoU activates JNK resulting in activation of AP-1 (a transcription factor) which up-regulates IL-8 synthesis [135]. Activation of the immune system in this way might aid pathogen colonisation by decreasing the number of resident gut microflora (as occurs in *Citrobacter* infections [136]). Down regulation of the immune response (as shown by EPEC [134]) may enable bacteria to reach a critical mass, disseminate/cause disease and spread to other organisms.

Many local and systemic changes in the host are controlled by small GTPases. These highly conserved molecular switches are involved in many aspects of eukaryotic cell biology [137] e.g Rho GTPases regulate actin cytoskeleton and microtubule dynamics as well as the cell cycle [138]. Their central role in eukaryotic biology and their ancient/conserved nature means that GTPases are often targeted by T3SS effectors [139] [140]. GTPases are regulated by GEFs (GTP exchange factors) and GAPs (GTPase activating proteins) that activate and inactivate GTPases respectively [137]. Activation of GTPases can have a variety of outcomes including up/down regulation of the immune response via the MAPK signalling pathway [141]. Numerous effectors mimic GAP/GEFs and activated forms of GTPases (see table 1.5).

Eukaryotic target	Effectors	Function	Outcome
Actin cytoskeleton	Tir (*Citrobacter rodentium*)	Binds Nck to activate N-WASP and recruit Arp2/3 [142].	Actin polymerisation and pedestal formation. Intestinal colonisation [144]
	VopL *(Vibrio)*	Arp2/3-independent actin assembly [143].	Disrupts actin homeostasis of host cells [145].
Microtubule cytoskeleton	VirA (*Shigella*)	Disrupts microtubules [146].	Enhanced intra- and intercellular spread [147].
Tight junctions	SopE (*Salmonella*)	Rho GEF [148].	Disruption of tight junction structure and function – contributes to diarrhoea [149].
Cell cycle	Cif (EPEC)	Inhibits mitosis inducer CDK 1 by preventing degradation of CDK inhibitors [150].	Inhibition of cell cycle at G2/M phase transition [151].
Mitochondria	Map (EPEC)	Disrupts mitochondrial membrane potential and morphology [152].	Mitochondrial swelling, damage and dysfunction [153] [154].
Signalling (Ubiquitination)	SseL (*Salmonella)*	Deubiquitinase [155].	Macrophage death [155].
	IpaH9.8 (*Shigella*)	Ubiquitin E3 ligase [156].	Disruption of host MAPK signalling pathways [156].
Signalling (Rho GTPases and MAPKs)	YopE (*Yersinia*)	Rho GAP [157] [158].	Inhibits Rac1 to inhibit phagocytosis and superoxide anion production [165].
	IpgB2 (*Shigella*)	Rho mimic [159].	Induces formation of actin stress fibres [159].
	SptP (*Salmonella*)	Rho GAP [160].	See text.
	SopE2 (*Salmonella*)	Rho GEF [160].	Bacterial uptake [148].
	Map (EPEC)	Cdc42 mimic [159].	Actin cytoskeleton rearrangement (filopodia) [159].
	YopJ (*Yersinia*)	MAPKK acetyltransferase [161].	Decreased chemokine production, apoptosis [166, 167].
	VopA (*Vibrio*)	MAPKK acetyltransferase [162].	Decreased cytokine production, apoptosis [162]
	OspF (*Shigella*)	MAPK phosphothreonine lyase [163].	Blocks activation of NF-κB responsive genes [168].
	ExoT (*Pseudomonas aeruginosa*)	Rho GAP [164].	Disrupts cytokinesis [169].

Table 1.5: Examples of T3SS effectors.

Effectors may function individually, synergistically or antagonistically with other effectors within the host cell to perturb normal cell processes. For example, EHEC Tir and TccP act together to disrupt and manipulate the actin cytoskeleton whereas others e.g. *Salmonella*'s SopE and SptP counteract each other. Such interactions between effectors can be important in the progression of infection through various stages. The antagonistic action of SptP (represses the activation of cellular GTPases caused by SopE [170]) restores normal cytoskeletal function thus preventing the expulsion of internalised bacteria. This promotes a transition between the invasive and intracellular growth stages of the *Salmonella* infection. T3SS effectors can also act alongside other secretion system proteins e.g. autotransporter proteins during infection processes such as dissemination. For example, *B. pseudomallei* uses the Bsa T3SS to invade host cells [102] and escape into the cytoplasm (via lysis of the endosomal membrane [102]). It then uses the T5SS protein BimA (induces actin polymerisation at the pole of the cell [171]) to facilitate cell-to-cell spread and dissemination within the host [171].

Individual T3SS effectors often exhibit multiple functions; for example, EPEC EspF targets mitochondria, the tight junction proteins Nexin-9 and occludin, and inhibits PI-3 kinase [172] [173]. Functional redundancy between effectors has also been demonstrated: EspF targets mitochondria, an organelle also targeted by another EPEC effector, Map [174].

Effector proteins, in contrast to T3SS structural proteins, are very diverse and do not show a high degree of sequence similarity between species [175] [176]. Similar strains of the same species can possess different repertoires of effectors. Over 300 different effectors are known

to exist [2]. However, despite T3SS effector diversity, there are common themes in the nature of the host cell processes that effectors manipulate and exploit. Many effectors target the host cell cytoskeleton and host cell signalling pathways. These are often the most conserved processes and pathways of eukaryotic cell biology. This is perhaps due to an ancient, long standing interaction between bacteria and eukaryotic cells. There is increasing evidence that T3SS may have evolved, not as virulence factors for bacterial pathogenesis in animals, but for other roles in microbial ecology such as survival within predatory amoebae [177]. Recently, the *Salmonella* T3SS encoded by SPI-2 was found to be essential for survival within *Acanthomoeba polyphaga* [177]. Other virulence factors including T2SS and T4SSs (in *Legionella pneumophila* [178] [179]) and toxins (Stx of EHEC O157:H7 [53]) have been shown to increase survival of bacteria in predatory protozoa.

The study of T3SS effectors allows us to better understand the interface between T3S organisms and their host, and may ultimately provide opportunities for therapeutic intervention [180] and vaccine design [181]. An increasing number of T3SS effectors and translocator proteins have been identified as T cell immunogens (i.e. vaccine candidates). For example, the *B. pseudomallei* effector BopE induced a BopE-specific T cell response in immunised mice [182] (although it provided incomplete protection against infection). *Yersinia* effector YopE also elicited a T3SS-specific T cell response in mice challenged with this effector [183].

T3SS effectors have also proved to be useful tools in the study of eukaryotic cell biology – allowing the molecular dissection of complex signalling pathways and cytoskeletal

processes [184]. In particular, our understanding of actin assembly in eukaryotic cells has greatly improved through the use of T3SS effectors [185]. For example, the minimal requirements for actin assembly in structures such as lamellipodia and filopodia can be defined using T3SS effectors from organisms such as *Shigella* and *Burkholderia* [186]. Novel connections and new classes of enzymes have also been discovered using T3SS effectors e.g. investigations into *Salmonella* effectors that modulate GTPase activity identified a novel connection between the Cdc42 and Slt2 mediated cell integrity pathway in yeast [160]. Structural studies of the *Shigella* effector IpaH led to the discovery of a novel class of E3 ubiquitin ligases [156, 187, 188].

1.6. T3SS in EHEC mediated disease

EHEC O157:H7 was shown by Tzipori *et al* to adhere to intestinal epithelia in a similar manner as EPEC and cause attaching and effacing (A/E) lesions in the intestines of infected piglets [189]. Knutton *et al* subsequently showed that the A/E lesion was characterised *in vitro* by bacterial microcolonies intimately adhering to the surface of cultured cells with cytoskeletal actin accumulation beneath the adherent bacteria [190] [191] [192] (see figure 1.2). Actin polymerisation beneath the site of adherence caused protrusions of the host cell plasma membrane known as pedestals [13]. Knutton *et al* used the actin-rich nature of the pedestals to devise a diagnostic test for EPEC infection using fluorescently labeled phalloidin (a toxin that binds filamentous (F)-actin) [13]. The fluorescent actin staining (FAS) test allowed infection with the EPEC pathotype to be diagnosed more quickly and easily than did O:H serotyping [13]. Formation of the EPEC A/E lesion was subsequently

shown by Jerse *et al* to be dependent on a gene, *eae* (*E. coli* attaching and effacing) [193] that was also found in EHEC O157:H7 [194]. McDaniel *et al* then showed that the *eae* gene was encoded within a 35-kb pathogenicity island and that this island was found in many enteric pathogens [195]. The pathogenicity island was shown to encode genes for attachment, a T3SS and T3S effector proteins [103, 195] and was termed the locus for enterocyte effacement (LEE) [196]. The LEE-encoded T3SS is an important virulence factor of EHEC and other A/E pathogens (e.g. *Citrobacter rodentium*, EPEC) [196]. The LEE is required for full virulence and for efficient colonisation of cattle by EHEC O157:H7 [197] and other EHEC serotypes [198]. The next section describes the LEE and associated T3SS effectors.

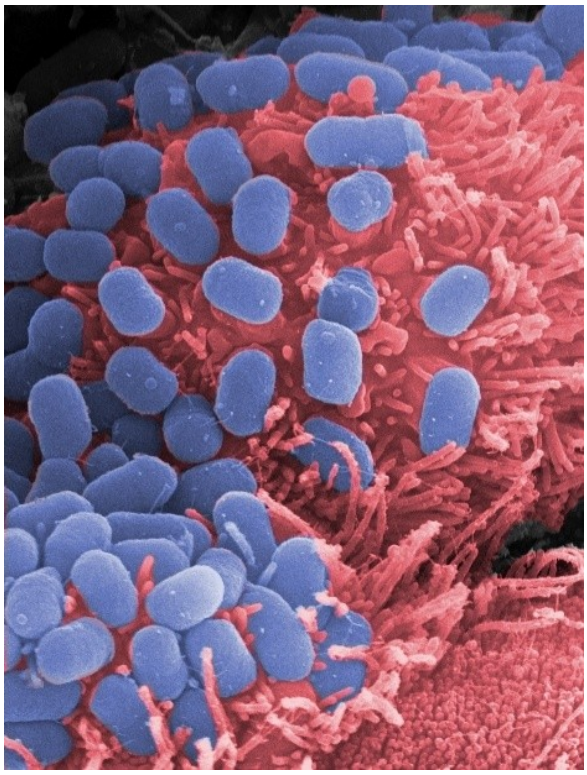

Figure 1.2: The attaching and effacing lesion. Scanning electron micrograph of EHEC O157:H7 intimately adhering to enterocytes. The LEE-encoded T3SS is required for the formation of the A/E lesion. Image used courtesy of Dr R. Shaw, University of Birmingham.

1.7. Locus of Enterocyte Effacement

1.7.1. The LEE is a pathogenicity island

T3SS are often encoded by pathogenicity islands; for example the *Salmonella* pathogenicity island SPI-1 encodes a T3SS required for invasion of intestinal cells [199]. PAIs are a subclass of genomic island that encode virulence factors and share many features [200] [201]. These features will be discussed with reference to the LEE:

- PAIs carry genes that encode virulence factors (e.g. adhesins, toxins, secretion systems); the LEE encodes a T3SS, T3SS effector proteins and an adhesin that produce the A/E lesion.
- PAIs are present in the genome of pathogenic bacteria but not in the genome of similar non-pathogenic members of the same species [201]. The LEE is found in attaching and effacing *E. coli* but not in non-pathogenic lab-strains of *E. coli* [202] [195].
- PAIs are relatively large: 10-100kb. The EHEC O157:H7 LEE is approximately 43kb in length [203].
- PAIs differ in G+C content and codon usage from the rest of the host genome. The EHEC O157:H7 LEE has a G+C content of approximately 40% whereas the rest of the chromosome has an average G+C content of 50.8% [201].
- PAIs are often flanked by directly repeated DNA sequences. The 5' end of the EHEC LEE contains a directly repeated sequence from a P4-like prophage [203].

- PAIs are often associated with tRNA genes. The EHEC O157:H7 LEE is inserted into the *selC* tRNA locus. However, *pheU* and *pheV* loci are also used in other *E. coli* strains [204-207].
- PAIs often carry mobility factors (although these are sometimes non-functional) e.g. integrases, transposases, insertion (IS) elements. The EHEC O157:H7 LEE carries a P4-like prophage element (933 L). The *C. rodentium* LEE contains many IS elements [201].
- PAIs represent unstable regions of DNA i.e. easily deleted/transferred/transmitted. Previous autonomous mobility of the LEE is suggested by the IS*600*-like insertion elements found at the ends of the LEE and a transposase-like peptide encoded by a small CDS [203]. The position of the LEE within A/E pathogen genomes also varies; suggesting that the LEE has been acquired independently, a number of times, by A/E pathogens [76, 208].

PAIs are acquired by horizontal gene transfer (HGT) [209]. HGT of PAIs via transduction, conjugation or transformation is significant – along with modification and loss of existing genes, acquisition of new genes is an important process in the evolution of bacterial pathogens [201]. Acquisition of PAIs enables bacteria to make a 'quantum leap' towards pathogenicity. This has been demonstrated by functional transfer of T3SSs encoded on pathogenicity islands. For example, transfer of the 'high pathogenicity island' of a virulent *Yersinia enterocolitica* strain to a less virulent strain resulted in increased virulence in the recipient strain [210]. Similarly, transfer of the EPEC LEE to *E. coli* strain K-12 conferred the ability to form A/E lesions on enterocytes [211]. There is evidence to support the hypothesis that the LEE was acquired independently via horizontal gene transfer by EPEC

and *Citrobacter rodentium* [208]. The insertion site of the EPEC LEE (inserted in the *selC* tRNA gene) is different from the insertion site in *C. rodentium* which is flanked by plasmid sequences [208]. The presence of plasmid sequences suggests that the LEE may have resided on a horizontally transferable plasmid [208]. Horizontal transfer of plasmids among A/E pathogens is plausible – plasmids pCRP3 of *C. rodentium* and p9705 of EHEC O157:H7 are very similar [208].

Many *E. coli* strains, including EHEC O157:H7, contain a PAI that encodes a degenerate T3SS [212] [213]. This PAI is known as *E. coli* type III secretion system 2 (ETT2). Although the secretion system encoded by ETT2 cannot be used for secretion of proteins due to frameshifts in the secretion apparatus genes [213], ETT2 retains a regulatory role in the secretion of proteins through the LEE-encoded T3SS [3].

1.7.2. Structure of the LEE

The EHEC O157:H7 Sakai LEE contains 54 coding sequences (CDS). 41 of these are common to the EPEC and *C. rodentium* LEE. The EHEC Sakai LEE is located at the *selC* tRNA gene [208]. The 13 CDSs found only in EHEC belong to a P4-like prophage that is inserted near the *selC* locus. The genes of the EHEC O157:H7 Sakai and EPEC LEE often show more than 98% homology and the order and orientation of the loci in both pathovars are identical [203]. The proteins encoded in the LEE include: T3SS structural proteins; translocators; the adhesin intimin; regulatory proteins; chaperones of effectors and a small number of effectors [214, 215]. LEE genes are ordered into 5 large polycistronic operons

(*LEE1-5*) and several smaller transcriptional units. Most of the structural proteins that make up the injectisome are encoded in *LEE1* and *LEE2*. *LEE3* encodes the ATPase (EscN), an effector protein (EspH) and a number of Sep proteins that are important in the control of secretion hierarchy. A variety of protein types are encoded in *LEE4* including the effector EspF, the chaperone CesD2 and the translocator proteins, EspA, EspB and EspD. *LEE5* encodes Map, Tir, the Tir chaperone CesT and intimin. The 41 LEE CDS common to EHEC and EPEC are shown in figure 1.3.

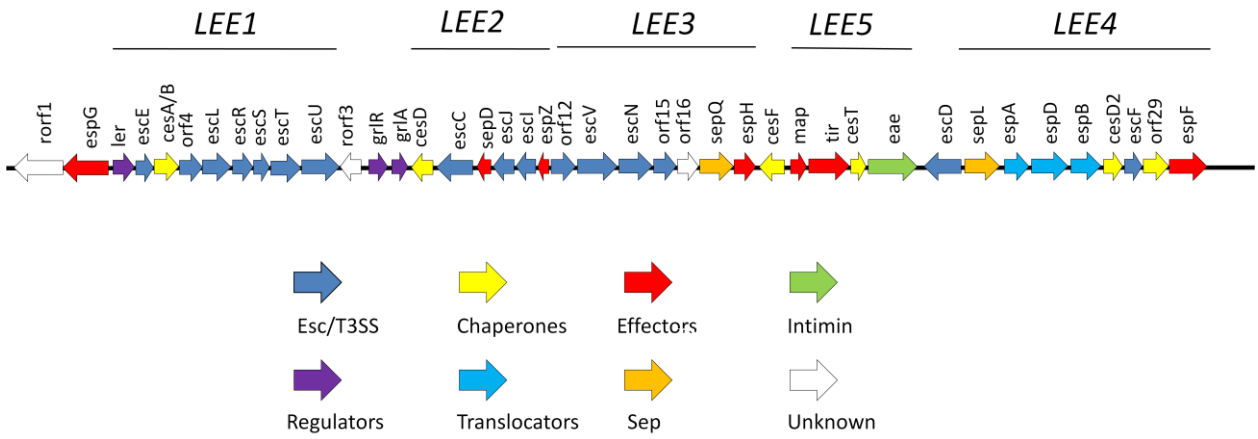

Figure 1.3: 41 CDS common to both EHEC and EPEC LEE regions. The function of proteins encoded by each CDS is indicated by colour. Operons *LEE1-5* are also indicated.

1.7.3. LEE-encoded translocators

The needle of the Esc injectisome, unlike other type III needles has a long flexible filament attached to the tip. The filament is composed of EspA and assists in initial attachment of bacteria to enterocytes [216-219]. EspA is probably homologous to flagellin [1] [218] [220] and polymerises to form a filament at the distal end of the needle with a channel of 25-Å diameter [221]. EspA is required for colonisation of the calf intestine by EHEC O157:H7. Due to its role in colonisation and its exposure on the surface of EHEC O157:H7 cells, EspA has been tested as a vaccine to prevent infection of cattle. However, results showed that despite the induction of a high-titre antigen-specific IgG and IgA response to EspA, calves were not protected against colonisation by EHEC O157:H7 [222]. Translocators EspB and EspD lie at the end of the EspA filament and form a pore in the host cell membrane through which effector proteins pass into the host cell [223] .

1.7.4. LEE-encoded chaperones

The LEE also encodes several chaperones that are required for efficient secretion of effectors and translocator proteins [224]. T3SS chaperones have various functions including; delivery of substrate to the secretion apparatus, prevention of substrate degradation and efficient secretion of the substrate [224, 225]. Chaperones may also maintain substrates in a secretion competent state (e.g. prevent polymerisation of EspA [226]), aid protein entry into the secretion machinery [225], and control the hierarchy of effector secretion [227] [228]. T3SS chaperones are divided into several classes. Class I chaperones are small acidic proteins that associate with effector proteins. There are three

class I chaperones encoded within the LEE; CesF [229], CesT [196] and Orf12 [Pallen, unpublished]. The effectors Tir, EspH, EspZ and Map require CesT for secretion [230] whereas EspF requires CesF [230]. Class II chaperones contain three tetratricopeptide repeat motifs and assist in the secretion of translocator proteins [231] [232]. The LEE-encoded protein CesD is a class II chaperone; CesAB and CesD2 appear to form two distinct classes of their own [233]. An additional chaperone of EspA encoded by LEE gene L0017 was recently characterised [234].

1.7.5. Regulation of LEE gene expression

Coordinated control of virulence gene expression in response to environmental changes is vital to pathogen survival [235]. T3SSs are costly in terms of the amount of substrates (e.g. amino acids) used in their construction; transcriptional regulation of T3SS expression is therefore tightly controlled. T3SSs are only expressed under specific environmental conditions or in response to certain stimuli e.g. quorum sensing [236]. For example, *Aeromonas salmonicida* A449 expresses its T3SS in response to a rise in temperature or salt concentration [237]. Regulation of the LEE is complex and a full discussion is beyond the scope of this introduction – the reader is directed to recent reviews for more information on transcriptional regulation of the LEE [236, 238]. Figure 1.4 provides a summary of LEE regulation. At least four regulatory inputs influence expression of LEE genes. These include:

- a regulatory hierarchy within the LEE involving LEE-encoded regulators Ler [239], GrlR and GrlA [196].

- global regulators and responses e.g. H-NS, IHF, FIS, quorum sensing and responses to host hormones e.g. noradrenaline [236].
- regulators encoded on mobile genetic elements [3].
- Post-transcriptional processing. Experimental evidence demonstrates that post transcriptional processing of *LEE4* may allow differential regulation of SepL and Esp protein production [240].

In EHEC O157:H7, environmental factors such as temperature, nutrient availability, ion concentrations and butyrate are evaluated by sensing systems [241] [242]. This results in expression of transcriptional regulators which then activate or repress virulence gene expression [243] [244]. For example, the stringent response in EHEC O157:H7 differentially regulates expression of phage/PAI encoded perC-homologous (pch) genes [245]. Pch genes are positive regulators of the LEE [246]. Activation of transcription of the *LEE1* operon by such regulators results in expression of Ler [246]. Ler is itself a transcriptional regulator that activates transcription of other LEE operons by antagonising repression caused by H-NS [247]. GrlA and GrlR are LEE-encoded regulators that act with Ler to regulate transcription of various LEE operons to coordinate construction of the T3SS [248]. Ler and GrlA are also involved in the regulation of non-LEE-encoded virulence factors e.g. enterohaemolysin [249] [250]. Cross regulation of T3SS and flagella in EHEC O157:H7 by GrlA/GrlR has also been demonstrated [251]. Other transcriptional regulators encoded on mobile elements such as EtrA and EivF that are encoded within the ETT2 locus in EHEC O157:H7 have been shown to negatively regulate transcription of the LEE [3]. Also, proteins encoded on pO157 including ToxB and Efa-1' are required for the expression of LEE genes and secretion of type III secreted proteins in EHEC O157:H7 [60].

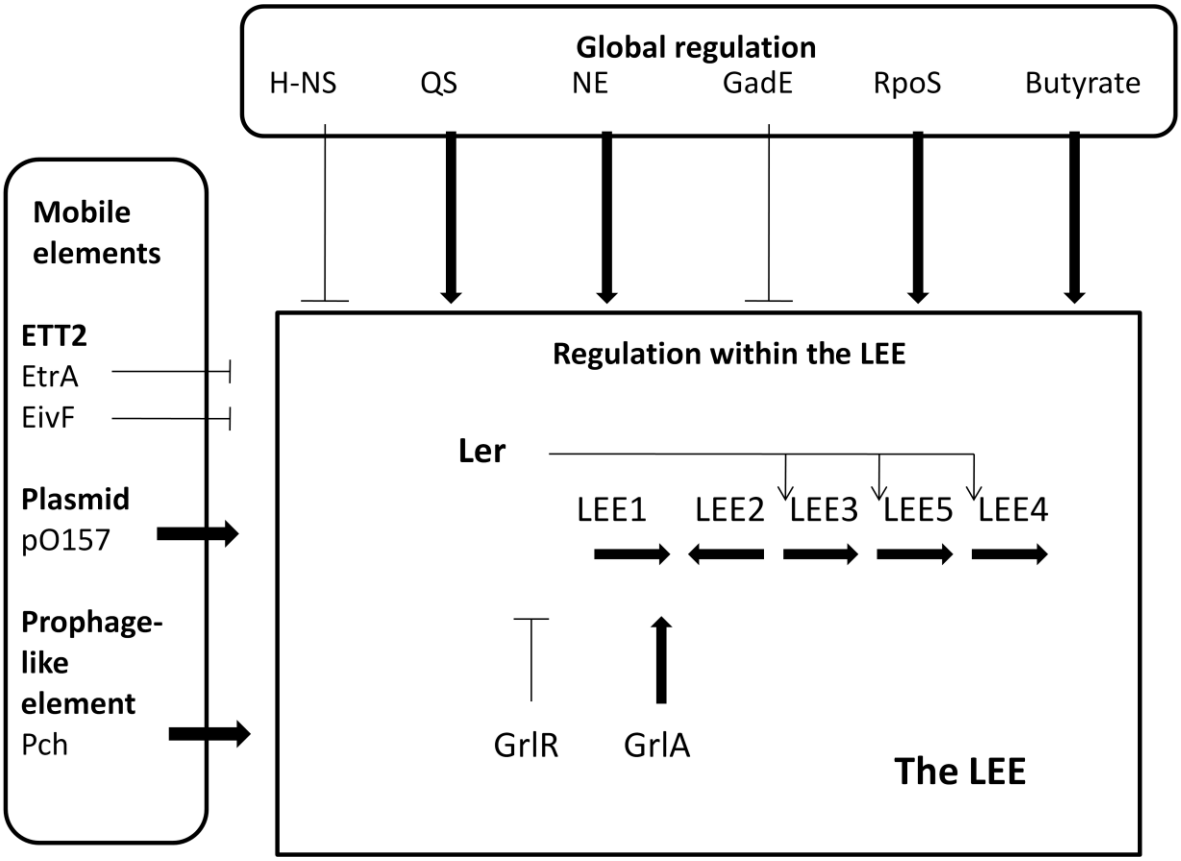

Figure 1.4: Regulation of the EHEC O157:H7 LEE. Transcription of the LEE is regulated by LEE encoded proteins, regulators encoded on mobile elements and global regulatory systems. QS, quorum sensing; NE, noradrenaline. Arrowheads indicate positive influences, crossbars indicate negative influences. Figure adapted from Zhang *et al*., 2004 [3].

1.7.6. LEE-encoded effectors

A small number of T3SS effectors are encoded within the LEE [196]. They include: Tir [58], EspF [252], EspG [253], EspH [254], EspZ [255], and mitochondrial associated protein (Map) [153]. The translocator EspB is also translocated into host cells [256].

The translocated intimin receptor (Tir) was the first T3SS effector of A/E pathogens to be characterised [58]. This effector is essential for formation of the A/E lesion [59] [257]. Without it, A/E pathogens are less able to colonise the host [196] [258]. As its name indicates, Tir acts as a receptor for the bacterial adhesin intimin, which is also encoded within the LEE [58]. After translocation into the host enterocyte, Tir inserts into the apical host cell membrane. Here it adopts a hairpin–like structure with both the amino- and carboxy-termini of the protein facing the host cell cytoplasm and the mid section of the protein exposed to the external milieu. The mid section forms the receptor for intimin present on the bacterial surface. The N-terminus of Tir interacts with cytoskeletal proteins such as α-actinin and cortactin [259, 260]. The C-terminus of Tir may be phosphorylated at a specific tyrosine residue (Y474) and bind Nck to indirectly activate host N-WASP or remain unphosphorylated and bind Tir cytoskeleton coupling protein, TccP [261, 262]. Alternatively, Tir may bind both Nck and TccP to activate N-WASP [263]. N-WASP then recruits host protein Arp2/3 which leads to actin polymerisation beneath the adherent bacterium and pedestal formation [261]. EHEC O157:H7 Tir binds TccP to activate N-WASP. However, pedestals were detected beneath an adherent EHEC O157:H7 *TccP* mutant in calves. Colonisation of the calves by the mutant was also unaffected whereas

pedestal formation *in vitro* was affected [257]. This suggests that*, in vivo*, actin assembly may occur by a distinct mechanism.

Map contains an N-terminal mitochondrial targeting sequence and causes mitochondrial swelling, damage and dysfunction by interfering with the mitochondrial membrane potential [153]. Map also binds EBP50/NHERF1 via a C-terminal TRL motif to induce filopodia formation and disruption of tight junctions [264].

EspF is a potent multifunctional effector. Studies have shown that it is able to disrupt host intestinal barrier function [265], coordinate host membrane trafficking [266] and cause apoptosis [267] [268]. EspF also inhibits sodium exchanger 3 activity [269], activates N-WASP [266] and causes redistribution of aquaporins [270].

EspG is homologous to VirA of *Shigella* [253]. The main function of EspG is disruption of microtubules [271] [272] [273]. Pertubation of microtubules by EspG has been shown to have various effects on the host cell including a decrease in the level of the anion exchanger DRA in host cell apical plasma membrane, mislocalisation of enterocyte aquaporins AQP2 and AQP3 [274] and alteration of paracellular permeability [275]. Disruption of chloride transport in the intestinal luminal membrane and redistribution of aquaporins may contribute to diarrhoea [276].

EspH modulates the actin cytoskeleton and when overexpressed causes elongation of pedestals and represses formation of filopodia [254].

EspZ is a hypervariable effector that, contrary to initial reports, does not mediate uptake of bacteria into host cells or affect phosphorylation of Tir [255]. EspZ is delivered at high levels (comparable with Tir) early in the infection process [230].

1.8. Non-LEE-encoded effectors

1.8.1. The LEE is not self-contained

Transfer of the EPEC LEE [211] but not the EHEC O157:H7 LEE [277] to *E. coli* K-12 is sufficient to confer the A/E phenotype on this non-A/E strain. This shows that the EHEC O157:H7 LEE is not self contained and that non-LEE-encoded effectors are required for A/E lesion formation. Whilst characterising the *C. rodentium* LEE, Deng *et al* discovered seven non-LEE-encoded effectors (Nle A-G) that were translocated through the LEE-encoded T3SS [196]. Other non-LEE-encoded effectors have since been discovered; TccP [278], EspJ [279], EspG2 and Cif [151]. This shows that the EHEC O157:H7 LEE, like other T3SS PAIs, does not act in isolation. For example, *Salmonella* encode many T3SS effectors outside their T3SS PAIs [280] and the plant pathogen *Pseudomonas syringae* encodes over 200 T3SS effector proteins outside the Hrp locus [281] [176].

The occurrence of numerous T3SS effector genes encoded outside the T3SS pathogenicity islands of many T3S organisms prompted Pallen and his research group (in collaboration with Toru Tobe and Gad Frankel) to investigate the presence of non-LEE effector genes in EHEC O157:H7. The rest of this section describes the work carried out by the aforementioned groups (author was a member of the Pallen group) in the search for non-LEE-encoded T3SS effectors in EHEC O157:H7 Sakai. The work documenting the discovery of novel non-LEE effectors was published in 2006 and is subsequently referred to as work by Tobe *et al* [2]. The genomic context of the novel effector genes is discussed. The final section of this chapter provides a discussion of model systems that have been used to investigate T3SS effector localisation and function to provide a background to the experimental work presented in subsequent chapters.

1.8.2. Non-LEE-encoded effectors in EHEC O157:H7

T3SS effector genes do not have common amino acid sequences such as secretion signal peptides (as found in other secretion systems such as T2SS) [282]. This means that T3SS effectors cannot be identified by simple analysis of protein sequences - one is forced to rely on the detection of homology to known effectors using more advanced sequence analysis such as Basic Local Alignment Sequence Tool (BLAST) and Position Specific Iterative (PSI)-BLAST.

PSI-BLAST is a powerful tool used to find distant homologies to an input query sequence [283] [284]. The original BLAST program calculates similarity and produces alignments

between a query sequence and sequences contained within a database [285]. BLAST also provides a statistical estimate as to the likelihood of two sequences being related by chance alone [285]. PSI BLAST is similar to BLAST except that a position-specific scoring matrix (PSSM) is used in place of the query sequence in an iterative process [283]. The PSSM allows patterns to be developed from an alignment of significantly similar sequences to the original query sequence. This is achieved by weighting the amino acid residues of each sequence according to the information provided by the residue [286]. The PSSM (pattern) is then used in subsequent iterations of PSI-BLAST to find more distant homologies. Tobe *et al* used PSI-BLAST and a query set of 300 known T3SS effectors from organisms such as *Shigella* and *Pseudomonas* to identify over sixty novel non-LEE-encoded T3SS effector genes in the EHEC O157:H7 Sakai genome [2].

Thirteen of the novel EHEC effectors were predicted to be pseudogenes. Pseudogenes encode proteins that are not functional due to alterations such as premature stop codons (resulting in a protein <80% length of functional counterpart), insertions, or frameshifts (altering more than 20% original amino acid sequence) [287].

1.8.3. Distribution of non-LEE-encoded effectors

The novel EHEC O157:H7 effectors were encoded in more than 20 exchangeable effector loci (EEL). EEL are found in many T3SS organisms. The loci contain different collections of effector genes in different strains of the same species and contain the hallmarks of horizontally transferred genes i.e. they contain phage genes, transposable elements, plasmid-

related sequences and usually show significantly different G+C content to the rest of the host genome [176]. For example, the EEL of *P. syringae* strains contain completely different collections of effector genes in each strain; this is in contrast to the conserved effector locus (CEL) that contains the same effectors in all strains and is necessary for pathogenesis [176] [288]. The EHEC EEL (containing novel effectors) included 14 non-phage EEL and nine EEL within lambdoid prophages [2].

Phage-encoded effectors were located within the passenger compartments of phage genomes. These compartments, known as 'morons' are downstream of phage tail fibre genes and can be transcribed independently from the rest of the phage genome owing to the presence of promoter and terminator regions [289]. It was found that EHEC phage EEL always contained more than one effector gene (Sp9 EEL harbours eight effector genes) and effectors typically showed low G+C content (*ca.* 30%) compared to their host phage. The location and structure of the EHEC O157:H7 phage EEL that contain novel T3SS effectors are shown in figure 1.5.

Non-phage EEL in EHEC contained one or a small number of effectors and were mainly lineage specific insertions. It is speculated that non-prophage encoded EEL represent ancient phage remnants or ETT2 substrates [2].

1.8.4. Novel effectors give insights into EHEC evolution

As discussed earlier, the acquisition of PAIs is an important process in the evolution of bacterial pathogens. PAIs and prophages are a major source of genomic diversity within *E. coli* [7] [290]. Much of the 1.4Mb of the EHEC O157:H7 genome that is absent from the *E. coli* K-12 genome [291] is comprised of horizontally transferred PAIs and prophages [292] - the lethal Shiga-like toxins of EHEC O157:H7 are encoded on lambdoid prophages [293]. It has been suggested that T3SS effectors present in EHEC O157:H7 prophages belong to a dynamic phage-based metagenome [2]. This hypothesis is supported by evidence of recombination between prophage of Sakai and EDL strains of EHEC O157:H7 i.e. both strains contain the same prophage at the same loci, but internally the phages are distinct [294]. As the number of sequenced EHEC genomes increases it is becoming clear that horizontal gene transfer, including HGT of T3SS effector genes, has been important in the evolution of EHEC [294].

1.8.5. Novel effector nomenclature

Novel EHEC effectors were classified into 20 families; the largest being NleG with 13 members. A scalable nomenclature similar to that used for *Pseudomonas syringae* effectors [281] was devised for the novel effectors. This was based on effector family and followed the *E. coli* secreted protein nomenclature (Esp) i.e. families were named EspK – EspR. Two further families were EspY (all effectors with a WEX5F motif) and EspX (all SopA-like effectors). Subfamilies and their individual members were defined using a numerical suffix e.g. NleG8-2 [2]. Novel effectors were found to include, amongst others, homologues of

effectors from plant pathogens e.g. *Pseudomonas syringae* effector HopPtoH; and homologues of *Shigella* 'Osp type' effectors.

Non-LEE-encoded effectors including those discovered by Deng *et al* have been investigated individually using various models. However, their functions remain poorly characterized with most having only been identified as being necessary for colonization of a murine infection model (Table 1.6). This study aimed to use a standardised approach to characterise novel EHEC O157:H7 Sakai effectors (focusing on those discovered by Tobe *et al*) using a range of model systems.

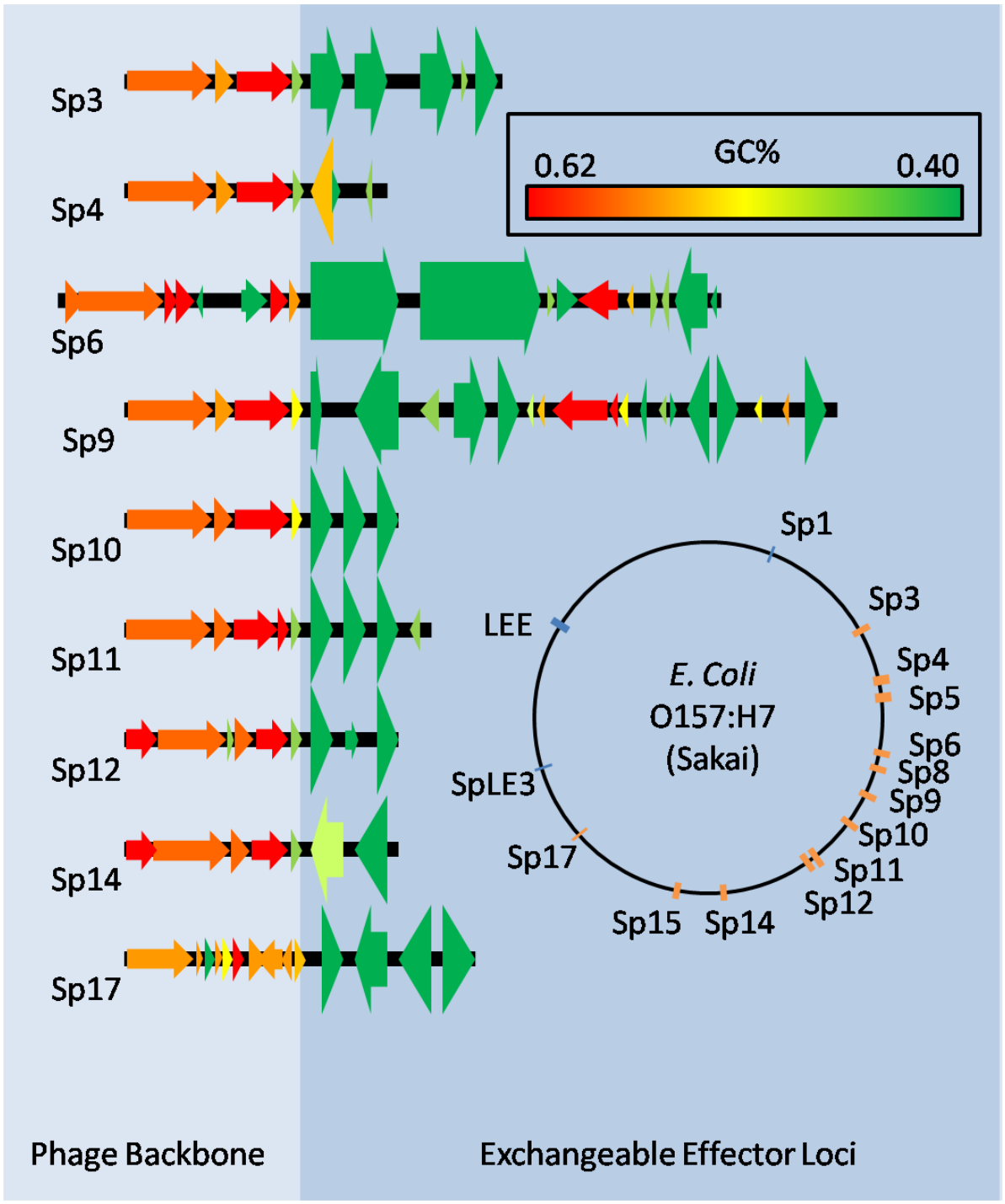

Figure 1.5: Exchangeable effector loci (EEL) of EHEC O157:H7 Sakai. Non-LEE T3SS effectors are indicated by tall arrows. The location of EELs within the EHEC (Sakai) genome is also shown. G+C content is indicated by colour. Figure used with permission from [2].

Non-LEE effector	Function
NleA	Localises to the Golgi apparatus. Required for virulence in a mouse model [295]
NleB	Associated with non-O157 EHEC outbreaks and HUS [296]. Necessary for colonisation and mortality of mice [297].
NleC	Not required for A/E lesion formation or colonisation [298].
NleD	Not required for A/E lesion formation or colonisation [298].
NleE	Required for colonisation and mortality of mice [299]. Associated with outbreaks (and HUS) of non-O157 EHEC [296].
NleF	Required for colonisation of mouse and piglet colon [299] [300].
NleG	Unknown.
NleH	Homologous to *Shigella* effector OspG. Involved in activation of NF-κB in host cells to optimise host gut inflammatory response [301]. Required for colonisation of mice [302].
EspJ	Homologous to *Pseudomonas syringae* effector HopF. Required for clearance of *C. rodentium* from the mouse colon [279]. Not required for A/E lesion formation *in vitro* or *ex vivo* [279].
EspK	Homologous to *Salmonella* T3SS effector GogB. Not required for A/E lesion formation [303]. Shows a diffuse cytoplasmic localisation in eukaryotic cells [303].
EspG2	Similar to EspG. Disrupts microtubule network [272] [304]. Alters paracellular permeability [275].
Cif	Inhibits cell cycle at G2/M-phase transition [305] [306]. Recruits focal adhesion plaques causing stress fibre formation [151].
TccP	Tir cytoskeleton coupling protein [307]. Promotes Nck independent actin assembly [278]. Required for actin assembly beneath adherent EHEC O157:H7 *in vitro* but dispensable for A/E lesion formation *in vivo* [257].

Table 1.6: Selected Non-LEE-encoded effectors (for a full list see Tobe *et al*.[2]).

1.9. Models of infection

Human challenge with live EHEC O157:H7, Stx or other EHEC virulence factors is unethical. Therefore, epidemiological evidence and models must be relied upon in order to investigate EHEC O157:H7 and its virulence factors. Unfortunately, there is no animal model that accurately represents the disease process of human infection with EHEC O157:H7 [308]. However, models have been developed that mimic, at least in part, the infection process of A/E pathogens – these, along with other model systems that have been used to investigate individual T3SS effectors of T3S organisms are discussed in the following sections.

1.9.1. Vertebrate models

1.9.1.1. Murine infection model

Citrobacter rodentium is an attaching and effacing pathogen of mice [309]. Possession of the LEE and other virulence factors also found in EHEC make this model system ideal for investigating EHEC pathogenesis [310]. Susceptible strains of mice die soon after infection with *C. rodentium*. However, colonisation and development of colonic hyperplasia can be investigated using resistant mouse strains or a streptomycin-treated mouse model [311]. Therefore, although clinical outcome of infection differs from EHEC O157 the effect of virulence factors can be assessed by monitoring disease progression. *C. rodentium* can be engineered to overexpress EPEC and EHEC effectors so that their effects can be monitored *in vivo*. Murine models have also been used in the study of other T3S pathogens including

B. pseudomallei – the T3SS translocator BipD and the effectors BopA and BopB were shown to be required for full virulence [94]. Modern techniques such as real-time bioluminescence imaging (BLI) can be employed when using a murine model to monitor tissue tropism and infection dynamics *in vivo* [301] [312].

1.9.1.2. Rabbit infection model

Rabbit EPEC (REPEC) is a natural rabbit pathogen. This infection model, in particular the use of infant rabbits, has been useful for characterising formation of the A/E lesion and localisation of EPEC colonisation. The model was used to demonstrate that intimin and Tir are both essential virulence factors [313].

1.9.1.3. Porcine infection model

Piglets have been used in the study of EHEC O157:H7 virulence factors (e.g. intimin). Intimin-based vaccines have also been tested using this model [314]. Caecal explants have recently been used to investigate the effects of noradrenaline on colonisation of the caecum and colon by EHEC O157:H7. Noradrenaline was shown to increase initial loose (T3SS-independent) attachment of EHEC O157:H7 to the porcine caecal mucosa [315].

1.9.1.4. Bovine infection model

As the main reservoir of EHEC O157:H7, cattle are an important part of research into EHEC O157:H7 disease and epidemiology. Due to the lack of effective treatment for infection with EHEC O157:H7, reducing carriage and transmission of the organism within

cattle is an important area of research. Preventing infection of cattle may help to prevent human infection with EHEC O157:H7 by decreasing contamination of meat, vegetable products and the farm environment [38]. Many important discoveries have been made regarding colonisation, mucosal immune response and significance of A/E lesion formation in the cattle intestine using the bovine infection model and bovine *in vitro* organ culture (bIVOC). Cattle have also been used in studies of vaccines that protect against colonisation by EHEC O157:H7 [117] [222]. Significantly, the cattle genome was recently sequenced [42] thus providing another resource for understanding the complex interaction between cattle and EHEC O157:H7.

Several studies using a bovine infection model have revealed mechanisms of colonisation of cattle by EHEC. For example, non-O157 serotypes demonstrated greater interaction with calf intestinal epithelium than EHEC O157:H7 [60] [316]. Efa-1 was found to mediate colonisation of the bovine intestine by non-O157 EHEC strains [60]. Signature-tagged transposon mutagenesis (STM) of EHEC O157:H7 has also been used in conjunction with the bovine infection model to identify bacterial factors (including LEE-encoded proteins) required for colonisation of the bovine intestine [317]. The site of specific adherence of EHEC O157:H7 to bovine intestinal epithelium was also determined using this model – EHEC O157:H7 bacteria were found to reside in the terminal rectum where they induced the formation of A/E lesions on, and cytokine release from, enterocytes [197] [40].

Bovine intestinal loops were found by Gerdts *et al* (2001) to be a valuable model for investigating the mucosal immune response [318]. Bovine ligated ileal loops have been used

to analyse the secretory and inflammatory response to different EHEC serotypes [60] and to identify host/bacterial factors that modulate such responses [319]. In accordance with results from bovine infection studies, non-O157 serotypes caused more fluid accumulation and neutrophil infiltration in the intestinal epithelium than EHEC O157:H7 [320]. This response was not affected by Tir, intimin or Stx-1 [321].

The use of large animal (bovine/ovine/porcine) models is expensive and, like other vertebrate models, time consuming and controversial. The use of vertebrate models is subject to legislation contained within the Animals (Scientific Procedures) Act 1986. Codes of practice and licences issued by the Home Office must be followed when using vertebrate models. There is therefore a need for alternative laboratory models.

1.9.2. Invertebrate models

1.9.2.1. *Caenorhabditis elegans*

C. elegans is a small free-living soil nematode that feeds on bacteria. *C. elegans* has tractable genetics and has been used to investigate several bacterial pathogens including *Salmonella* and *Pseudomonas aeruginosa*. Paralysis and death of worms can be monitored to indicate toxicity of strains or toxins. More recently *C. elegans* has been used to identify virulence factors e.g. tryptophanase in EHEC and EPEC, and to demonstrate that EPEC requires the LEE for full virulence [322].

1.9.2.2. *Galleria mellonella*

The greater wax moth caterpillar *Galleria mellonella* is used as a model to study the insect immune system [323]. Proteins encoded in PAIs of *Photorhabdus asymbiotica* and *Pseudomonas aeruginosa* T3SS effectors have been screened using *G. mellonella* [324] [325]. The T3SS effector study found that the *G. mellonella* system was as reliable as the *C. elegans* model and that there was a high correlation between cytopathology assays using *G. mellonella* and Chinese hamster ovary (CHO) cells [325].

1.9.3. Eukaryotic organ and cell culture

1.9.3.1. *In vitro* organ culture

IVOC has been described as 'the gold standard for investigating EPEC infection of the human intestinal mucosa' [326] and has been used to study interactions of EPEC, EHEC [327] [328] [329] and *Salmonella* [330] with human intestinal tissue. Intact intestinal biopsy tissue is cultured in either petri dishes on foam supports or in specifically designed plates to maintain the luminal and serosal compartments [331]. The mucosal side of the tissue is then infected with the A/E pathogen. Inflammatory responses and changes associated with disease can then be monitored through sampling of the growth medium (cytokine release etc.) and immunofluorescence/electron microscopy [326]. IVOC has advantages over *in vitro* cell culture models as intestinal membrane integrity is maintained i.e. cells maintain a 3-dimensional tissue architecture with cell-cell and cell-matrix interactions that are more representative of tissues *in vivo*. This allows research into the significance of apical and basolateral distributions of bacterial and host cell proteins during infection. A major

disadvantage of the human IVOC model is the difficulty of obtaining sufficient tissue samples to perform experiments [330]. Bovine IVOC models have been developed [332] [333] and used to investigate A/E lesion formation by EHEC O157:H7 and non-O157 EHEC strains [258] on bovine intestinal epithelium. The bIVOC model provides a more practical alternative to the bovine infection model as it allows the study of bacterial pathogenesis, host response and host specificity without requiring the specialist infrastructure (and associated cost) needed to accommodate large animals at high containment [258].

1.9.3.2. Cell culture

Cell culture is one of the most convenient and widely used model systems for investigating T3SS effectors. Numerous studies have used infection and transfection of various immortalised human and murine cell lines to investigate T3SS effector function and localisation [255]. Cell culture was instrumental in the development of the fluorescent actin staining (FAS) test for EPEC and EHEC infection [13]; observation of actin accumulation beneath adherent bacteria on cultured cells allowed A/E pathogens to be easily identified and diagnosed. Epithelial cell types can be used to investigate how T3SS effectors affect the cytoskeleton and cell signalling pathways. Polarised enterocytes such as Caco-2 cells can provide insights into overall intestinal 'barrier function' during infection as well as information regarding changes in transepithelial resistance and position of tight junction proteins [174]. The use of cultured epithelial cells revealed that LEE-negative STEC use an autoagglutinating adhesin (Ssa) and a novel type IV pilus in adherence to eukaryotic cells [334] [335].

1.9.4. *Saccharomyces cerevisiae*

The budding yeast *Saccharomyces cerevisiae* has been used as a model of eukaryotic cellular processes for many years. Much of our knowledge of fundamental processes such as transcription, translation, cell signalling and cytoskeletal dynamics has been gained through the use of this organism [336]. Yeast is both easy to genetically manipulate and easy to grow in the laboratory making it an ideal model organism – even compared to other popular model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, yeast represents a simple system.

Over the past decade, researchers have increasingly used the yeast cell model to investigate the functions of T3SS effectors from a variety of T3SS organisms [336] [337]. As mentioned earlier, T3SS effectors have a diverse range of effects within mammalian and plant cells but tend to target conserved eukaryotic cell processes and protein assemblages such as the cytoskeleton, small GTPase regulated signaling cascades and ion transport [140]. As these processes are conserved throughout eukaryotes, yeast can be used as a model of these processes without the need for expensive and time consuming mammalian organism/cell culture [157]. Yeast can also be used to study effector targets involved in the immune system. Many of the signalling pathways that regulate unique multicellular processes such as the immune response are conserved in yeast. This means that even though it is a single-celled organism, yeast can give us insights into multicellular specific processes [336] [163]. An increasing volume of research also shows that bacterial virulence factors may be directed against predatory protozoa and single-celled organisms [53] – EHEC O157:H7 virulence factor targets may therefore include yeast and yeast cell processes.

1.10. Aims of this thesis

Given the identification of a previously unsuspected T3SS effector repertoire in EHEC O157:H7, two important tasks remained: confirming that putative effectors are indeed translocated and identifying and characterising their effects on eukaryotic cells.

The aims of the research described in this thesis therefore are:

a) To confirm that selected putative effectors of EHEC O157:H7 Sakai are translocated into eukaryotic cells, using a beta-lactamase translocation assay.
b) To characterise the effects of selected effectors on eukaryotic cells (mammalian and yeast cells), focusing on:
    (i) Effects on cell growth.
    (ii) Effector localisation and effects on cell shape (as determined by microscopy).
    (iii) Disruption of cellular physiology (as determined by transcriptomics and a suppression screen).
c) To dissect sequence-activity relations for selected effectors.

CHAPTER TWO

General Materials and Methods

2.1. Suppliers

Chemicals, media and reagents were supplied by Sigma, Oxoid or Invitrogen unless otherwise stated.

2.2. Bacterial media

All media were sterilised at 121°C, 15psi for 15 minutes unless otherwise stated. Antibiotics and sugar solutions used to supplement media were sterilised by filtration through a 0.22 μm filter (Millipore, UK).

Luria-Bertani (LB) broth/agar was used for the routine culture of all *E. coli* strains. This media consisted of 10g tryptone, 5g yeast extract, 10g NaCl dissolved in 1 litre distilled water. One litre LB agar contained 15g bacto-agar in addition to the ingredients stated above. SOC broth was used for the recovery of bacteria after transformation. This media consisted of 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose.

For the expression of the LEE-encoded T3SS, bacteria were cultured in low-glucose Dulbecco's modified Eagles medium (DMEM) (Invitrogen, UK) supplemented with appropriate antibiotics.

Antibiotics were added to the media as required to a final concentration of: ampicillin 100 µg/ml; kanamycin 50 µg/ml; chloramphenicol 34 µg/ml; tetracycline 10 µg/ml.

2.3. Yeast media

YPD complete medium was used for the growth and maintenance of *S. cerevisiae* strains FY833 and BY4741. This consisted of 10 g Bacto-yeast extract, 20 g Bacto-peptone, 20 g glucose dissolved in 1 l dH_2O. 20 g Bacto-agar was added to make solid medium.

Synthetic complete media (SC) lacking uracil (SC-Ura) or SC lacking uracil and leucine (SC-Ura-Leu) were used for the selection, growth and maintenance of transformed *S. cerevisiae*. SC-Ura consisted of 6.7 g bacto-yeast nitrogen base without amino acids (Appleton Woods, UK) and 1.92g of yeast synthetic drop-out media supplement without uracil (Sigma) dissolved in 900 ml dH_2O. SC-Ura-Leu consisted of 6.7g bacto-yeast nitrogen base without amino acids, 1.4 g yeast synthetic drop-out media supplement without histidine, leucine, tryptophan and uracil (Sigma), 20 mg histidine and 20 mg tryptophan dissolved in 900ml H_2O. For solid media, 20 g bacto-agar was also added. Media were supplemented with sterile water to a final concentration of 2% (v/v) glucose, galactose or raffinose as appropriate after autoclaving.

2.4. Bacterial and Yeast strains

2.4.1. Bacteria

TOP10 chemocompetent *E. coli* cells (Invitrogen, UK) were used for the routine propagation of bacterial plasmids including entry vectors and recombined destination vectors. DB3.1 chemocompetent *E. coli* were used for the propagation of original destination vectors. The genotypes of each strain are listed in table 2.1. Other bacterial strains are listed in the appropriate chapter.

Strain	Genotype	Source
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *φ*80*lacZ*Δ M15 Δ*lac*X74 *rec*A1 *ara* Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen™
DB3.1	F- *gyr*A462 *end*A1 Δ(sr1-recA) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(SmR) *xyl*-5 *λ*- *leu mtl*1	Invitrogen™

Table 2.1: Bacterial strains used in this study.

2.4.2. Yeast

Saccharomyces cerevisiae strains FY833 and BY4741 were used in this study. Details of the strains are listed in table 2.2.

Strain	Genotype	Source
FY833	*MATα his3Δ ura3Δleu2Δ lys2Δ trp1Δ GAL2*	S. Dove, University of Birmingham
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	S. Dove, University of Birmingham

Table 2.2: Yeast strains used in this study.

2.5. Growth conditions

2.5.1. Bacterial growth conditions

E. coli was grown aerobically in liquid culture or on solid agar. For liquid cultures, the culture vessel was at least 5 x volume of the media. Cultures were incubated at either 30°C or 37°C on an orbital shaker or shaking waterbath. For growth on solid media, plates were incubated in a static incubator overnight at 37°C. Liquid cultures were stored long term at -80°C in cryovials, which contained 250 µl of 60 % (v/v) glycerol and 750 µl of an overnight liquid culture.

2.5.2. Yeast growth conditions

Broth cultures were grown aerobically at 30°C in a shaking waterbath (200 rpm). Culture vessels were at least 5 x the volume of the media to ensure good aeration. For yeast growth on solid media plates were sealed with parafilm (Millipore) to prevent desiccation. Plates were incubated at 30°C in a static incubator. Liquid cultures were stored long term at -80°C in cryovials, which contained 250 µl of 60 % (v/v) sterile glycerol and 750 µl of an overnight liquid culture.

2.6. Human cell culture

HeLa cells were grown in minimal Eagle's medium (MEM) without antibiotics, foetal calf serum 10% (v/v) was used to supplement the media. Cells were grown in 75 cm^2 tissue culture flasks by incubating at 37°C in 5% CO_2 until 80% confluence was achieved.

2.7. Gateway system

2.7.1. High-throughput cloning

A high-throughput DNA cloning system was required to increase throughput and efficiency when characterising effectors using numerous model systems. This study makes use of the Gateway system of cloning (Invitrogen). This system allows a gene of interest (GOI) to be amplified and easily cloned into a myriad of expression vectors for use in various systems. Many types of destination vectors are available including bacterial, mammalian and yeast expression plasmids. This means that the Gateway system is very versatile. The use of the system gives the potential to explore the localization and function of the novel effectors in diverse systems and model organisms. The Gateway system of cloning differs from conventional cloning in that the GOI is amplified once and is transferred to expression vectors through a process of recombination rather than through restriction digestion and ligation. An overview of the system is shown in figure 2.1.

2.7.2. Polymerase chain reaction

PCR was used to amplify genes of interest (GOI) for cloning. Primers were designed to have similar melting temperatures and to contain approximately 50 % G/C. As per the requirements of the Gateway cloning system, forward primers were designed with a CACC oligonucleotide motif at their 5' end. Primers were synthesized by Eurogentec (Belgium) or Alta Biosciences (UK) and were supplied at a concentration of 100 µM. Those primers used in Quikchange reactions were purified by high performance liquid chromatography (HPLC). Primers used in this study are listed in appropriate chapters. For the amplification of EHEC O157:H7 Sakai effector genes PCR mixtures consisted of 1 x ExTaq buffer, 1.5 mM $MgCl_2$, 2 nM dNTPs, 1 µM each primer, genomic DNA (EHEC O157:H7 Sakai) and 2 U of ExTaq polymerase in 50 µl total volume (dH_2O was used to make up reaction volume). Reaction mixtures were vortexed and centrifuged briefly. Amplification of DNA took place in a Primus thermocycler. The thermocycling program used is shown below. PCR products were then analysed by gel electrophoresis.

95°C 2 minutes

95°C 30 seconds

Annealing temperature (40-60°C) 30 seconds

72°C 30 seconds

Cycle
x 30

72°C 2 minutes

4°C until use

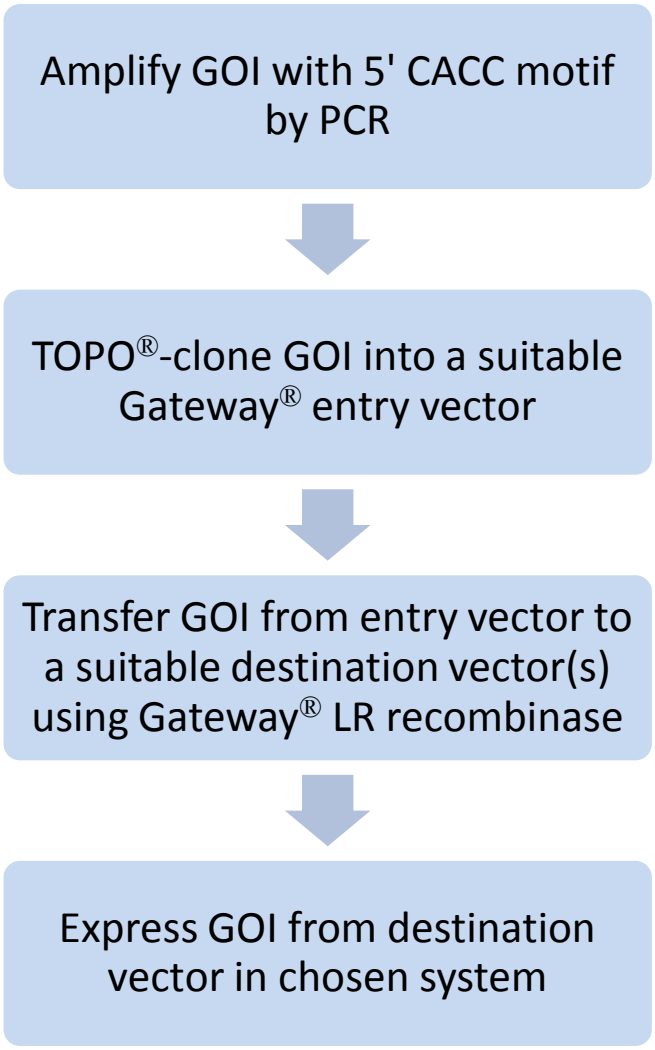

Figure 2.1: Overview of the Gateway system of cloning. GOI; gene of interest.

2.7.3. Electrophoresis of DNA

Buffers for DNA electrophoresis

TAE buffer: A 50 x stock solution of TAE buffer was prepared and diluted fifty-fold to create a working solution. The stock solution consisted of 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) per litre of dH_2O.

Sample loading buffer: a 6 x buffer contained 0.25% (w/v) Bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol in dH_2O.

DNA electrophoresis

DNA was analysed by electrophoresis on 0.8% agarose gels. Agarose was dissolved in TAE buffer and melted. Ethidium bromide was added (100 ng/ml) once the agar had cooled to 50°C. Gels were prepared using Hybaid moulds and combs. The gel was submerged in TAE buffer in a Hybaid electrophoresis tank. Samples were mixed with DNA loading buffer and 5 µl was added to each well. 5 µl hyperladder I (Bioline) was added to the left-most lane as a size and quantity marker. Samples were run at 90 volts. DNA was then visualised using an ultra-violet transilluminator of a Biorad gel documentation system.

Gel extraction and purification of DNA

DNA was visualized in the agarose gel after electrophoresis using a UV transilluminator and was excised from the gel using a clean scalpel. DNA was purified using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

2.7.4. TOPO cloning

A schematic of the TOPO cloning process is shown in figure 2.4. The PCR product containing the GOI is cloned into the 'entry vector' using TOPO cloning. Entry vectors were obtained from Invitrogen (UK) and are shown in figures 2.2 and 2.3. Entry vectors used in this study are listed in the appropriate chapter. The entry vector contains a 3' GTGG overhang that is covalently attached to *Vaccinia* topoisomerase I protein. The overhang of the entry vector invades the 5' end of the PCR product. The 5' hydroxyl group of the PCR product then attacks the phosophotyrosyl bond between the vector DNA and topoisomerase enzyme which releases the enzyme. The GTGG motif of the entry vector anneals to the complementary CACC motif of the PCR product and the PCR product is stabilised in the entry vector in the correct orientation.

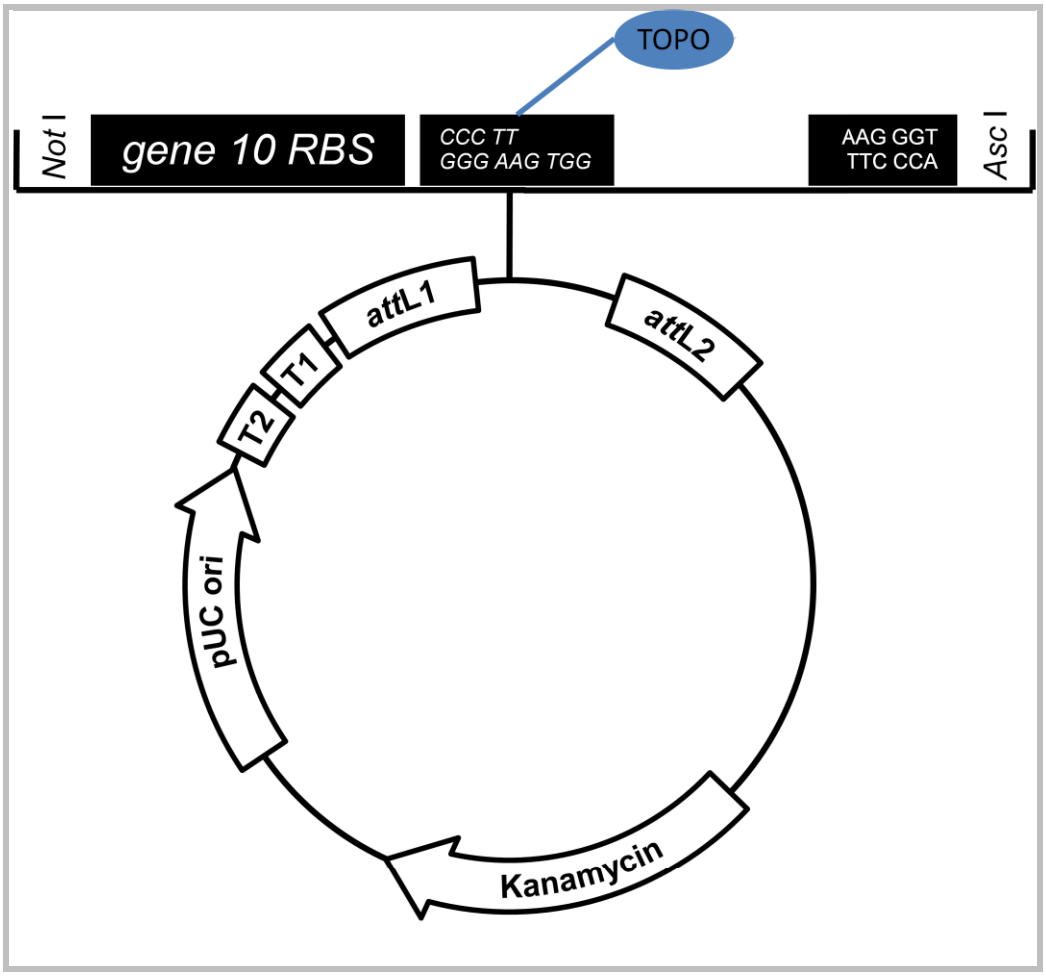

Figure 2.2: pENTR-SD/D-TOPO. Gateway entry vector (with Shine-Dalgarno sequence). TOPO indicates *Vaccinia* topoisomerase I.

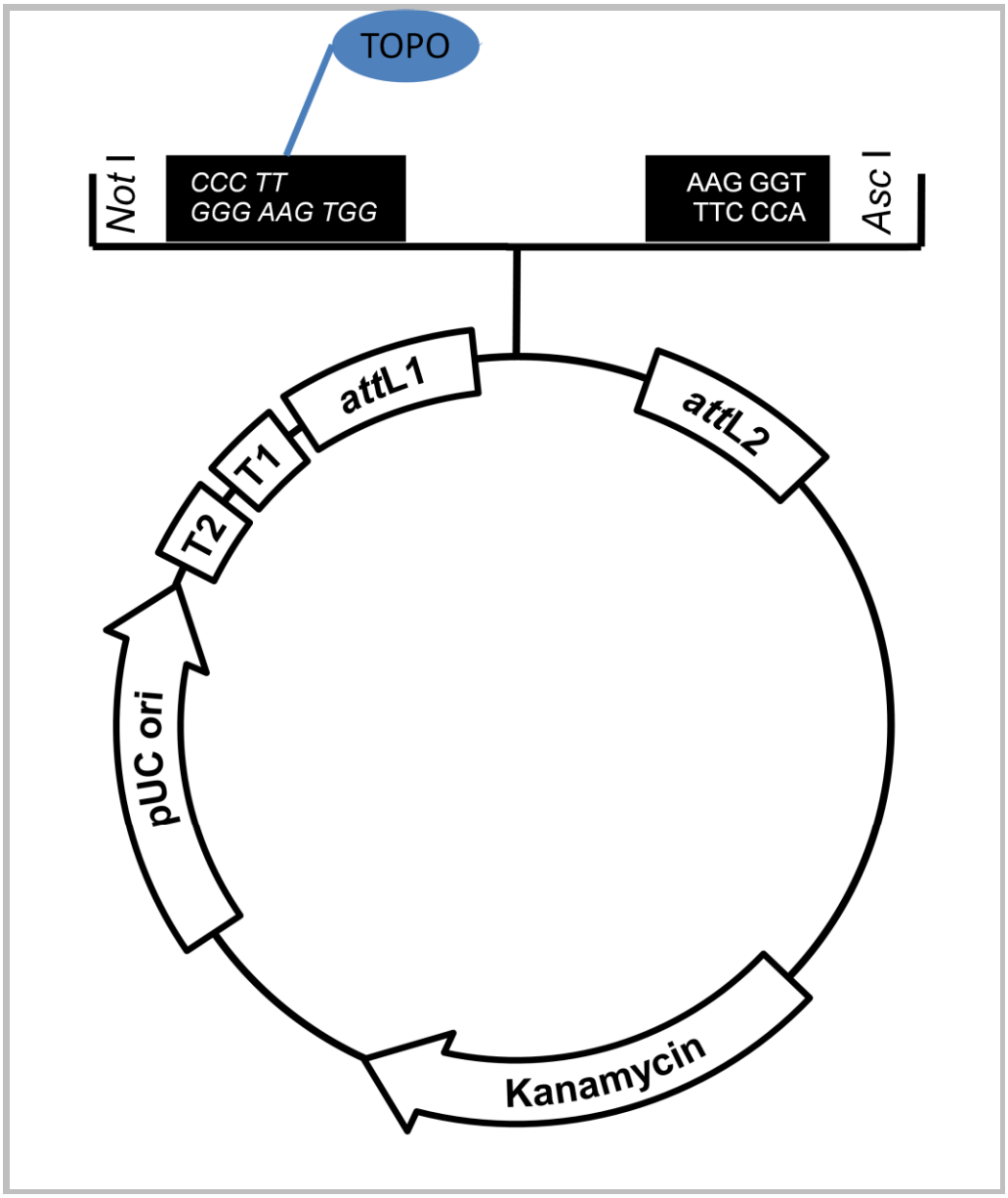

Figure 2.3: pENTR-D-TOPO. Gateway entry vector.

TOPO indicates *Vaccinia* topoisomerase I.

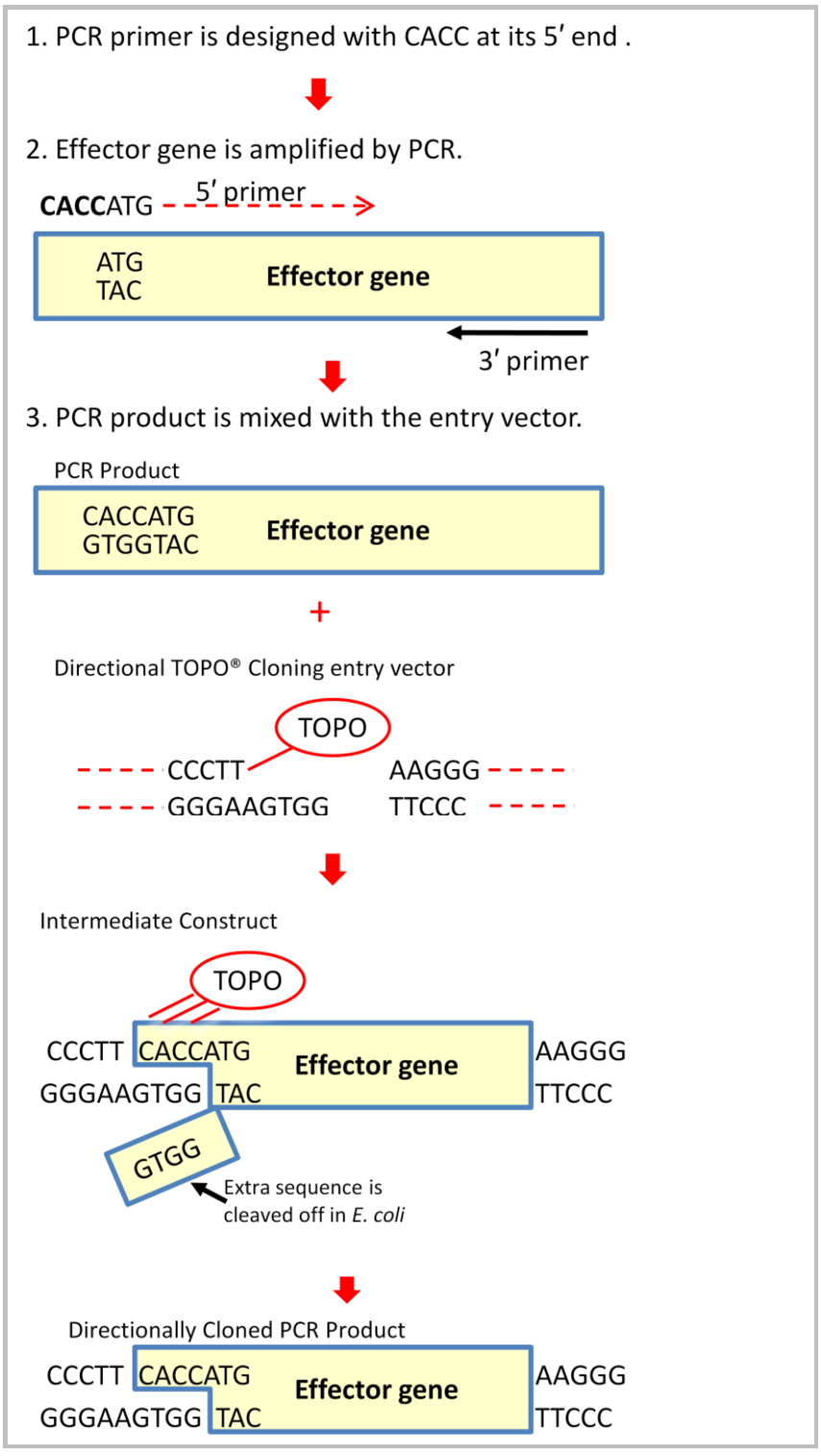

Figure 2.4: Directional cloning of an effector gene into a TOPO entry vector.

Covalently bound topoisomerase 1 is represented in a red oval. Figure adapted from Invitrogen [338].

Insertion of the GOI PCR product into the entry vector was performed: 1 µl of TOPO entry vector (Invitrogen), 10-40 µg PCR product, 1 µl salt solution (1.2M NaCl, 0.06 $MgCl_2$) and H_2O (added to a make a final volume of 5 µl) was incubated at room temperature for 5 minutes. The reaction mix was then incubated on ice or stored at -20°C until used in transformation of competent *E. coli*.

2.7.5. Transformation of *E. coli*

Heat shock transformation

10 µl chemically competent TOP10 *E. coli* cells (Invitrogen) were transformed with 1 µl desired plasmid by incubating the mixture on ice for 30 minutes. The cells were then heat shocked in a water bath at 42°C for 30 seconds. 200 µl SOC medium was then immediately added to the cells and the cells grown statically at 37°C for one hour. Transformants were then plated out onto LB agar (supplemented with appropriate antibiotics) and grown overnight at 37°C.

Electroporation

Electrocompetent *E. coli* were first prepared: an overnight culture of *E. coli* was diluted 1 in 100 into fresh LB broth (supplemented with appropriate antibiotics) and grown at 37°C until mid-log phase (OD_{600} between 0.5 and 0.7). Bacteria were chilled on ice. The culture was then centrifuged at 3000 x g for 10 minutes. The supernatant was discarded and the cells resuspended in 24 ml sterile, ice cold 10 % (v/v) glycerol. Cells were washed twice more, each time the volume of glycerol solution used to resuspend the cells decreased by half (i.e.

12 ml then 6 ml). After the final centrifugation, the cells were resuspended in 200 µl sterile dH_2O. For electroporation, 50 µl cell suspension was aliquoted into ice cold, sterile electroporation cuvettes (Geneflow). 2 µl of plasmid was then added to the cuvette. Cells were incubated on ice for 30 minutes. Cells were electroporated with a Biorad electroporator using 2.5 kV. Immediately after electroporation, 250 µl SOC medium was added to the cuvette and cells were transferred to a microfuge tube. Cells were incubated with shaking (200rpm) at 37°C for 1 hour before being plated onto LB agar containing the appropriate antibiotic/s. Plates were incubated overnight at 37°C.

2.7.6. Isolation of DNA from bacterial cells

Single colonies of transformed *E. coli* were picked and grown overnight in 5 ml LB (containing appropriate antibiotic) at 37°C with shaking (200 rpm). The bacterial culture was then centrifuged at 13,000 rpm for 5 minutes. Plasmid DNA was obtained by a standard alkaline-lysis method using commercially available miniprep kits following the manufacturer's instructions (Qiagen).

2.7.7. DNA sequencing

Plasmid DNA was extracted as above using miniprep kits (Qiagen). The orientation and sequence of the GOIs in entry vectors (and destination vectors) was confirmed by automated DNA sequencing. The single primers used in the sequencing of each destination vector are listed in the appropriate chapter. The universal primers M13 Forward (-20) and M13 Reverse were used to sequence entry vectors. Plasmid products were sequenced by the Functional Genomics Laboratory, School of Biosciences, University of Birmingham. Sequencing reactions contained 300 nM primer and 200 – 500 ng of plasmid template DNA in a final volume of 10 µl (made up with dH_2O). The sequencing process used a Big Dye Terminator kit (Applied Biosystems) and an MWG Primus HT 96 well PCR machine. The sequences of products were analysed by capillary electrophoresis on an ABI Prism 3700 DNA analyser. FinchTV software was used to analyse sequence data.

2.7.8. LR recombination reaction

Once the sequence and orientation of the GOI in the entry vector had been confirmed, expression constructs containing the GOI were generated by performing an LR recombination reaction between the entry vector and Gateway destination vector of choice. Destination vectors used in this study are listed in the appropriate chapter.

Stocks of non-recombined destination vectors were made by transforming non-recombined destination vectors into *E. coli* strains (i.e. DB3.1) that are resistant to the effects of CcdB encoded by the *ccd*B gene contained within the destination vector. Transformation of commercially available chemo-competent *E. coli* DB3.1 (Invitrogen, UK) with non-recombined destination vectors was via the heat-shock transformation protocol as described above.

Bacteriophage λ recombinase was used to transfer the GOI from the entry vector into the destination vector through recombination. Recombination replaces a chloramphenicol resistance cassette and the lethal *ccd*B gene (contained between the attR sites in the destination vector) with the GOI (contained between attL sites in the entry vector). Depending on the destination vector used this creates an in-frame translational fusion to a detectable protein tag e.g. GFP. Selection of vectors containing the GOI is possible using ccdB negative selection.

The recombination reaction mix consisted of 100-300 ng entry clone, 150 ng destination vector, 2µl 5 x LR Clonase reaction buffer, 1 µl LR clonase enzyme II and 1 µl dH_2O. The reagents were vortexed and incubated at room temperature for 1 hour. After incubation, 2 µl of Proteinase K solution was added to the mix and the mix incubated for 10 minutes at 37°C. The mixture (containing recombined destination vectors) was then transformed into chemocompetent TOP10 *E. coli* cells. Transformants were plated onto LB agar plates supplemented with ampicillin (100 µg/ml). Plasmid DNA was obtained from transformed bacteria as above. The sequence and orientation of the GOI within the destination vector

was confirmed by DNA sequencing as above. Stocks of successful transformants were stored at -80°C. Purified destination vectors were transformed or transfected into appropriate hosts as listed in the appropriate chapter.

2.8. Protein analysis

2.8.1. SDS-Polyacrylamide gel electrophoresis

Buffers and solutions for SDS-PAGE

Resolving buffer contained 1.5 M Tris-HCl and 0.4% (w/v) sodium dodecyl sulphate (SDS) adjusted to pH 8.8. Stacking buffer contained 0.5 M Tris-HCl and 0.4% (w/v) SDS adjusted to pH 6.8. SDS-running buffer (10 x stock solution) contained 30.3 g Tris-HCl, 144 g glycine and 10 g SDS per litre H_2O. The stock solution was diluted 10-fold to make a working solution i.e 50 ml of 10 x stock solution was added to 450 ml dH_2O. Sample loading buffer (2x) consisted of 100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) Bromophenol blue and 20% (v/v) glycerol, 10% 2-mercaptoethanol.

SDS-PAGE gels were stained with ProtoBlue Safe (National Diagnostics) colloidal Coomassie G-250 stain. A working solution for 1 mini-gel consisted of 2 ml absolute ethanol and 18 ml ProtoBlue Safe.

SDS-Polyacrylamide gel electrophoresis

Gels were made using Ultrapure ProtoGel containing 30% w/v acrylamide and 0.8% w/v NN'-methylenebisacrylamide. Resolving gel consisted of 12.5 ml Ultrapure ProtoGel, 7.5 ml resolving buffer, 150 µl 10% (w/v) ammonium persulphate (APS), 30 µl N, N, N', N'-tetramethylethylenediamine (TEMED) and 10 ml dH_2O (mix sufficient for 3 gels). Stacking gels consisted of 1.3 ml Ultrapure ProtoGel, 2.5 ml stacking buffer, 100 µl 10% (w/v) APS, 20 µl TEMED and 6.1 ml dH_2O.

12% polyacrylamide gels (1mm thick) were used to separate proteins for analysis. Resolving gel was poured between two glass plates (Biorad) until 2.5 cm from the top of the plate. 200 µl butanol was added to the top of the gel to prevent oxygen (inhibits polymerisation) reaching the gel. Once the resolving gel had set, butanol was removed by washing with dH_2O. Excess water was removed with filter paper and the stacking gel added on top of the resolving gel. An 8 or 15 well comb was inserted into the stacking gel to create wells (30 µl volume). Protein samples were mixed with SDS-loading buffer (10% β-mercaptoethanol) and boiled for 10 minutes before being loaded into the wells of the stacking gel. Separation was achieved by the application of 200V for 45 minutes. After separation, gels were removed from the glass plates and washed twice in dH_2O. 20 ml ProtoBlue Safe Coomassie stain was added to the gel in a small tub, the tub covered with film and gels left to stain overnight on a rotating platform (60rpm). Gels were then washed twice (10 minutes each) in dH_2O to remove excess stain before being scanned using a computer.

2.8.2. Immunoblotting

Electrophoretic transfer of proteins

Transfer buffer consisted of 5.8 g Tris-HCl, 2.9 g glycine and 0.37 g SDS in 20 ml methanol and 800 ml dH_2O. Proteins were separated by SDS-PAGE as above, with a pre-stained protein marker run alongside. The SDS-PAGE gel, two sheets of 3 mm Whatman filter paper and two sponge pads were soaked in transfer buffer for 10 minutes. A gel-sized sheet of Hybond™-P polyvinylidene difluoride (PVDF) membrane (GE Life Sciences, UK) was washed in absolute methanol for 30 seconds and then washed in transfer buffer for 5 minutes. A gel-membrane 'sandwich' was made by placing a sponge pad onto the clear side of the Biorad cassette, followed by one piece of filter paper, the membrane, the gel, another piece of filter paper and the final sponge pad. The cassette was closed and placed in a cassette holder in the tank. An ice pack was placed in the tank to prevent overheating. Transfer buffer was added to the tank until the cassette was submerged. Proteins were then electrophoretically transferred from the gel onto the membrane by application of 200 mV for 1 hour. Transfer buffer in the tank was stirred continuously during the transfer process by a magnetic stirrer.

Western blotting

Blocking buffer consisted of 1 g bovine serum albumin (1% w/v) dissolved in 100 ml 1 x phosphate buffered saline (PBS) supplemented with 0.05% (v/v) Tween-20.

After transfer, the membrane was washed twice in dH_2O before being incubated at 4°C overnight in blocking buffer to block non-specific sites from reacting with antibody probes. Membranes were probed with primary antibody in fresh blocking buffer for 2 hours at room temperature with gentle shaking. Membranes were then washed three times (10 minutes each) in fresh blocking buffer before the secondary antibody (a horseradish peroxidase conjugate) was added (in 20 ml fresh blocking buffer). Membranes were incubated at room temperature for 1 hour with gentle shaking before being washed 3 times (10 minutes) in fresh blocking solution. Membranes were developed using chloro-1-napthal as a substrate for horseradish peroxidase using the Opti-4CN (Biorad) detection system. Developing solution consisted of 9 ml dH_2O, 1 ml diluent and 200 µl substrate. Bands were allowed to develop at room temperature (up to 10 minutes). Membranes were then washed twice in dH_2O and scanned using a computer.

CHAPTER THREE

Translocation of candidate effectors into eukaryotic cells

3.1 Introduction

3.1.1. Background

The work presented in this chapter describes a connected programme of research that spanned three research groups: the Pallen group in Birmingham, the Frankel group at Imperial College, London and the Tobe group at the University of Osaka, Japan. Before the experimental work described here was performed a bioinformatics-based survey of the EHEC O157:H7 Sakai genome within the Pallen group had identified 49 candidate T3SS effectors [2]. I exploited a β-lactamase translocation assay, one of four methods used to provide experimental evidence of type III secretion of the predicted EHEC O157:H7 T3SS effectors. This work contributed to a publication [2]. The introduction to this chapter describes the methods used by my collaborators to confirm effector status. The methods, results and discussion sections describe use of the β-lactamase translocation assay by myself and others in the Pallen and Frankel groups to confirm that selected putative effectors were translocated into eukaryotic cells.

3.1.2. Investigating type III secretion and translocation

Several methods can be used to confirm secretion and translocation of T3SS effectors. For example, secretion of effectors into the growth medium can be revealed by SDS-PAGE and mass spectroscopy [2] whereas translocation of effectors into host cells can be detected by immunoblotting host cell lysates [339], immunofluorescence microscopy of infected cells

[340] and enzymatic-based translocation assays [341] [282] [230]. In this study, four methods were used to monitor type III secretion and translocation

- EHEC O157:H7 secretome analysis,
- an immunofluorescence-based translocation assay,
- an adenylate cyclase translocation assay
- a β-lactamase translocation assay.

3.1.3. Secretome analysis

Our collaborators in the Tobe laboratory began experimental confirmation of the predicted effectors using a proteomics approach. They compared the proteins secreted into the growth media by a Δ*sepL* Sakai isolate with those proteins secreted by a non-type-III-secreting isogenic Δ*sepL* Δ*escR* Sakai isolate. A Δ*sepL* Sakai isolate was used as it secretes effectors into the culture supernatant at higher levels than wild type EHEC Sakai [196]. The Sakai strain used in this study also exhibited enhanced expression of the Esc-Esp T3SS due to high level expression of the LEE transcriptional activator PchA (provided on a multicopy plasmid). Proteins secreted into the culture supernatant by each isolate were separated by SDS-PAGE and slices of gel (representing the whole lane) were trypsinised. The peptides created by trypsinisation were then analysed by mass spectroscopy. Comparison of the proteins secreted by each strain allowed the identification of proteins that were secreted through the T3SS i.e. those proteins secreted by Δ*sepL* but not Δ*sepL* Δ*escR* isolates. 31 proteins from the bioinformatics survey were found to be secreted by the T3SS using this method (thereby confirming the effector status of 31 predicted effectors) [2]. The validity of

this approach was confirmed by the presence of nine known T3SS proteins including Map, EspF and EspG in the EHEC O157:H7 T3SS secretome [2].

3.1.4 Immunofluorescent staining

Our collaborators in the Tobe laboratory also used immunofluorescent staining to confirm the effector status of effector candidates. In this approach, candidate effectors were tagged with a small epitope tag (FLAG). Non-toxigenic EHEC O157:H7 Sakai and non-type-III-secreting isogenic EHEC harbouring FLAG-fusion plasmids were used to infect Caco-2 cells. Immunostaining using anti-FLAG antibodies enabled translocated effectors to be visualised within host cells by microscopy. Immunofluorescent staining of FLAG-tagged effectors in Caco-2 cells demonstrated that 12 of the candidate effectors were translocated via the T3SS [2].

In studies of other putative T3SS effectors, translocation of individual effectors has been confirmed by immunofluorescent staining using antibodies to the effector in question e.g. confirmation of T3S of BopE by *B. pseudomallei* [342]. However, the high cost associated with obtaining antibodies reactive for each putative effector in this study was prohibitive. The methods described so far also involve numerous time-consuming processes (e.g. secretome analysis requires SDS-PAGE, trypsinisation and mass spectroscopy [2] whereas immunofluorescence analysis requires cell fixation, permeabilisation and immunostaining [340]). Therefore, in order to maintain an efficient throughput during initial screening of effector translocation we decided to use enzymatic translocation assays.

3.1.5. Translocation assays

T3SS permissible substrates such as small peptide tags (e.g. tetracysteine) or enzymatic reporter fusions can be used effectively to determine whether a protein is translocated into a host cell [282]. Translocation assays include FlAsH labelling (see below), the adenylate cyclase (CyaA) assay and the β-lactamase (TEM-1) fusion assay.

FlAsH/tetracysteine labeling

Fluorescein arsenical helical binding (FlAsH) labeling is a newly developed method that can be used to track effector translocation without the need for immunostaining [343]. FlAsH is a small biarsenical molecule that passively enters eukaryotic cells and binds specifically to a 12-amino-acid tetracysteine motif [343]. The tetracysteine motif can be incorporated into effector genes on the chromosome so that effector gene expression occurs under physiological conditions [170]. Once bound to the tetracysteine motif, FlAsH emits a green fluorescence signal. Translocation of effectors carrying the motif can be monitored *in situ* and in real time using time-lapse fluorescence microscopy [170] [344]. FlAsH translocation assays were recently used to visualise real-time *Salmonella* effector delivery into HeLa cells [170].

Adenylate cyclase assay

The adenylate cyclase (CyaA') domain derived from *Bordetella* toxin CyaA causes an increase in the levels of cyclic AMP (cAMP) in eukaryotic host cells in response to host cell calmodulin [345]. In this assay, CyaA is fused to the protein of interest i.e. a T3SS effector.

Translocation of the fusion into host cells is detected by an increase in cAMP in the host cell [346] or loss of cell viability [347]. The increase in intracellular cAMP can be detected by enzyme-linked immunosorbent assay (ELISA) [2], radioimmunoassay [345] or colourimetric assay [347]. An increase in host cell cAMP confirms translocation of the CyaA'-fusion. This assay has been used successfully to confirm T3SS of effectors from *P. syringae* [348], EPEC [265], *Erwinia chrysanthemi* [111], *R. solanacearum* [349], *Salmonella enterica* [350] and to investigate the secretion signals of YscP of *Yersinia enterocolitica* [351]. Colleagues at Osaka University used the adenylate cyclase translocation assay with an ELISA based detection system to confirm the effector status of 10 of the predicted EHEC O157:H7 effectors [2].

β-lactamase fusion assay

The β-lactamase fusion assay was developed in 2004 to identify the secretion and translocation domain of the EPEC and EHEC T3SS effector, Cif [282]. This assay uses the fluorescent substrate CCF2/AM to demonstrate the action of a β–lactamase (TEM-1) fused to the protein of interest e.g. T3SS effector. CCF2/AM consists of a coumarin moiety and a fluorescein moiety joined together by a CCF2 β–lactam ring. CCF2/AM passively enters eukaryotic cells. Excitation at 409 nm results in fluorescence resonance energy transfer (FRET) from the coumarin to the fluorescein which causes the fluorescein moiety to emit a green fluorescence signal (520 nm). Translocation of the TEM-1 fusion protein into the eukaryotic cell induces catalytic cleavage of the CCF2 β-lactam ring. This disrupts FRET and causes emission of a blue fluorescence signal. Translocation of the TEM-1-effector fusion can easily be detected in live host cells by blue CCF2/AM fluorescence [282]. The

principle of this assay is summarised in figure 3.1. This assay has been successfully used to demonstrate translocation of EspJ [279], EspK [303], NleC and NleD [298] into HeLa cells.

A β–lactamase fusion assay was used in this study to confirm the effector status of the candidate EHEC O157:H7 T3SS effectors. TEM-1-effector fusion plasmids were created and transformed into EPEC and an isogenic EPEC Δ*escN* mutant at the University of Birmingham. Seventeen translocation assays were performed by the author, S.A. Beatson and C. Bailey under the supervision of O. Marches at Imperial College, London.

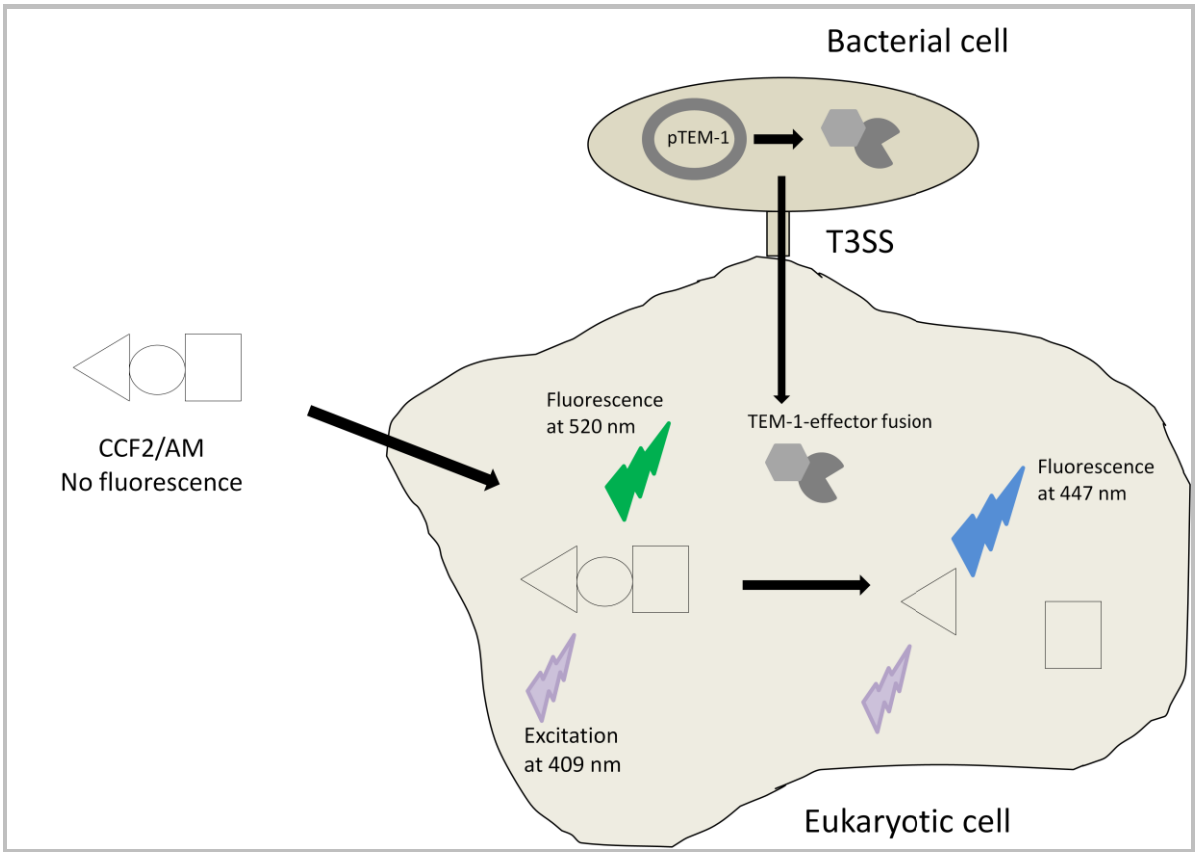

Figure 3.1: β-lactamase fusion assay used to study translocation of T3SS effectors. Non-fluorescent CCF2/AM passively enters the eukaryotic cell where it is esterified by cellular esterases which results in charged, fluorescent CCF2. FRET from the coumarin moiety (triangle) to the fluorescein moiety (square) upon excitation at 409 nm leads to emission of a green fluorescence signal at 520 nm. Type III mediated translocation of a TEM1-effector fusion protein (expressed from pTEM-1) into the CCF2-loaded cell leads to cleavage of the β-lactam ring (circle) of CCF2. FRET is therefore disrupted causing the coumarin moeity to emit a blue fluorescence signal at 447 nm (upon excitation at 409 nm). Figure adapted from Charpentier *et al* [282].

3.2 Methods

3.2.1. Gateway DNA cloning

PCR

Novel effectors were amplified by polymerase chain reaction by S.A. Beatson, A. Fivian, R. Younis and C.M. Bailey as described in chapter 2. Primers used are shown in table 3.1.

TOPO Cloning

Seventeen effectors were cloned into pENTR-SD/D-TOPO (performed by S.A.Beatson, A. Fivian and R. Younis) as described in chapter 2. Entry vectors used in this study are shown in table 3.2.

LR recombination

Seventeen effectors were recombined into pCX340gw (i.e. Gateway compatible pCX340 β-lactamase expression vector) via the LR recombination reaction by S.A.Beatson as described in chapter 2. Destination vectors created and used in this study are shown in table 3.3. The author confirmed destination vectors by DNA sequencing as detailed in chapter 2 using primers GatewayM13F (entry vectors) and TEM1_seqprimer2 (destination vectors).

Primer	Sequence (5'-3')
DNA sequencing	
GatewayM13F	GTAAAACGACGGCCAG
TEM1_seqprimer2	GGCACTCGACCGGAATTAT
Effector gene amplification	
ECs0061gwF	CACCATGAAAGTATCAGTTCCA
ECs0061gwR	TTCAATAATTGCGTTGTC
ECs0847.gwF2	CACCATGAAAATTCCCTCATTACAGTCC
ECs0847.gwR2	TCATTGCTCATTGTGTTTGTCC
ECs0850.gwF	CACCATGCGCCCTACGTCC
nsGW.0850r	AAGCAATGGATGCAGTCTTACC
ECs0876gwF	CACCATGGATTGTTCAAAATGC
ECs0876gwR	CCATGCGTCTGGCGTCCA
ECs1127gwF	CACCATGAGCGGAACCTCAGGT
ECs1127gwR	CAAAAAAGATTGGGGAGG
gw1567f	CACCATGCCATTTTCAATC
nsGW.1567r	TTCTTTTGTGTTGTGTATCTC
ECs1568.gwF	CACCATGCTTCCTACATCG
nsGW.1568r	AGAATATTTATATGTGG
ECs1814gwF	CACCATGTTATCGCCCTCTTCT
ECs1814_new_rev	TTATATCTTACTTAATACTACAC
ECs1815gwF	CACCATGTTACCAACAAGTGGT
ECs1815gwR	TCCACATTGTAAAGATCC
ECs2226gwF	CACCATGCTTCAATTATCTTCA
ECs2226gwR	TTTACAATGCTGTTTCTG
ECs3488gwF	CACCATGGAGAGAAGGGCT
ECs3488gwR	TTAAGCATCACTTGCAAC
ECs4561gwF	CACCATGCCTATTGGTAATCTT
ECs4561gwR	GACGAAACGATGGGATCC
ECs4564gwF	CACCATGTCGTTATCAGGAGCG
ECs4564gwR	TAATACGCTATAAGAGGA
ECs4571gwF	CACCATGGAAGCAGCAAATTTA
ECs4571gwR	GGCATATTTCATCGCTAA
ECs4590gwF	CACCATGATACTTGTTGCCAAA
ECs4590gwR	AGTGTTTTGTAAGTACGT
ECs4653gwF	CACCATGGTCGCTAAATTAAAAC
ECs4653gwR	AGCCTGGGTTATATTTTG
ECs5021gwF	CACCATGTTAGGTCATATCTCA
ECs5021gwR	AATACCTTTATTCTTTAT

Table 3.1: DNA primers used in this study

Plasmid	Details	Source
pCX340gw	Gateway compatible TEM-1 expression plasmid based on pCX340 [282].	Tobe *et al* [2].
pENTR-SD/D-TOPO	Gateway entry vector.	Invitrogen (UK)

Table 3.2: Gateway /Gateway-compatible entry and destination vectors used in this study.

Effector	Sakai ID	Entry vector (pENTR-SD/D-TOPO)	TEM-1 destination vector (pCX340)	Source
EspY1	ECs0061	pSB9.47B	pSB16.23A	S.A. Beatson, this study
NleC	ECs0847	pRY2-ENTR	pRY2-DEST	R. Younis, this study
NleD	ECs0850	pRY3-ENTR	pRY3-DEST	R. Younis, this study
EspX2	ECs0876	pSB10.4	pSB16.27	S.A. Beatson, this study
EspV'	ECs1127	pSB9.41A	pSB16.20A	S.A. Beatson, this study
EspO1-1	ECs1567	pAF14	pAF14D	A. Fivian, this study
EspK	ECs1568	pAF5	pAF5D	A. Fivian, this study
NleH1-2	ECs1814	pSB9.46B	pSB16.22A	S.A. Beatson, this study
NleF	ECs1815	pSB10.11B	pSB16.28A	S.A. Beatson, this study
NleG7'	ECs2226	pSB9.33B	pSB16.13A	S.A. Beatson, this study
NleG6-3'	ECs3488	pRY12-ENTR	pRY12-DEST	R. Younis, this study
Tir	ECs4561	pSB9.13C	pSB16.6A	S.A. Beatson, this study
EspH	ECs4564	pSB9.15B	pSB16.7A	S.A. Beatson, this study
EspZ	ECs4571	pSB9.16B	pSB16.8A	S.A. Beatson, this study
EspG	ECs4590	pSB9.5A	pSB16.2A	S.A. Beatson, this study
EspY4	ECs4653	pSB10.22B	pSB16.32A	S.A. Beatson, this study
EspX4	ECs5021	pSB11.5A	pSB16.35A	S.A. Beatson, this study

Table 3.3: Entry vectors and TEM-1-effector expression vectors created and used in this study.

Transformation of EPEC

Electrocompetent EPEC 2348/69 and an isogenic non-type III secreting EPEC strain (EPECΔe*scN*) were prepared by the method described in chapter 2. 2 µl purified destination vector was transformed into each strain by electroporation as described in chapter 2 (transformation of wild type EPEC strain was performed by S.A. Beatson; the author performed transformation of EPECΔ*escN*). Transformants were selected on LB agar containing 10 µg/ml tetracycline.

3.2.2. Translocation assay

Solutions

The solutions used in the β-lactamase translocation assay are as follows: Hanks' Balanced Salt Solution; HBSS (Invitrogen), Dulbecco's modified eagles medium; DMEM (Sigma), Hepes modified DMEM (Sigma), CCF2/AM loading solution (Invitrogen, UK) comprising 6 µl solution A, 60 µl solution B, 934 µl solution C.

β-lactamase translocation assay

The day before infection 4 x 10^4 HeLa cells were seeded into each well of an 8-well labtek culture slide (BD Falcon). 500 µl DMEM was added to each well and the HeLa cells were grown overnight at 37^{o}C with 5% CO_2. 3 ml LB broth (supplemented with 10 µg/ml tetracycline) was inoculated with a fresh colony of the appropriate EPEC strain. The culture was incubated overnight at 37^{o}C with shaking (200 rpm). The next day the bacterial culture

was diluted 1:100 in DMEM (Hepes modified) that had been preheated to 37^{o}C. The culture was incubated statically at 37^{o}C for 2.5 hours. DMEM was removed from the HeLa cells and replaced with 500 µl of the bacterial culture. Cultures were incubated for a further 30 minutes at 37^{o}C with 5% CO_2. 1 mM IPTG was then added to each well to induce expression of TEM1-effector fusion and the cells incubated for one hour at 37^{o}C 5% CO_2. DMEM was removed from the cells and the HeLa cells were washed three times with HBSS. After removing the last wash, 200 µl of HBSS and 40 µl of the CCF2/AM loading substrate were added to the HeLa cells. The cells were incubated in the dark at room temperature for 90 minutes and then washed three times in HBSS. Excess HBSS was removed from the slide with Whatman 3 mm filter paper and the chamber partitions were removed from the slide. Wells were covered with two square (2 cm^2) glass coverslips. Live cells were viewed using a Leica DMRE fluorescence microscope with a 4',6'-diamidino-2-phenylindole (DAPI) filter set (340 - 380 nm excitation and 425 nm long-pass emission). Images were taken using a true colour digital camera.

3.3 Results

Thirteen EHEC O157:H7 T3SS effector candidates and 4 confirmed LEE encoded effectors were successfully cloned into the TEM-1 expression vector pCX340gw (performed by S. Beatson). Wild type EPEC 2348/69 and an isogenic non-T3S EPEC Δ*escN* were then transformed with the recombined TEM-1 fusion expression plasmids. The author together with S.A. Beatson and C.M. Bailey performed 17 β-lactamase translocation assays under the supervision of O. Marches at Imperial College, London.

Figure 3.2 shows an example of the blue and green fluorescence seen in the translocation assays. Blue fluorescence was seen in HeLa cells infected with wild type EPEC expressing TEM-1-fusions of LEE encoded effectors Tir, EspH, EspZ and EspG. Blue fluorescence was also seen for 11 candidate effector proteins indicating translocation into eukaryotic cells (Table 3.4). Green fluorescence was seen in HeLa cells infected with EPEC Δ*escN* expressing the same effectors indicating that translocation of the putative effectors was dependent on the T3SS. This confirmed the effector status of 11 candidate effectors. Green fluorescence was seen in cells infected with EPEC expressing EspV' and EspX4.

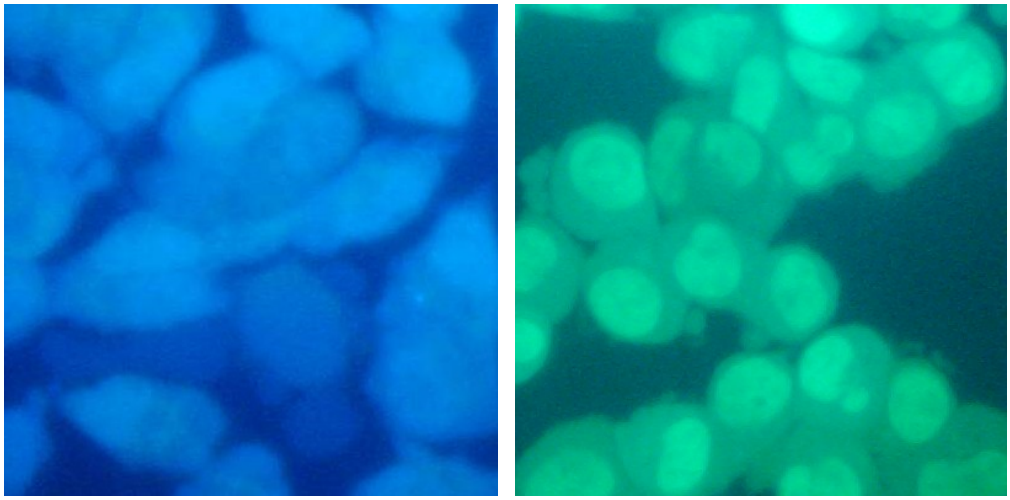

Figure 3.2: β-lactamase translocation assay. HeLa cells infected with EPEC2348/69 (a) or EPEC Δ*escN* (b) expressing TEM-1 EspO1-1. Blue fluorescence indicates translocation of fusion protein. Green fluorescence indicates no translocation. Images used with permission from Tobe *et al*., 2006 [2].

Effector	Sakai ID	Result
EspY1	ECs0061	Positive
NleC	ECs0847	Positive
NleD	ECs0850	Positive
EspX2	ECs0876	Positive
EspV’	ECs1127	Negative
EspO1-1	ECs1567	Positive
EspK	ECs1568	Positive
NleH1-2	ECs1814	Positive
NleF	ECs1815	Positive
NleG7’	ECs2226	Positive
NleG6-3’	ECs3488	Positive
Tir	ECs4561	Positive
EspH	ECs4564	Positive
EspZ	ECs4571	Positive
EspG	ECs4590	Positive
EspY4	ECs4653	Positive
EspX4	ECs5021	Negative

Table 3.4: Results of the β-lactamase translocation assays

Effector	Sakai ID	Evidence
EspY1	ECs0061	B
NleC	ECs0847	B
NleH1-1	ECs0848	SC
NleD	ECs0850	B
EspX2	ECs0876	B
EspX7	ECs1560	S
EspN	ECs1561	S
EspO1-1	ECs1567	SCFB
EspK	ECs1568	SFB
NleG2-1*	ECs1810/1	S
NleA	ECs1812	S
NleH1-2	ECs1814	SCFB
NleF	ECs1815	SB
NleG	ECs1824	SF
EspM1	ECs1825	SF
NleG2-2	ECs1994	SCF
NleG6-1	ECs1995	SCF
NleG5-1	ECs1996	SCF
NleG5-2	ECs2154	S
NleG6-2	ECs2155	S
NleG2-3	ECs2156	S
NleG7*	ECs2226	B
EspJ	ECs2714	SC
TccP	ECs2715	S
EspM2	ECs3485	SF
NleG8-2	ECs3486	SCF
EspW	ECs3487	SC
NleG6-3*	ECs3488	B
EspL2	ECs3855	SF
NleB1	ECs3857	SCF
NleE	ECs3858	SF
EspF1	ECs4550	S
EspB	ECs4554	S
Tir	ECs4561	B
Map	ECs4562	S
EspH	ECs4564	SB
EspZ	ECs4571	SFB
EspG	ECs4590	SB
EspY4	ECs4653	B

Table 3.5: Evidence supporting effector status. B = positive result in β-lactamase assay, S = present in EHEC O157:H7 Sakai T3S secretome, C = positive result in CyaA translocation assay, F = positive result in FLAG tag staining, * = predicted pseudogene. Table adapted from [2].

3.4 Discussion

Forty-nine novel T3SS effectors were discovered in the EHEC O157:H7 genome by a bioinformatics survey (performed by M. Pallen and S.A. Beatson). In order to confirm the effector status of the candidate effectors, T3S-dependent secretion and translocation of effectors needed to be demonstrated.

Four independent methods were used to demonstrate the T3S-dependent secretion and translocation of the candidate effectors. Three approaches were carried out by colleagues at the University of Osaka, Japan. In the first approach 31 effectors were shown to be secreted into the culture supernatant by the T3SS using secretome analysis. In the second approach translocation of 12 novel effectors was demonstrated using FLAG tagged effectors and immunofluorescence. The third approach, an adenylate cyclase translocation assay, confirmed the translocation of 10 predicted effectors. Work carried out at the University of Birmingham and Imperial College, London demonstrated translocation of candidate effectors using a β-lactamase translocation assay. Predicted effectors were expressed as TEM-1 fusion proteins within wild type EPEC 2348/69 or an isogenic non-T3SS EPEC strain. HeLa cells were infected with EPEC strains (expressing novel effector-TEM-1 fusions). Translocation of effectors was monitored using the fluorescent substrate CCF2/AM. Results showed that 11 novel effectors and 4 LEE encoded effectors were translocated into HeLa cells via the T3SS.

Table 3.5 shows the results of each assay for each effector. Results from this study confirm that the effector repertoire of EHEC O157:H7 Sakai is much larger than previously imagined. The total number of T3SS effectors in this strain now stands at 39. The results of the β-lactamase assay together with the results of assays performed by collaborators confirm that homologues of *Shigella* Osp-type effectors i.e. EspL (a homologue of OspD), EspO1-1 (a homologue of OspE) and NleH1-2 (a homologue of OspG) are translocated by the LEE T3SS. Significantly translocation of EspW, a homologue of HopW from the plant pathogen *P. syringae* was confirmed in this study. This implies that effectors target conserved aspects of eukaryotic cell biology and that the association between type-III secretion and eukaryotic subversion is ancient [2].

3.4.1 Non-prophage encoded effectors

As discussed in chapter one, effector genes encoded in non-phage encoded EELs are predicted to be substrates of the ETT2 T3SS (a second T3SS defunct in the Sakai strain). Two such candidate effectors (EspY1 and EspY4) were shown to be translocated through the LEE encoded T3SS using the β-lactamase assay. This shows that these putative effectors are genuine effectors (or at least were effectors of the ETT2 T3SS).

3.4.2 Pseudogenes encode T3SS-translocated proteins

Interestingly two effectors (NleG6-3' and NleG7') encoded by pseudogenes caused blue fluorescence in HeLa cells indicating that they were translocated via the T3SS. This shows

that the cryptic T3SS secretion signal remains intact despite the prediction that these effectors can no longer function. It is possible however that these effectors modulate the function of other effectors and thus play a role in cellular subversion. This thesis concentrates on effectors that are not encoded by predicted psuedogenes. However, further studies into pseudogene-encoded novel effectors (especially those that are translocated into host cells) may provide valuable insight into EHEC pathogenesis and evolution. Translocation could not be demonstrated for two of the 17 effectors tested (EspV' and EspX4). EspV' is a pseudogene, it is likely that the T3SS secretion signal has been disrupted by a frameshift or by mutations thereby preventing T3S of this effector.

3.4.3 Future T3SS effector discovery and confirmation

In addition to expanding the repertoire of T3SS effectors in EHEC O157:H7, the results of this study confirm that homology searches can be used to predict T3SS effectors reliably. Homology searches of the recently sequenced EPEC 2348/69 genome have revealed the presence of 21 putative T3SS effector genes [352]. Previous experimental studies of T3SS effectors have often relied upon transposon mutagenesis and other laborious techniques to discover individual novel T3SS effectors [350]. This study highlights the effectiveness of an alternative approach in which bioinformatics and mid-throughput translocation assays are used to identify novel T3SS effectors and confirm their translocation into host cells. Such approaches are becoming more relevant as the availability of next generation sequencing technology means that there is likely to be a huge increase in the number of sequenced bacterial genomes (including many T3SS organisms) in the near future [353] [354]. To exploit the vast quantities of information provided by increased genome sequencing e.g. for

defining the effector repertoires of numerous strains of pathogens/clinical isolates, researchers will need to identify novel T3SS effectors quickly, easily and accurately [355] [356]. The approach to T3SS effector discovery and confirmation demonstrated by Tobe *et al* [2] will most likely enable this.

The Gateway system of cloning enabled fast and efficient cloning of candidate effectors into a β-lactamase expression vector. Entry vectors created in this study can be used in recombination reactions with other destination vectors and other model systems in the future – thereby reducing the need for further amplification of effector genes by PCR.

The β-lactamase translocation assay was relatively straightforward to perform and allowed translocation of T3SS effectors to be investigated in live cells. Results appear to be reliable as they are consistent with studies on the secretion/translocation of LEE-encoded effectors [196]. However, there are limitations to the approaches used. For example the reporter proteins (e.g. adenylate cyclase) fused to the effectors were large and may have interfered with secretion through the T3SS apparatus. This may have caused false negative results. Also, translocation of individual effectors was tested using EPEC. There is thus no hierarchy of secretion between EHEC effectors in this model – false negative results may have occurred if secretion of the effector in question relies upon or is regulated by another EHEC-specific effector or protein. Translocation should therefore be tested using strains of EHEC in which individual effectors are deleted. This would provide a more physiologically relevant model of translocation of T3SS effectors during EHEC infection.

CHAPTER FOUR

Expression of effectors in mammalian cells

4.1. Introduction

The second aim of this thesis was to characterise the effects of novel EHEC O157:H7 T3SS effectors on eukaryotic cells. This chapter documents work carried out by the author and R. Shaw to define the localisation of selected novel effectors in mammalian cells.

4.1.1. Localisation as a clue to effector function

Following translocation into host cells, effectors are often targeted to specific organelles and cell structures where they perform their role in cellular subversion [357]. For example, EPEC Map is targeted to the mitochondria [152] whereas Tir localises to the plasma membrane to function as the receptor for intimin [58] [358]. Protein sequence analysis and predicted secondary structure e.g. transmembrane domains, may give clues as to effector targeting in host cells. Often however, no such clues are available – YopM of *Yersinia* localises to the host cell nucleus despite containing no classical nuclear localisation signal [359]. Numerous studies of T3SS effectors have investigated the localisation of individual effectors within mammalian cells [254] [359] [360]. In addition to following effector localisation directly, observation of cell processes such as cytoskeletal rearrangements and vesicle trafficking can also be useful for gaining an insight into effector function as disruption of these processes may indicate interference by the effector [254] [361]. For example, phalloidin, a toxin from the death cap mushroom that binds to filamentous actin [362] can be used to probe for actin stress fibres and other actin-rich cytoskeletal formations. Such changes in the actin cytoskeleton may indicate that the effector interacts with actin or disrupts small GTPase signalling [159].

T3SS effectors can be introduced into mammalian cells by infection or transfection [363] [364]. Localisation of the effectors may then be monitored by immunofluorescence microscopy or immunoblotting of cell fractions.

4.1.2. Infection

Infection of cells with type III-secreting pathogens allows effectors to be studied during the course of a physiologically representative infection i.e. all T3SS components (e.g. chaperones) are present. The advantages of this method include; delivery of effectors into host cells through the T3SS and maintenance of the correct hierarchy of secretion (may allow effectors to act in concert e.g. SopE and SptP of *Salmonella*). However, redundancy between effectors and the co-operative nature of many T3SS effectors means that it can be difficult to characterise the effects of one effector when that effector is translocated into host cells alongside other effectors through the same T3SS [357]. Transient transfection allows effectors to be expressed individually within the eukaryotic cell, making it easier to determine the contribution of a particular effector to cellular subversion [365]. This study used transfection of HeLa cells to examine the localisation of GFP-tagged EHEC O157:H7 T3SS effectors.

4.1.3. Transfection

Transfection introduces nucleic acids into eukaryotic cells through a non-viral mechanism e.g. electroporation, microinjection [366], calcium phosphate [367], cationic polymer- and cationic lipid-mediated transfection [368]. The method of microinjection was considered for

this study whereby a microneedle is used to inject DNA directly into the eukaryotic cell. However, microinjection is a process that requires much skill and time as each cell is transfected individually [366]. Cells may be damaged by microinjection and throughput is very low [366]. Therefore, transfection mediated by cationic lipids was used as it provided a high-throughput system whereby effectors could be screened quickly and easily with existing laboratory equipment. In this method, DNA is delivered to the eukaryotic cell in the external milieu. Uptake of DNA by the eukaryotic cell is aided by cationic lipid molecules that bind to/condense DNA to produce a positively charged DNA-liposome transfection complex [369]. This complex interacts with the negatively charged host cell membrane [370]. Transfection complexes are then thought to be taken up by the eukaryotic cells by endocytosis [369]. Cells are not damaged by the delivery of DNA and the throughput is much higher than microinjection as many cells can be transfected simultaneously [368]. Transient transfection of mammalian cells has been used to investigate the functions and localisation of effectors from many T3SS organisms including *Yersinia* [371], *Pseudomonas* [361], EPEC [254], *Salmonella* [372] and *Shigella* [373].

4.1.4. Protein tags

Protein localisation within cells can be followed using antibodies to the protein of interest. However, when screening a large number of proteins it is laborious and expensive to obtain antibodies to each protein of interest. Therefore the protein of interest is often tagged with an epitope tag; usually a small peptide often derived from a viral protein e.g. myc, haemagglutinnin (HA), V5, or FLAG [374]. Antibodies for a range of tags are commercially

available at low cost. Therefore, protein tags provide an easier and more cost-effective method for following proteins of interest by immunofluorescent microscopy.

Green fluorescent protein (GFP) is a 25kDa chromoprotein derived from the jellyfish *Aequoria victoria* [375]. GFP is photoactivatable, insensitive to pH and tolerates fusions – it is therefore a useful biological reporter protein and has been used to study protein expression, movement, localisation and protein interactions in a range of cell types [376] [377]. However, GFP cannot pass through the T3SS [225] so cannot be used in infection studies. GFP is useful in transfection studies of T3SS effectors where delivery of the effectors by the T3SS is not required. Fusion to GFP has also been shown to increase the overall stability of fusion proteins [378] [379].

In this study, effector proteins were expressed as GFP fusions within HeLa cells. The Gateway vector pcDNA-DEST53 (Invitrogen) was used for transfection of mammalian cells with effector genes. Recombination between entry vectors and pcDNA DEST53 allowed expression of GFP–effector fusion proteins. The localisation of GFP-effector fusion proteins within HeLa cells was followed by fluorescence microscopy. Fluorescent tetramethylrhodamine isothiocyanate (TRITC) phalloidin was also added to transfected cells following fixation to probe for actin stress fibres and other actin-rich cytoskeletal formations.

4.1.5. Site-directed mutagenesis

Another aim of this thesis was to dissect sequence-activity relations for selected effectors. To investigate the significance of individual amino acid residues in individual effectors, i*n vitro* site-directed mutagenesis was used. Site-directed mutagenesis is a useful technique as it allows specific amino acid residues/motifs of a protein to be mutated [380]. This study made use of the commercially available QuikChange II Site-Directed Mutagenesis Kit (Stratagene, USA) to quickly, easily and reliably mutate effector genes. This method uses a double-stranded methylated DNA vector with the insert of interest (effector gene) as a template. Mutagenic primers containing the desired mutation are then used to generate a mutated plasmid through thermocycling and extension of the primers by PfuUltra DNA polymerase. The methylated parent plasmid is digested using *Dpn* I (an endonuclease specific for methylated DNA). The mutated plasmid is selected for as it is not methylated and therefore not affected by *Dpn* I. Mutated plasmids are then transformed into and propagated within *E. coli* [381].

4.1.6. Targeting residues of EspM2 for mutagenesis

Amino acids that are highly conserved between homologous proteins are likely to be important for function and/or structure of a protein [382]. Conserved residues can be identified by producing a multiple sequence alignment of homologous proteins [383]. Figure 4.1 shows a multiple sequence alignment of representative members of the IpgB family of effectors including EspM [384], Map [153], EspT [385], IpgB1 and IpgB2 [159]. An alignment of 24 members of the family can be seen in Alto *et al* (2006) [159]. The

multiple sequence alignment reveals a number of conserved amino acid residues: Trp70, Glu74, Ser80, Leu118 and Asp119 (figure 4.1). In this study, Ser80, Leu118, Asp119 and Phe195 (non-conserved) were mutated by site directed mutagenesis to determine the contribution of these residues to effector function and targeting. The residues were substituted with alanine as it is a small amino acid, likely to preserve protein folding by conserving steric and hydrophobicity properties. Therefore any effect on protein function is more likely due to loss of amino acid side chain and not due to disruption of overall protein structure [386].

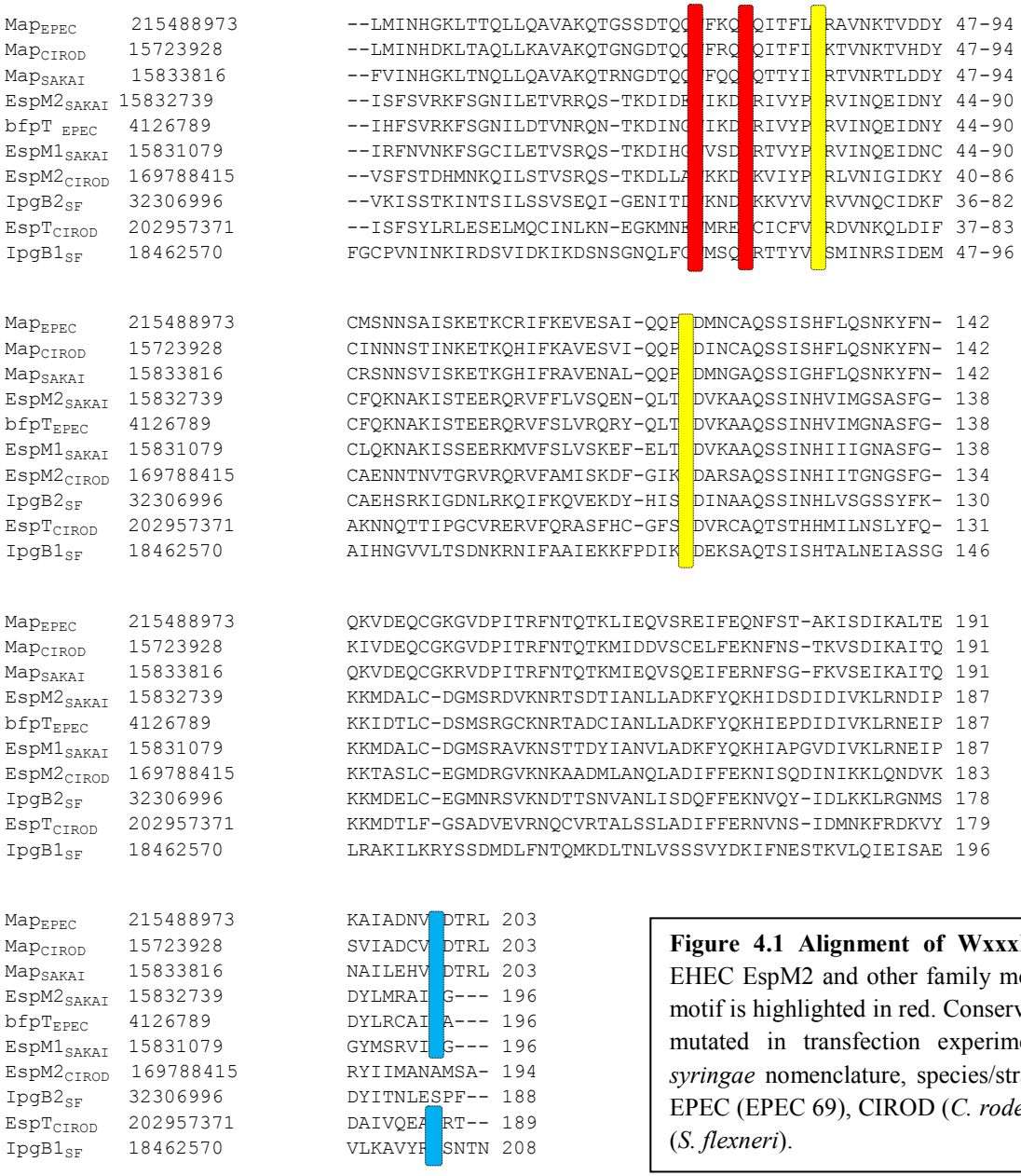

Figure 4.1 Alignment of WxxxE family members. Clustal alignment of EHEC EspM2 and other family members. Genbank ID# is listed. The WxxxE motif is highlighted in red. Conserved (yellow) and non-specific (blue) residues mutated in transfection experiments are highlighted. Consistent with *P. syringae* nomenclature, species/strain names are shown as subscript suffixes: EPEC (EPEC 69), CIROD (*C. rodentium*), SAKAI (EHEC O157:H7 Sakai), SF (*S. flexneri*).

4.2. Methods

4.2.1. Gateway DNA cloning

PCR

Thirteen effectors were amplified by PCR (author and R Younis performed five reactions each, S.A. Beatson performed three reactions) as described in chapter 2. Primers used in this study are listed in table 4.1.

TOPO cloning

Thirteen effectors were cloned into pENTR-D-TOPO (no Shine-Dalgarno sequence). Author performed five Topo cloning reactions as described in chapter 2 (R. Younis performed five reactions, S. A. Beatson performed three reactions). Entry vectors used in this study are listed in table 4.2

LR recombination

Thirteen effectors were recombined (as described in chapter 2) into Gateway mammalian cell expression vector pcDNA-DEST53 (figure 4.2). Destination vectors used in this study are listed in table 4.2. The author performed eight successful LR reactions, R. Younis performed five. Destination vectors were confirmed by DNA sequencing according to standard procedures described in chapter 2. Details of growth media, chemical transformation of TOP10 *E. coli* and plasmid preparation can also be found in chapter 2.

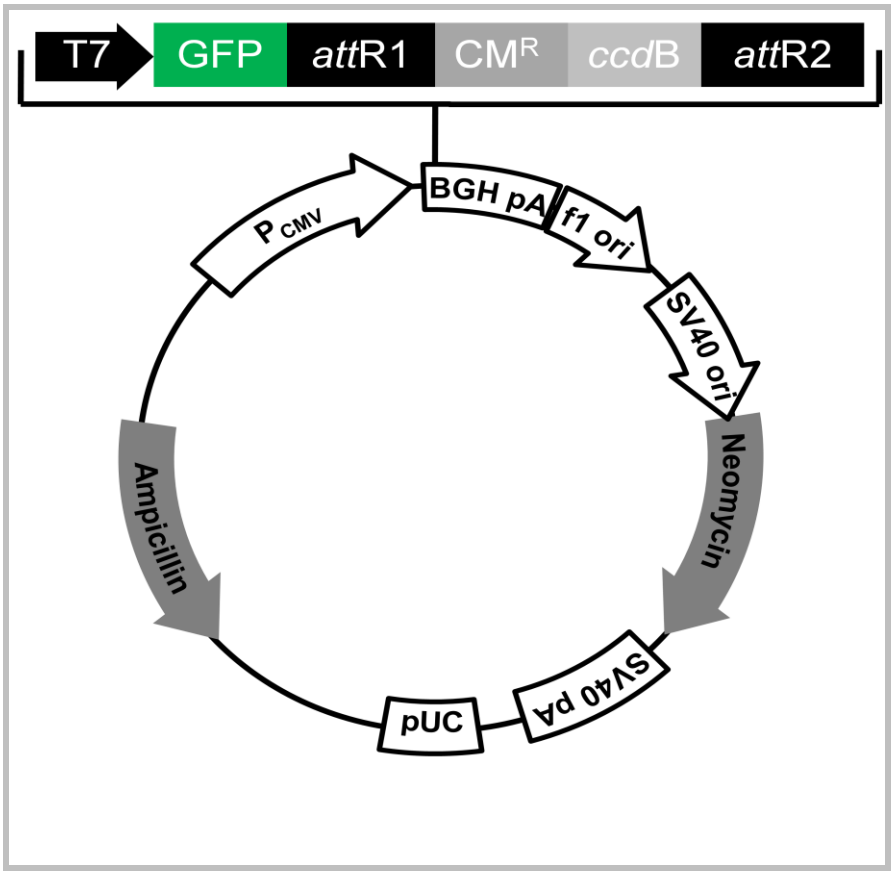

Figure 4.2: pcDNA DEST53. Gateway destination vector for mammalian expression of N-terminal GFP fusion. Expression of the GFP-effector fusion was controlled by a cytomegalovirus (CMV) promoter.

Primer	Sequence (5'-3')
DNA sequencing	
GatewayM13F	GTAAAACGACGGCCAG
gwpDEST53GFPf	GAGTTTGTAACTGCTGCTGGG
gw.pDEST53_R2	GCTGATCAGCGGGTTTATCTT
gw.pDEST53_R3	CCACTTTGTACAAGAAAGC
Amplification of effector genes	
ECs0850.gwF	CACCATGCGCCCTACGTCC
nsGW.0850r	AAGCAATGGATGCAGTCTTACC
ECs1812.gwF	CACCATGAACATTCAACCGACC
ECs1812.gwR	GACTCTTGTTTCTTGGAT
ECs1815gwF	CACCATGTTACCAACAAGTGGT
ECs1815gwR	TCCACATTGTAAAGATCC
ECs1824.gwF	CACCATGCCTGTCATATTAAAC
ECs1824.gwR	AATTCTAGTGCATATATTTTG
ECs1994gwF	CACCATGCCATTAACCTCAGAT
ECs1994gwR	ATTACCCTTTATAACGAA
ECs1825.gwF	CACCATGCCAGTAAATGCG
nsGW.1825r	CCCCTGTATAACACGACTC
ECs2714.gwF	CACCATGTCAATTATAAAAAAC
ECs2714.gwR	TTTTTTGAGAGGATATATG
ECs3485.gwF	CACCATGCCGATGAATACT
nsGW.3485r	TCCCTGTATAGCACGCATC
ECs3486.gwF	CACCATGCCAGTCATATTAAAT
ECs3486.gwR	AATACTGTTTTGTTGAAG
ECs3487.gwF	CACCATGCCCAAAATATCATCAG
ECs3487.gwR	ATTTCTAACCAAGGGGTCC
ECs3488.gwF	CACCATGGAGAGAAGGGCT
ECs3488.gwR	TTAAGCATCACTTGCAAC
ECs3857.gwF	CACCATGTTATCTTCATTAAATG
ECs3857.gwR	CCATGAACTGCAGGTATAC
ECs3858.gwF	CACCATGATTAATCCTGTTAC
ECs3858.gwR	CTCAATTTTAGAAAGTTTAT
ECs4562.gwF	CACCATGTTTAGTCCAATGACAATG
ECs4562.gwR	CAATCGGGTATCCTGTAC

Table 4.1: DNA primers used in this study

Effector	Sakai ID	Entry vector	Entry vector source	GFP Destination vector (pcDNA DEST53)	Destination vector source
Map	ECs4562	SME14a	This study	SM14.14a	This study
NleE	ECs3858	SME20a	This study	SM14.20	This study
NleB	ECs3857	SME13.19a	This study	pDEST3857	R. Younis, this study
NleG6-3	ECs3488	pENTR-D-3488	This study	pDEST3488	R. Younis, this study
EspW	ECs3487	pENTR-D-3487	This study	pDEST3487	R. Younis, this study
NleG8-2	ECs3486	pENTR-D-3486	This study	pDEST3486	R. Younis, this study
EspM2	ECs3485	pR1.26b	R. Younis, this study	pDEST3485	R. Younis, this study
EspJ	ECs2714	SME4.3	This study	SM16.4.3b	This study
NleG2-2	ECs1994	SME37a	This study	SM14.37a	This study
NleG	ECs1824	pSB2.12A	S.A.Beatson, this study	SM3.6	This study
NleF	ECs1815	pSB10.10B	S.A.Beatson, this study	SM16.11a12	This study
NleA	ECs1812	pSB2.10B	S.A.Beatson, this study	SM14.10b	This study
NleD	ECs0850	pR1.27a	R. Younis, this study	pDEST0850	This study

Table 4.2: Vectors used in this study.

Plasmid	Purpose	Source
pDEST/GW-53/CAT	GFP control	Invitrogen
pRK5 myc	Expression of Rho (actin stress fibre formation)	L. Machesky, University of Glasgow

Table 4.3: Control vectors used in this study.

4.2.2. Site directed mutagenesis

Primer design

Mutagenic primers were designed using the primer design guidelines issued by Stratagene or by using the Quikchange Primer Design Program available at www.stratagene.com. Briefly, both mutagenic primers contained the mutation (in the middle of the primer) and annealed to the same sequence on opposite strands of the plasmid; primers were between 25 and 45 bases long with a melting temperature >78°C and a minimum G+C content of 40%. Mutagenic primers (purified by polyacrylamide gel electrophoresis) were obtained from Eurogentec. Mutagenic primers used in this study are shown in table 4.4.

ECs3485 mutagenesis (performed by R. Younis)

Template plasmid DNA was obtained from a dam^+ *E. coli* strain to ensure selection for mutated plasmids following treatment with *Dpn* I. The reaction mixture consisted of 5 µl of 10x reaction buffer, 40 ng dsDNA template (pDEST53-3485), 125 ng each mutagenic primer, 1 µl dNTP mix, 1 µl PfuUltra DNA polymerase (2.5 U/µl) and 50 µl dH_2O.

The following reaction cycle was used:

95°C 30 seconds
95°C 30 seconds
55°C 1 minute
68°C 3 minutes
4°C until use

Cycle x 16

Dpn I digestion

10 U Dpn I restriction enzyme was added to each reaction mix after amplification. The mixture was mixed by pipetting and then incubated for 1 hour at 37°C to digest the nonmutated, parental DNA. *Dpn* I treated DNA was then transformed into chemocompetent *E. coli* (strain XL-1 Blue) as outlined in chapter 2. Mutagenesis was confirmed by DNA sequencing as outlined in chapter 2.

Primer pair	Sequence	Mutation/s introduced
3485.2afS	5′ GATAGTATATCCC**GCA**AGGGTGATCAACCAG 3′	S80A
3485.2arS	5′ CTATCATATAGGG**CGT**TCCCAGTAGTTGGTC 3′	
3485.3afQ	5′ GATGCGTGCTATA**GCA**GGATGAGGTGGGCGC 3′	Q195A
3485.3arQ	5′ CTACGCACGATAT**CGT**CCTACTCCACCCGCG 3′	
3485.4xfLD	5′ TCAACTAACC**GCAGCA**GTTAAGGCAGCACAAAG 3′	L118A D119A
3485.4xrLD	5′ AGTTGATTGG**CGTCGT**CAATTCCGTCGTGTTTC 3′	

Table 4.4: Mutagenic primers used in site directed mutagenesis of EspM2.

Effector	Destination vector	Source
$EspM2_{S80A}$	pDEST53 3485 2a	R. Younis, this study
$EspM2_{Q195A}$	pDEST53 3485 3a	R. Younis, this study
$EspM2_{L118D119A}$	pDEST53 3485 4x	R. Younis, This study

Table 4.5: Mutated EspM2 vectors created and used in this study.

4.2.3. Transfection

Preparation of DNA for transfection (DNA maxiprep)

To ensure high transformation efficiency, DNA used in mammalian cell transfection was required to be of a high quality, high concentration and endotoxin-free. Endofree Maxiprep (Qiagen, USA) kits were used for the preparation of plasmid DNA (all reaction vessels used for preparation of DNA were new/endotoxin-free). Briefly, single colonies of transformed TOP10 *E. coli* were picked and grown overnight (37°C, shaking at 200rpm) in 5 ml of LB broth supplemented with 100 μg/ml of ampicillin. 250 μl of this overnight culture was used to inoculate 250 ml LB broth supplemented with 100 μg/ml ampicillin. Cells were grown overnight at 37°C with shaking (200rpm). The bacterial culture was then centrifuged at 6000 x g for 15 minutes at 4°C. Plasmid DNA was obtained by a standard alkaline lysis method using a commercially available maxiprep kit (Qiagen, USA) following the manufacturer's instructions. Plasmid DNA was eluted in 300 μl elution buffer.

Preparation of Lipofectamine-DNA complexes

Transfection solutions were prepared by adding 6 μl Lipofectamine 2000 (Invitrogen, UK) to 10μl PBS. Endotoxin-free DNA stocks of 0.025 μg/μl were prepared in PBS. 16 μl of this stock was added to the Lipofectamine solution and mixed by gentle pipetting. Lipofectamine is a cationic lipid with a positively charged head group that interacts with and condenses DNA [369] [370]. Lipofectamine-DNA complexes interact with negatively charged cell membranes to promote endocytosis of the complex [368]. Prior to transfection, Lipofectamine-DNA complexes were allowed to form for 15 minutes at room temperature.

Immediately before transfection, 200 μl serum free DMEM was added to the Lipofectamine-DNA solution and mixed gently.

Lipofectamine transfection

HeLa cells were seeded on borosilicate glass coverslips (VWR, US) in 6-well tissue culture dishes (Invitrogen) and grown for 48 hours at 37°C to provide approximately 1 x 10^5 cells for transfection. Immediately before transfection, cells were washed twice with serum-free DMEM. 800 μl of serum-free medium was then added to the cells. 232 μl of the transfection solution containing 0.5 μg DNA complexed to Lipofectamine was gently added to each well. Cells were then incubated for 6 hours at 37°C 5% CO_2. The transfection medium was then removed and the cells washed twice in DMEM (10% FCS). 2.5ml DMEM (10% FCS) was added to each well and the cells incubated overnight at 37°C 5% CO_2. R. Shaw and author performed 60% and 40% (respectively) of transfections of HeLa cells.

4.2.4. Immunostaining

Coverslips were washed 3 times in PBS before being fixed in 3% paraformaldehyde for 15 minutes. Coverslips were then washed again (3 times in PBS) before cells were permeabilised for 4 minutes in PBS/0.1% Triton X-100. Coverslips were washed three times in PBS, quenched for 15 minutes in 50mM NH_4Cl and washed again (3 times in PBS). Coverslips were placed on dental wax and 5 μg/ml of tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin was added to each to stain the actin cytoskeleton. Phalloidin is light sensitive and extremely toxic therefore great care must be taken when handling.

Coverslips were incubated in the dark for 40 minutes at room temperature. Coverslips were transferred to a darkened 24-well plate and washed 3 times with PBS. Coverslips were then mounted on microscopy slides using 2 µl Citifluor AF1 (Citifluor, UK) and sealed with nail varnish.

4.2.5. Microscopy

All samples were examined using a Leica DMR immunofluorescence microscope equipped with a DC200 digital camera. Green (GFP) and red (TRITC-Phalloidin) images were analysed and superimposed using Leica IM1000 software. This allowed visualisation of GFP-effector proteins as green and actin as red.

4.3. Results

4.3.1. Gateway vectors

Thirteen pENTR-D-TOPO entry vectors and pcDNA-DEST53 mammalian expression vectors containing selected effector genes were successfully created. Entry vectors will be able to be used in LR recombination reactions with alternative destination vectors in future studies.

4.3.2. NleB, NleF, EspM2 and EspW diffusely localise within the host cell cytoplasm

Human epithelial (HeLa) cells were transiently transfected with the Gateway mammalian expression vector pcDNA-DEST53. This allowed expression of individual GFP-effector fusion proteins within the mammalian cell. GFP localisation was followed using fluorescence microscopy. TRITC-phalloidin was also used to visualise the actin cytoskeleton of transfected cells. Transfections were repeated 3 times and 20 images of transfected cells were recorded per transfection. Representative images of transfected cells are shown in figures 4.3 to 4.14 (transfections and image acquisition were performed by the author unless otherwise stated). HeLa cells were transfected with pDEST/GW-53/CAT plasmid as a control. This plasmid allows expression of GFP alone and was used to determine transfection efficiency and the effects of GFP expression on the mammalian cell. In cells transfected with the control plasmid, GFP was diffusely localised throughout the cytoplasm and nucleus with areas of higher concentrations of GFP in vesicle-like structures. Cells transfected with the control also showed no other changes compared to untransfected

cells. EHEC O157:H7 T3SS effector Map was used as a positive control as this effector has been well characterised and is known to localise to mitochondria and induce filopodia [153]. GFP-Map showed a punctate perinuclear localisation and filopodia were induced (figure 4.3). In cells expressing EspW, NleB1, NleD, NleF and EspM2, GFP was diffusely localised throughout the cytoplasm (figures 4.4 - 4.8, 4.13). Cells expressing NleD showed diffuse cytoplasmic GFP localisation (80% cells) and, occasionally, punctate GFP aggregates (20% cells) (figure 4.7). No changes in the actin cytoskeleton were observed in cells expressing NleF, EspW, NleB or NleD.

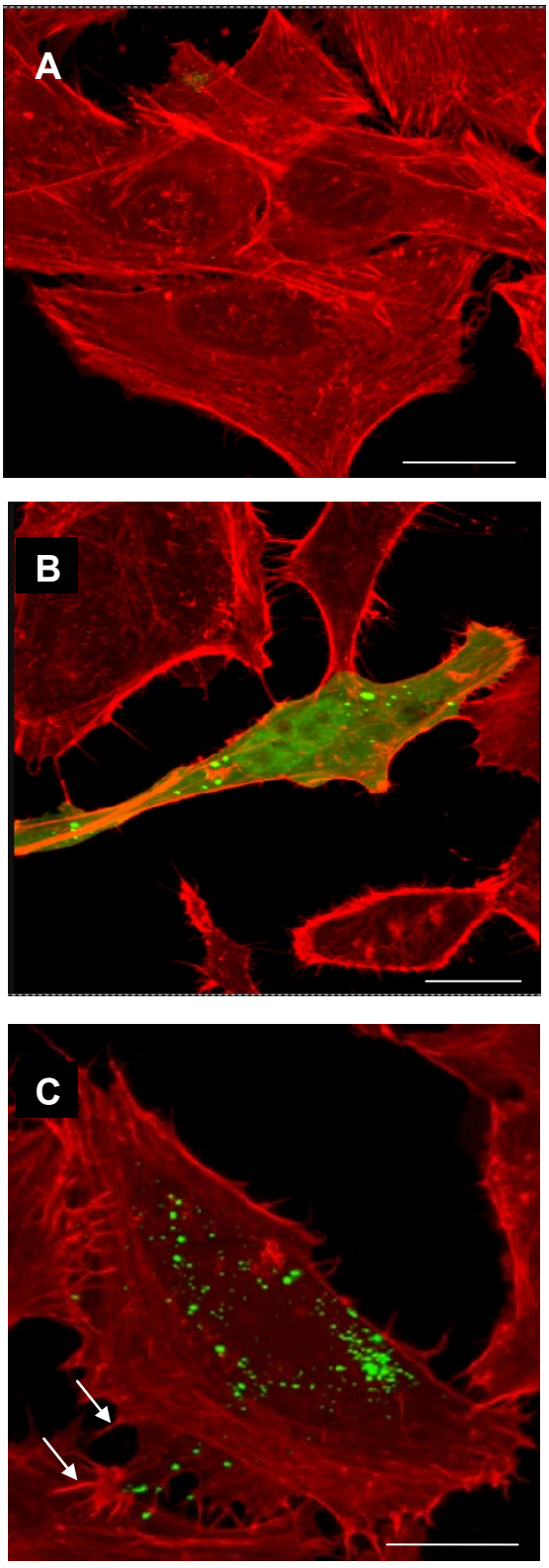

Figure 4.3: HeLa cell control transfections, stained with TRITC-phalloidin (A) non-transfected cells, (B) cells transfected with pcDNA CAT GFP, (C) cells transfected with Map (performed by R. Shaw). Filopodia are indicated. Scale bar represents 20 µm.

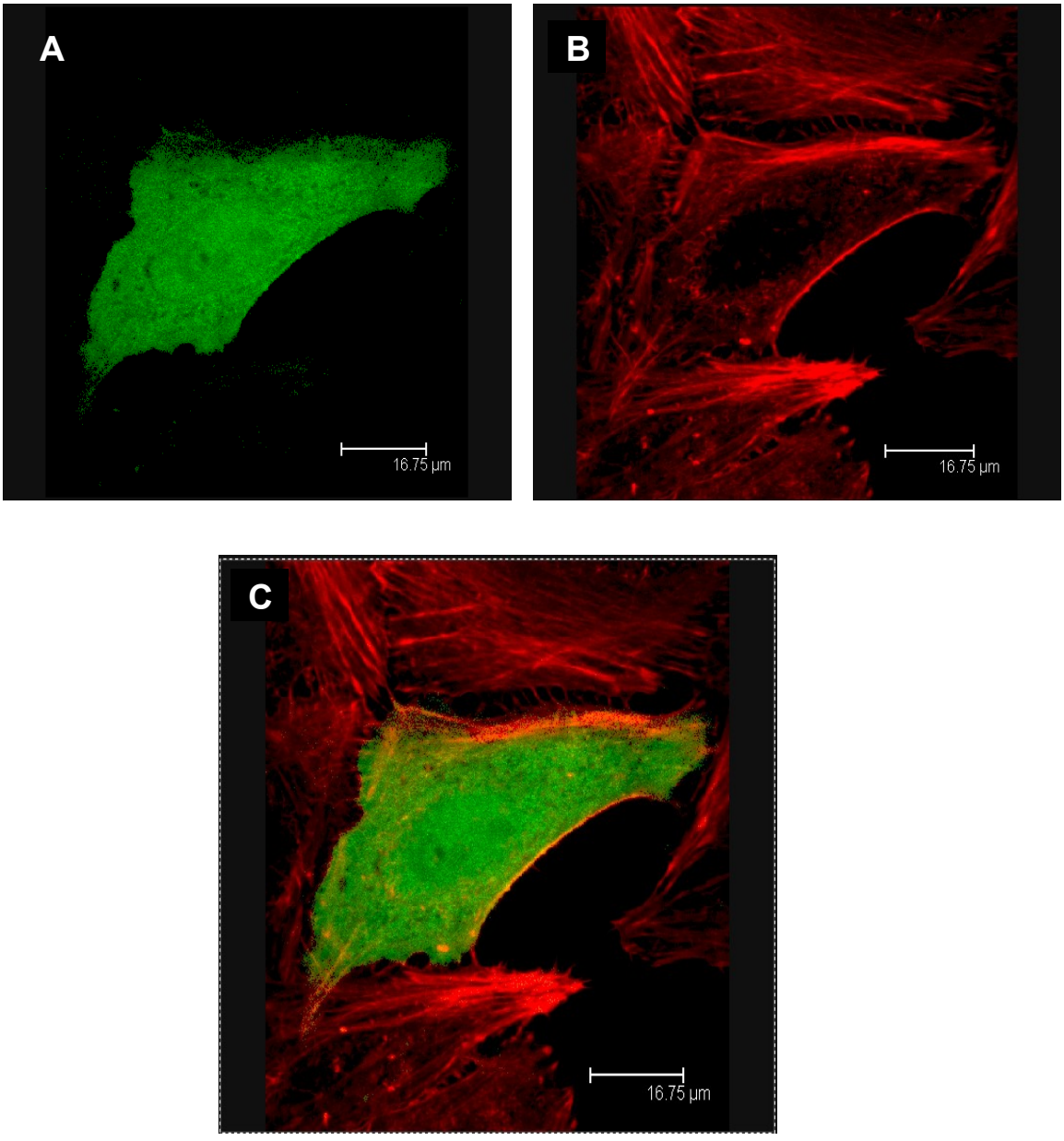

Figure 4.4: HeLa cells transfected with EspW (ECs3487) (A) GFP only, (B) TRITC-phalloidin, (C) merged image.

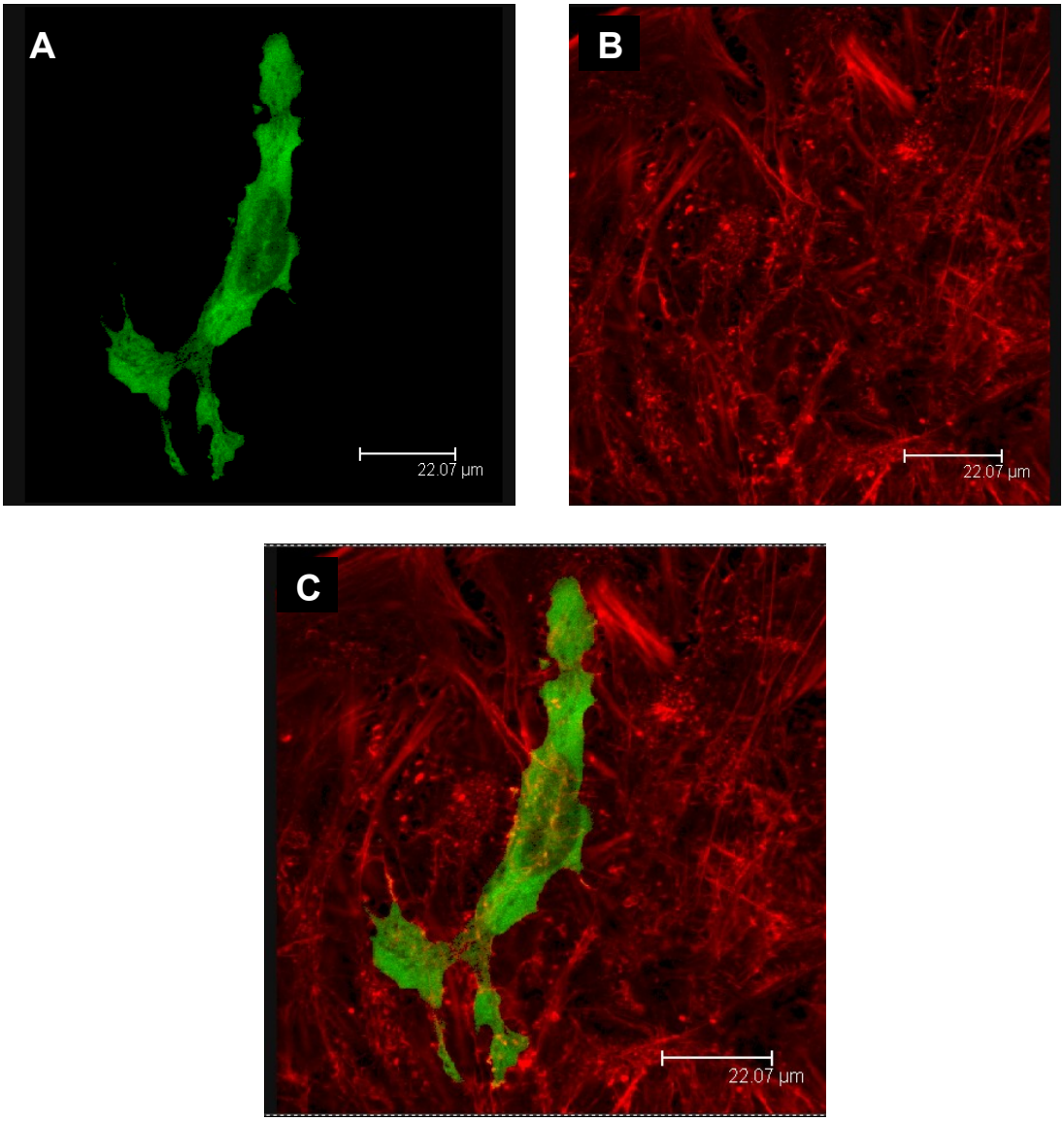

Figure 4.5: HeLa cells transfected with NleB1 (ECs3857). (A) GFP only, (B) TRITC-phalloidin actin stain, (C) merged image.

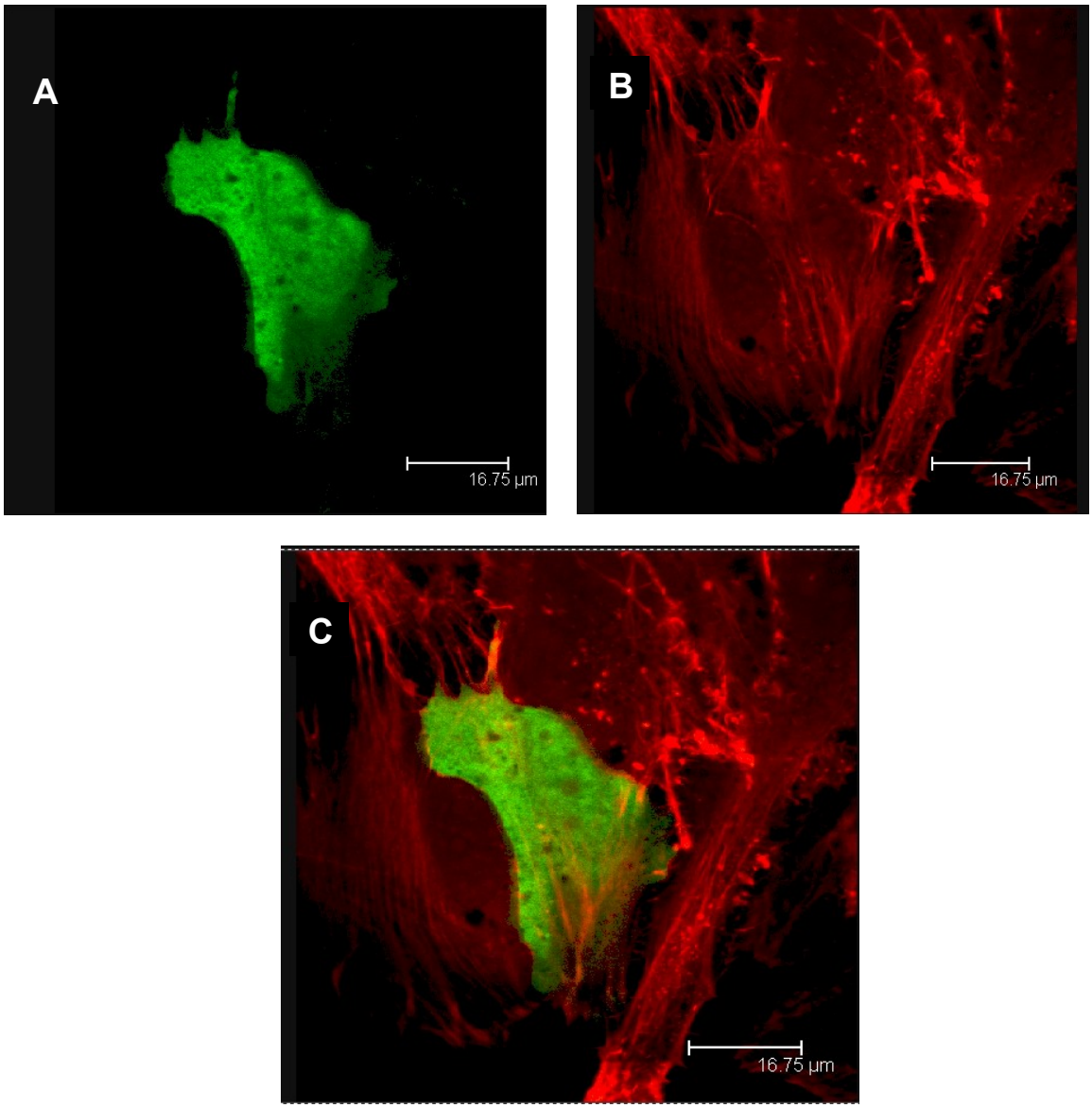

Figure 4.6: HeLa cells transfected with NleB1 (ECs3857). (A) GFP only, (B) TRITC-phalloidin actin stain, (C) merged image.

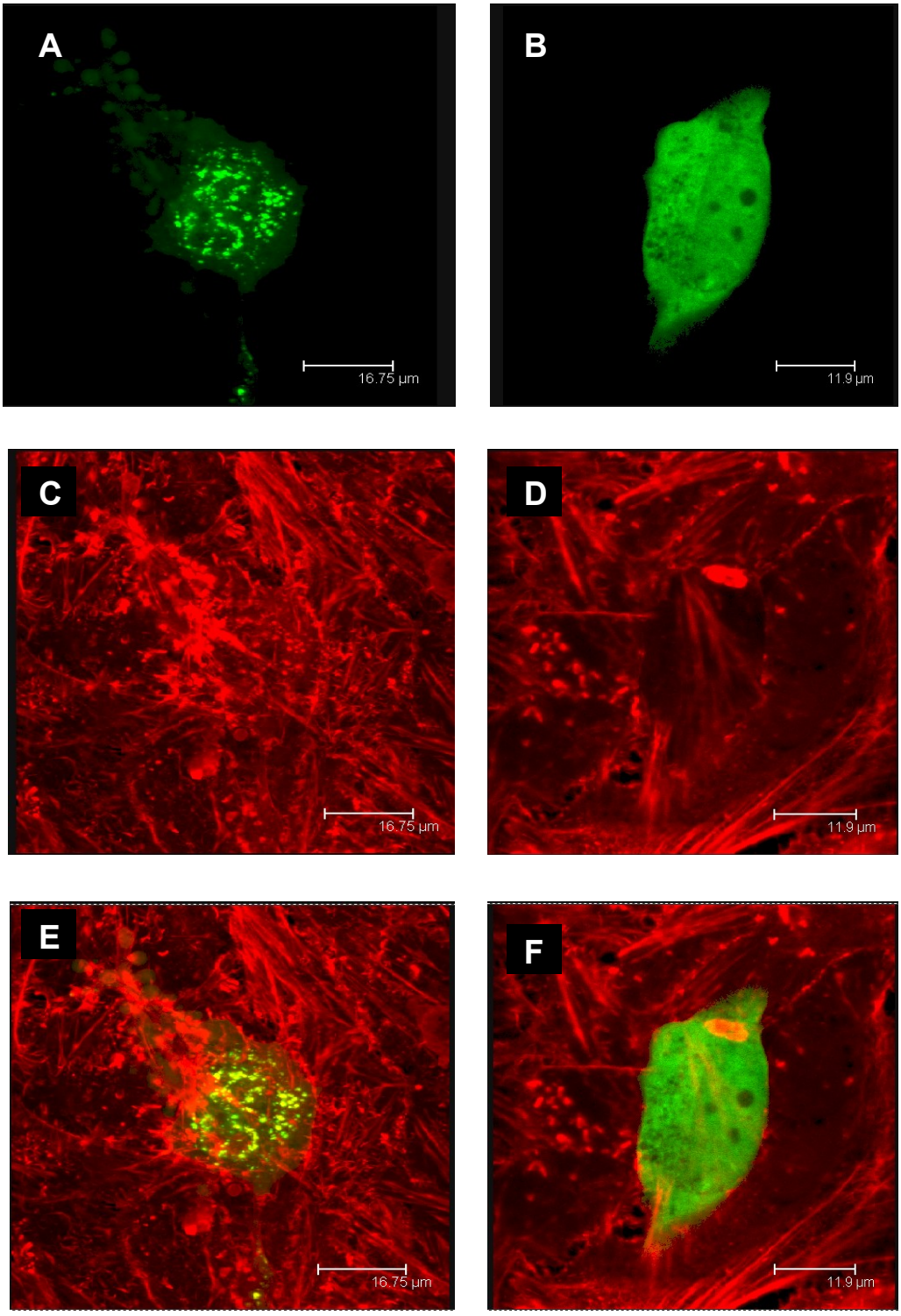

Figure 4.7: HeLa cells transfected with NleD (ECs0850). (A, B) GFP only, (C,D) TRITC-phalloidin actin stain, (E,F) merge showing differences in localisation patterns observed for the same effector.

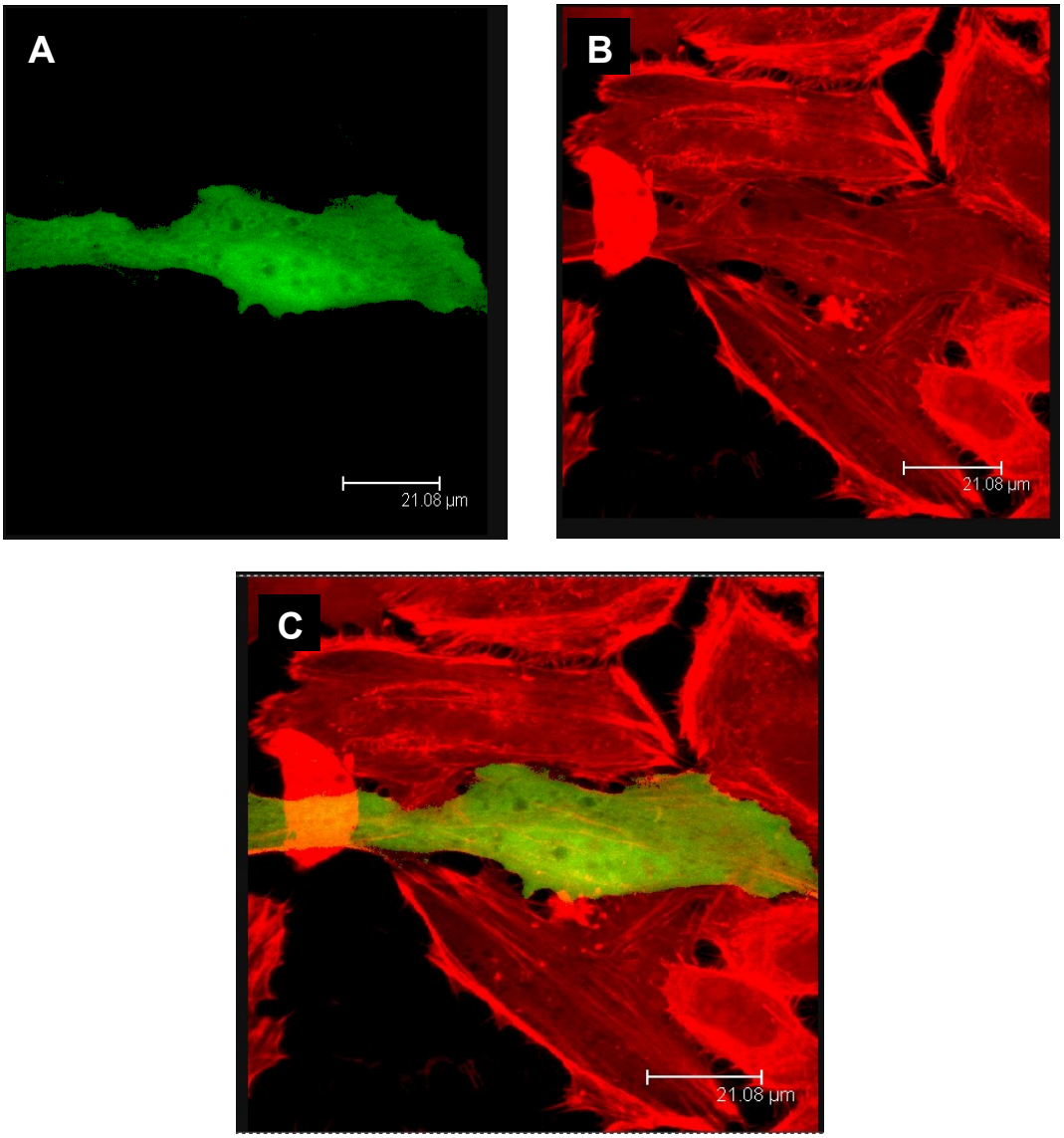

Figure 4.8: HeLa cells transfected with NleF (ECs1815). (A) GFP only, (B) TRITC-phalloidin, (C) merge.

4.3.3. NleA, NleE, NleG and EspJ show punctate localisation within the mammalian cell cytoplasm

In contrast to the diffuse localisation patterns of EspM2, NleB1, NleD, NleF and EspW, several effectors: NleA, NleE, NleG and EspJ showed punctate localisation within the cytoplasm of transfected cells. EspJ appeared in spots around the cell nucleus suggesting that this effector may be targeted to a perinuclear organelle such as the ER or to secretory vesicles.

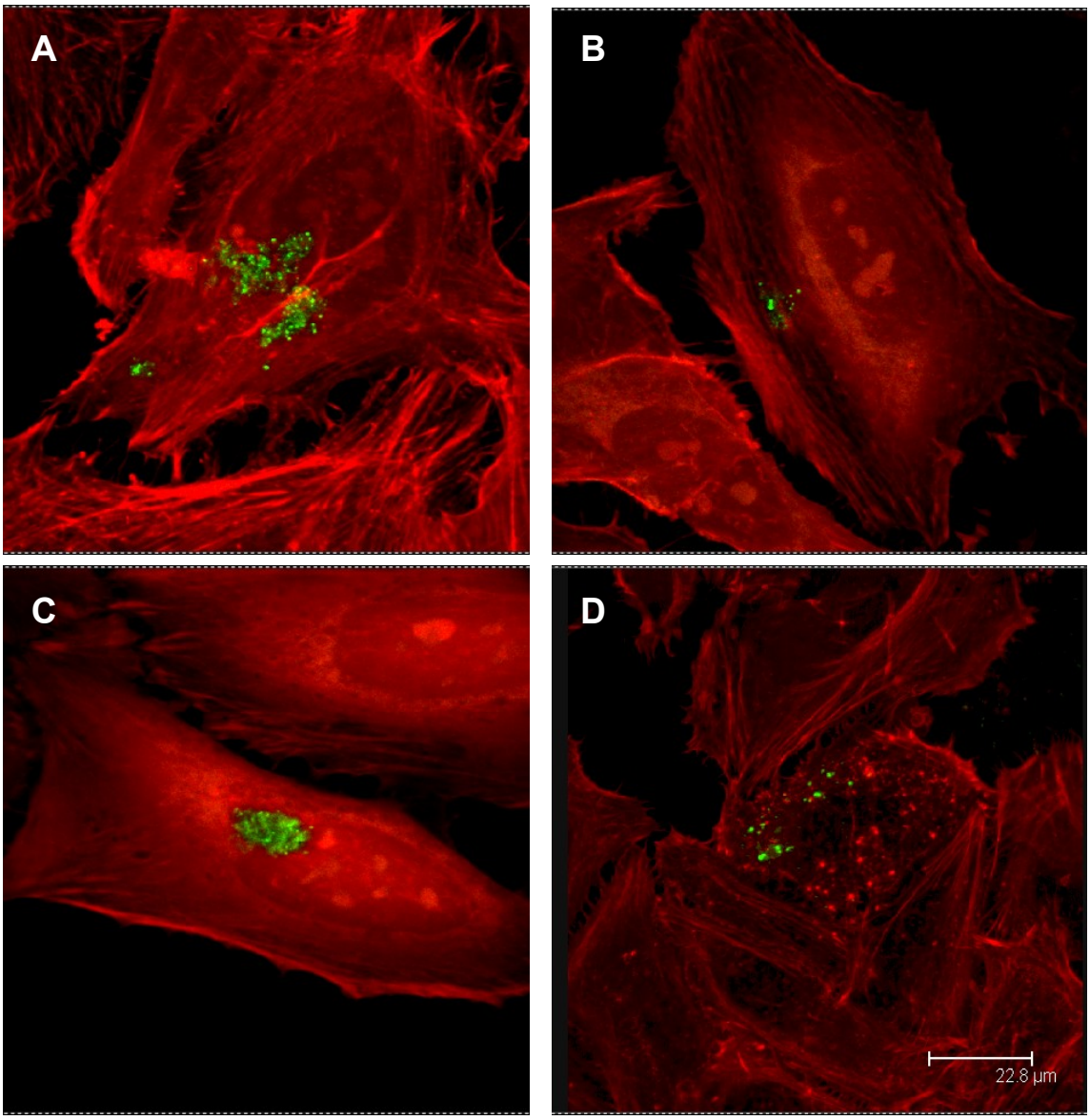

Figure 4.9: HeLa cells transfected with NleA (ECs1812). Transfection and images A, B and C performed by R. Shaw.

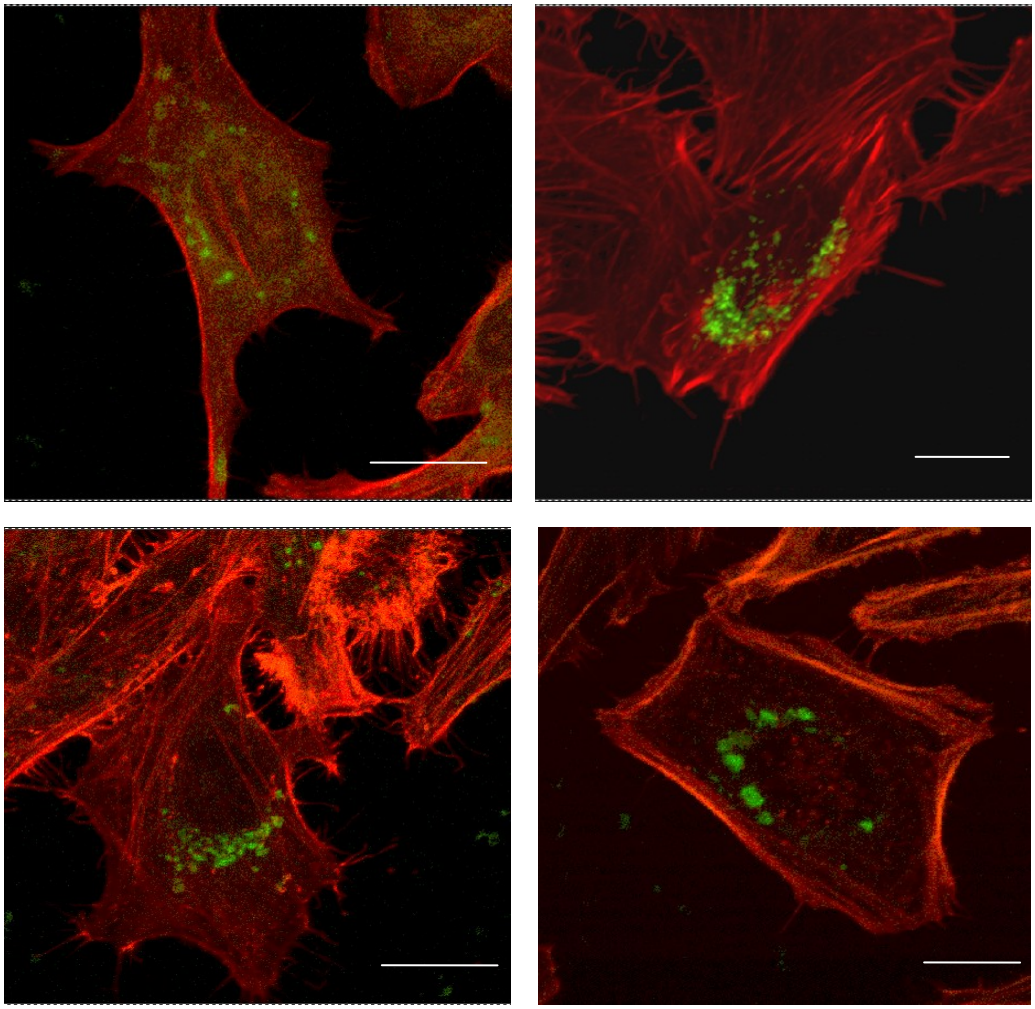

Figure 4.10: HeLa cells transfected with EspJ (ECs2714) (performed by R. Shaw). Scale bar represents 20 µm.

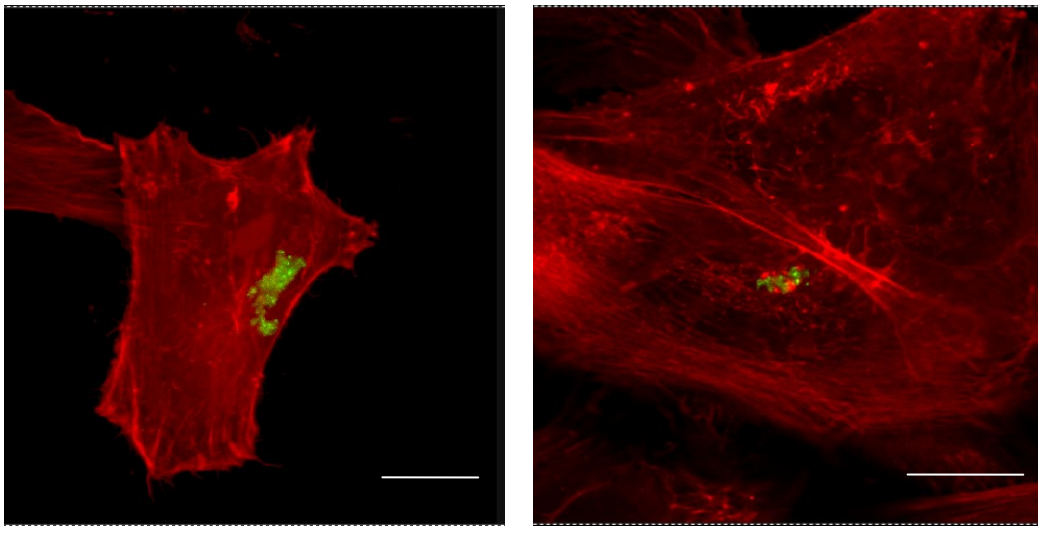

Figure 4.11: HeLa cells transfected with NleE (ECs3858) (performed by R. Shaw). Scale bar represents 20 µm.

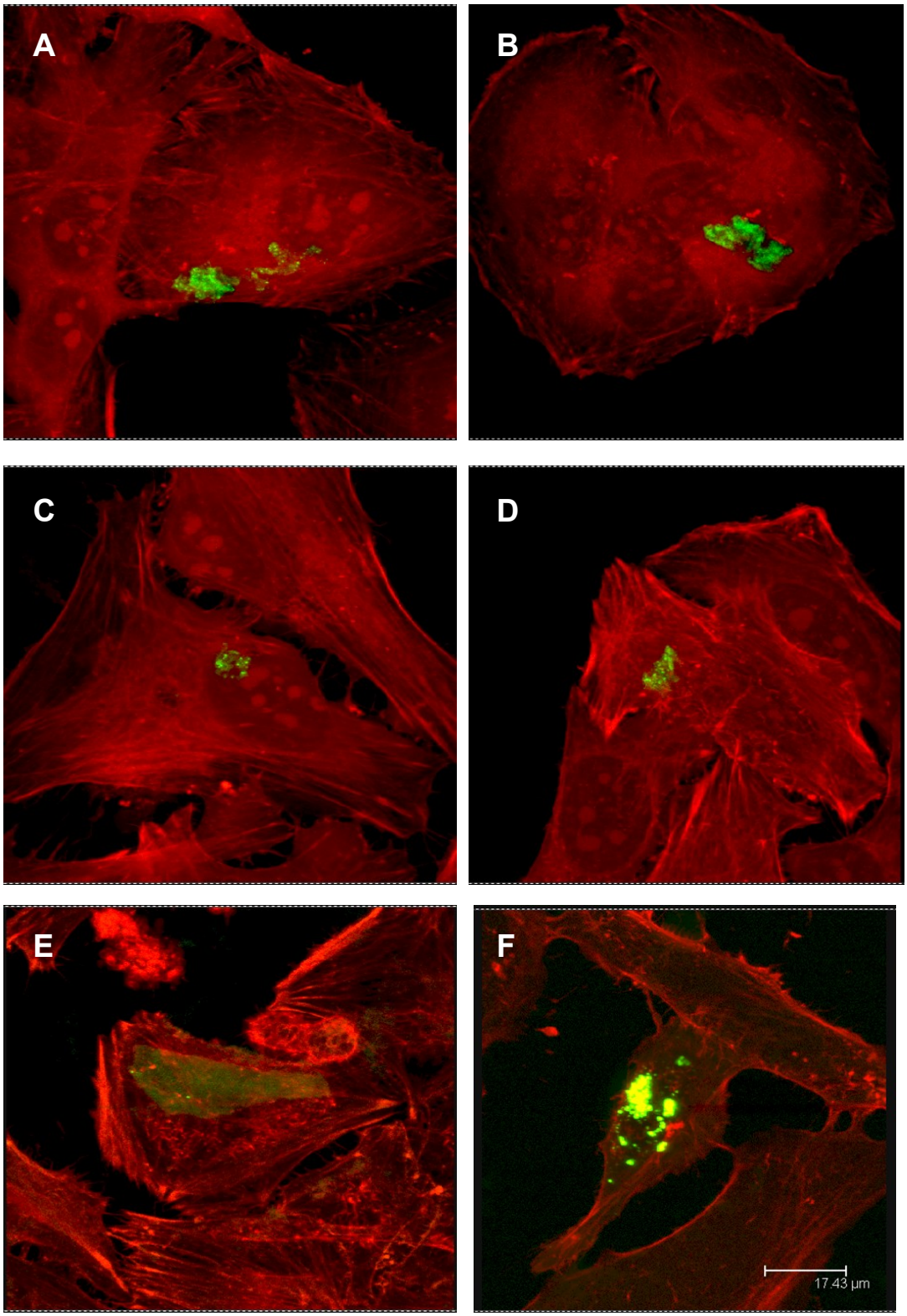

Figure 4.12: HeLa cells transfected with NleG. (A, B) NleG8-2 (ECs3486), (C, D) NleG6-3 (ECs3488), (E) NleG2-2 (ECs1994), (F) NleG (ECs1824). Transfections A-E performed and images taken by R. Shaw.

4.3.4. EspM2 promotes actin stress fibre formation

TRITC-phalloidin staining showed that actin stress fibres were formed in HeLa cells expressing wild type EspM2 (figure 4.13). Levels of actin stress fibre formation were comparable to cells expressing Rho, a small GTPase that regulates actin stress fibre assembly [387] as shown using Hela cells transfected with pRK5 myc (a Rho expression vector).

4.3.5. EspM2 mutants show altered patterns of localisation and decreased stress fibre formation

HeLa cells were transiently transfected with mutated EspM2. Localisation of $EspM2_{S80A}$ was similar to wild type i.e. diffuse cytoplasmic localisation. Actin stress fibres were also seen in cells expressing $EspM2_{S80A}$ mutant (comparable to levels caused by expression of wild type EspM2 and Rho). However, cells expressing $EspM2_{Q195A}$ and $EspM2_{L118A/D119A}$ showed altered localisation of GFP. GFP was no longer diffusely localised throughout the cytoplasm but appeared as small cytoplasmic aggregates (figure 4.14). No actin stress fibres were seen in cells transfected with $EspM2_{Q195A}$ and $EspM2_{L118A/D119A}$ (figure 4.14).

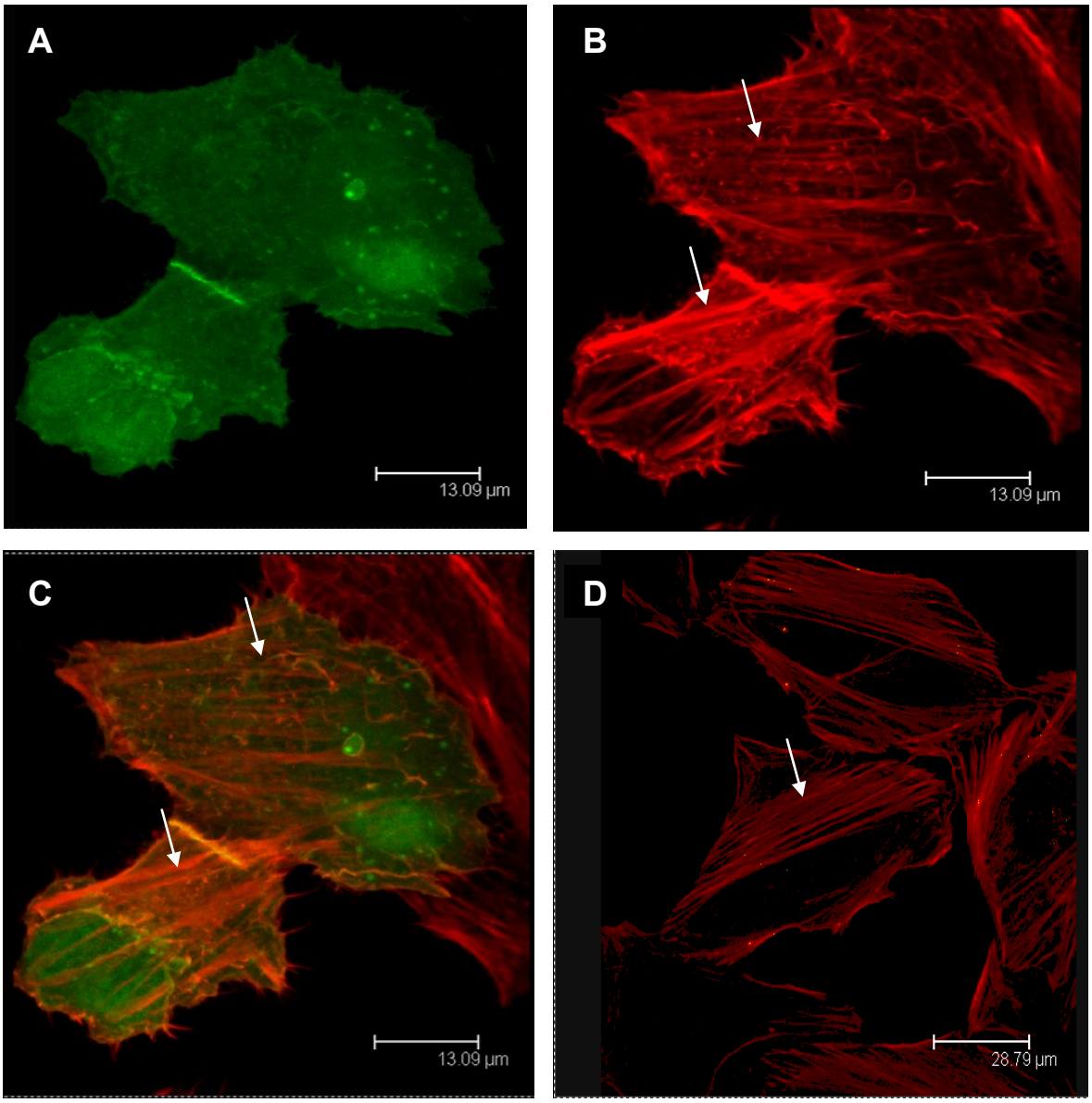

Figure 4.13: Hela cells transfected with EspM2. (A) GFP-EspM2 localisation, (B) TRITC-phalloidin actin stain, (C) merged image, (D) Hela cells transfected with pRK5-Myc to induce actin stress fibres. Actin stress fibres are indicated.

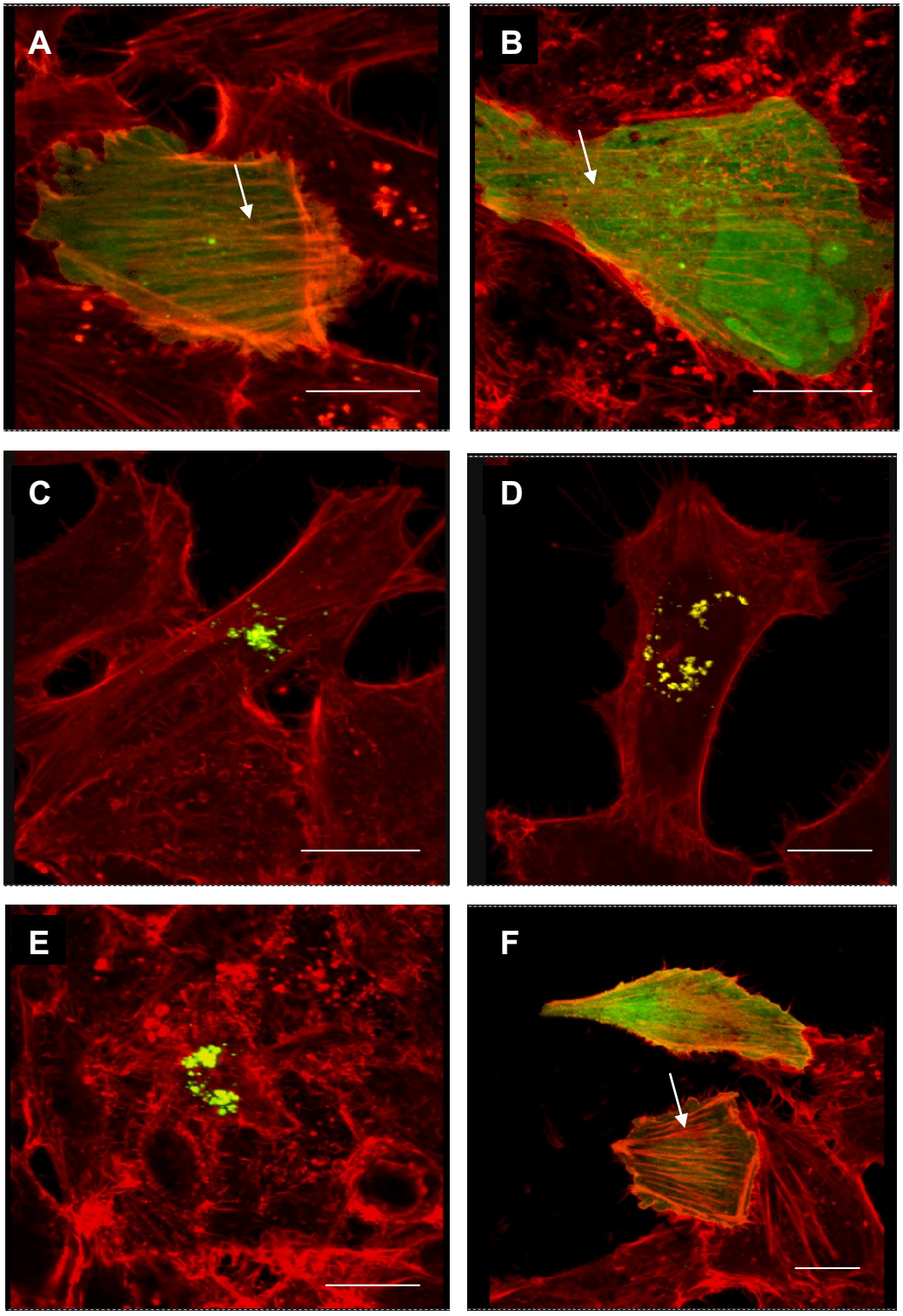

Figure 4.14: Hela cells transfected with EspM2 mutants. (A, B) $EspM2_{S80A}$, (C, D) $EspM2_{L118/D119A}$, (E) $EspM2_{Q195A}$, (F) $EspM2_{wt}$. Actin stress fibres are indicated. Scale bar represents 20 µm.

4.4. Discussion

Transfection has been used successfully to study many T3SS effectors. In this study, transient transfection was used to study the localisation of selected novel EHEC O157:H7 effectors. Effectors were expressed as N-terminal GFP fusions within human epithelial (HeLa) cells. The localisation of GFP was followed using fluorescence microscopy. Four effectors; EspM2, NleB1, NleF and EspW were diffusely localised within the host cell cytoplasm whereas seven effectors including effectors from the NleG family, NleA, NleE and EspJ were not diffusely localised and showed punctate localisation patterns within the host cell.

Diffuse localisation of EspM2, NleB1, NleF and EspW suggests that these effectors are not targeted to a specific organelle or cellular compartment. Further studies of EspM2 [388] and NleF [300] have confirmed cytoplasmic localisation of these effectors.

The non-diffuse patterns of localisation of NleA, NleE, EspJ and NleG are difficult to interpret as GFP localisation may indicate that the effectors are targeted to specific organelles or that there was poor transfection/expression or misfolding of the effectors within the mammalian cell. To determine the exact localisation of the effectors, cell compartment-specific dyes/antibodies could be used e.g. MitoTracker (Invitrogen) for visualising mitochondria, DAPI for visualising DNA/nucleus. In agreement with the punctate localisation patterns seen in this study, NleA has been shown to localise to the Golgi [295] whereas a study by Zurawski *et al* (2008) showed that OspZ (a *Shigella* NleE

homologue) demonstrates similar localisation to EHEC NleE in infected cells [389]. The perinuclear punctate localisation of GFP-EspJ suggests that EspJ may localise to the ER/secretory vesicles [390]. These findings support those of Marches *et al* (2008) who also found that EspJ-FLAG showed punctate, endomembrane localisation in transfected COS-7 cells. EspJ has since been shown to inhibit opsono-phagocytosis – possibly by blocking membrane/membrane-borne regulator trafficking [390].

Transfection of cells with NleD (ECs0850) demonstrated inconsistent results i.e. diffuse patterns of GFP were seen in 80% of cells whereas aggregates of GFP were present in 20% of cells. This may have been due to degradation of the GFP-effector fusion protein perhaps due to cell stress caused by toxicity of the effector. Studies have shown that effectors introduced by transfection do not always localise to the same place as those introduced by bacteria. For example, SseG (*Salmonella*) targets the endoplasmic reticulum and microtubules when delivered by bacteria [391] [392] but targets the Golgi of transfected cells [393]. Therefore, transfection is not wholly reliable and may not accurately reflect effector localisation during an infection – infection studies should be undertaken to confirm the localisation of effectors. Infection studies would eliminate the possibility that localisation patterns are due to poor transfection or expression of the effector within HeLa cells.

Transfected cells were stained with TRITC-phalloidin to visualise the actin cytoskeleton. There were no actin cytoskeletal changes observed in transfected cells except those expressing EspM2. In these cells, there was an increase in actin stress fibre formation.

Similar actin stress fibres are induced in cells over-expressing the small GTPase, Rho [394] [395]. This suggests that EspM2 might function by interfering with actin directly or by modulating actin cytoskeletal dynamics through activation of small GTPases. In similar experiments by Alto *et al*, a homologue of EspM2, IpgB2 from *Shigella*, induced the formation of actin stress fibres in transfected HEK293 cells. Alto *et al* showed that EspM2 (and EspM1) belong to a family of effectors that contain an invariant Trp-xxx-Glu (WxxxE) motif. This family contains T3SS effectors from *Shigella* (IpgB1, IpgB2), *Salmonella* (SifA, SifB) and EPEC (Map) [159]. Work by Alto *et al* [159] has shown that these effectors appear to mimic the GTP-active forms of Rho-family GTPase. Map mimics Cdc42, IpgB2 mimics RhoA and IpgB1 mimics Rac-1 to promote filopodia, stress fibre formation and membrane ruffles respectively [159]. Arbeloa *et al* have since confirmed that EspM2 causes global parallel stress fibre formation in a RhoA-dependent mechanism [388].

Mutation of Leu118/Asp119 and Phe195 to alanine affected GFP-effector localisation and stress fibre formation within HeLa cells. This may have been caused by protein misfolding and degradation. However, Arbeloa *et al* found that mutagenesis of L114 and D115 of EspM3 resulted in the formation of local parallel stress fibres instead of radial stress fibres [388]. Leu118 and Asp119 of EspM2 may therefore be important in controlling the conformation of actin stress fibres in host cells. Arbeloa *et al* also showed that mutagenesis of other conserved residues in EspM3 did not have any effect on stress fibre formation – similarly, results of this study showed that mutation of Ser80 to alanine did not affect effector localisation or stress fibre formation. Structural studies may help to elucidate the mechanism by which EspM2 promotes the formation of stress fibres.

In evaluation, transfection of Hela cells with GFP-effector fusions has provided an insight into the localisation of twelve EHEC O157:H7 non-LEE encoded T3SS effectors. Transfection also provided an insight into the function of EspM2. However, the localisation pattern of a small number of effectors, especially NleD, was also inconsistent. This highlights some of the limitations of the approach used. For example, expression of effectors was driven by a powerful viral promoter (CMV) – it is possible that high level expression of effector proteins may have led to aberrant targeting within the transfected cell. Also, localisation of effectors was observed at only one time point (12 hours) after transfection. A number of T3SS effectors are known to move between cellular compartments e.g. YopM moves to the nucleus by a mechanism that requires vesicular trafficking [359]. The subcellular localisation of the novel effectors should therefore be investigated at more time points after transfection e.g. at 6 and 24 hours to confirm that the correct subcellular localization (or movement of the effector through the cell) has been observed. As mentioned in chapter 3, there are limitations to studying T3SS effectors in isolation. During infection, effectors may interact inside host cells which may affect the localisation of each individual effector e.g. Tir and EspFu co-localise to the pedestal during infection [263]. The localisation of novel effectors should therefore also be investigated and confirmed using other methods (e.g. infection) and model systems. Yeast provides an ideal alternative model in which to study T3SS effectors – yeast cell culture is simple and less time-consuming that mammalian cell culture and there are many techniques that can be used to investigate T3SS effector function. Chapter 5 documents investigations into effector localisation and function using a yeast cell model.

CHAPTER FIVE

Expression of effectors in *Saccharomyces cerevisiae*

5.1. Introduction

The *Saccharomyces cerevisiae* model is increasingly being used to study T3SS effectors as it is simple and can provide valuable insights into effector localisation and function within eukaryotic cells. There are a variety of assays and techniques using the yeast cell model that have been used to dissect T3SS effector function. In this chapter, selected effectors were screened using a yeast growth inhibition assay to identify effectors with deleterious effects on yeast. The localisation of selected effectors within yeast cells was investigated using yeast reporter strains. The effect of effector expression on chitin localisation and cell morphology was also investigated. Finally, an attempt was made to dissect sequence-activity relations for effectors EspM2 and NleG8-2.

5.1.1. Expression of proteins in yeast

De novo expression of proteins e.g. T3SS effectors, in yeast is relatively straightforward as yeast can be easily transformed with plasmid DNA in the form of shuttle vectors that are easy to propagate in *E. coli* and transform into yeast [159, 396]. In this study, effector proteins were expressed as V5/6x His fusions from the Gateway shuttle vector pYES DEST52 (Invitrogen, UK). This high copy number plasmid is able to replicate in both *E. coli* and yeast. Yeast are transformed with the vector by an established lithium acetate method [397]. Expression of effector-V5/6xHis fusion proteins from pYES-DEST52 is controlled by the *GAL1* promoter. This promoter is repressed by glucose [398] and activated by galactose [399]. Therefore, expression of effectors can be induced by the addition of galactose to the growth media. Effector-V5/6xHis fusion proteins can be detected using

commercially available anti-V5 [400] or anti-His [401] antibodies. V5 is a small (4 kDa) epitope tag derived from Simian virus 5 [400]. The His tag is comprised of 6 histidine residues and is useful in protein purification [402].

5.1.2. Yeast growth as an indicator of protein toxicity

Yeast growth inhibition due to expression of T3SS effectors has been shown to be a sensitive and specific indicator of bacterial protein activity [403] [378]. Experimental evidence has shown that very few non-translocated bacterial proteins affect yeast growth [403] [378] whereas T3SS effectors from EPEC [404] [405], *Salmonella* [157, 406] [407], *Shigella flexneri* [159, 378], *Yersinia* [408] [157], *Pseudomonas aeruginosa* [409] [410] [411], *Pseudomonas syringae* [412] [413] and *Chlamydia trachomatis* [414] inhibit yeast growth. Slagowski *et al* (2008) showed that 19 translocated *Shigella* proteins significantly inhibited yeast growth whereas none of the 20 non-translocated *Shigella* proteins tested had an effect on yeast growth [378].

Growth inhibition caused by expression of proteins confined to the bacterial cytoplasm appears to be rare [336]. However, when this does occur it does not appear to be due to a non-specific effect of overexpression of a bacterial protein but is more likely due to interference with conserved cellular processes [336]. For example, in a study by Campodonico *et al* (2005), expression of *Legionella* sterol desaturase inhibited yeast growth – this was likely due to the bacterial protein being highly similar to an essential yeast protein, ERG25, required for membrane synthesis [403].

Yeast growth assays are inexpensive and simple to set up and measure. Therefore, the approach is amenable to high-throughput screens and has been used to screen >1000 *Francisella* proteins for toxicity [378]. There are several methods that can be used to monitor yeast growth including plating serial dilutions of yeast culture onto inducing media [157] [414], measuring the optical density of liquid cultures [378] or using dyes to measure cellular respiration [414]. In this study, yeast growth was monitored by plating serial dilutions of saturated culture onto solid media and comparing the growth of yeast on inducing and non-inducing media.

Differential yeast growth is the basis of many assays including overexpression screens and pathogenic genetic arrays (see Chapter 6). It has also been used to screen drugs/identify drug targets [415] and screen novel small molecule inhibitors of type-III secretion and T3SS effectors [415]. Inhibitors of type-III secretion can be identified by applying small molecules to yeast (that are expressing an effector that causes growth retardation): if growth is restored, the small molecule is likely to inhibit the effector (and not damage the eukaryotic cell itself). This screening technique was recently used to identify six compounds (from an initial 56,000) that inhibited the *P. aeruginosa* effector ExoS [415].

5.1.3. Localisation of effectors in yeast gives clues as to effector function

Many eukaryotic cell processes and components are highly conserved between yeast and mammalian cells [416] – therefore it is predicted that effector targeting will also be conserved when effectors are expressed *de novo* in yeast [336]. There is mounting evidence

to suggest that localisation of effectors in yeast is representative of effector localisation during the course of an infection. For example, effectors that localise to the actin cytoskeleton [405], nucleus [157], plasma membrane [414] and mitochondria [359] in mammalian cells also localise to these compartments within yeast [417]. Therefore, localisation of effectors within yeast cells can give valuable clues as to effector targeting within host cells. However, localisation is not as specific as growth assays – Siggers *et al* observed that a small number of bacterial proteins localised to specific yeast cell compartments even though the proteins do not normally contact host cells [336].

Visualisation of effector proteins in fixed/live yeast has been achieved using indirect immunofluorescence and fluorescent tags/fusions (e.g. GFP) [418]. This study uses indirect immunofluorescence of fixed yeast cells to determine the localisation of selected effector-V5 fusion proteins. Fluorescein isothiocyanate (FITC)-conjugated anti-V5 antibodies (Invitrogen, UK) were used to stain effector-V5 fusions to determine the subcellular localisation of the effectors. FITC is a fluorescent molecule (fluoresces at 530 nm) often used for fluorescence microscopy [419]. In addition to fluorescent antibodies, yeast reporter strains that constitutively express monomeric red fluorescent protein (mRFP) fusion proteins were used to help determine the localisation of effectors in the yeast cell. Monomeric RFP fluoresces at 605 nm; therefore fluorescence of RFP and FITC can be easily distinguished [420]. There are many yeast strains available that express RFP-fusions that localise to specific subcellular compartments/structures e.g. nucleolus, Golgi apparatus, lipid particles etc. [421]. This study makes use of four reporter strains (outlined in table 5.5) in which the RFP-reporter protein localises to the actin cytoskeleton, endoplasmic reticulum (ER) membrane, spindle pole body or microtubule cytoskeleton. These reporter strains were

chosen as they would allow visualisation of cell components/processes e.g. cytoskeleton, secretory pathway, that are often targeted by T3SS effector proteins.

5.1.4. Morphology of yeast cells can give clues as to effector function

Observing the overall morphology of the yeast cell, budding patterns, organelles and cytoskeleton using differential interference contrast (DIC) microscopy can also give clues as to effector function [405]. For example, vacuole fragmentation in cells expressing the *P. aeruginosa* effector, ExoU, led investigators to believe that the effector disrupted vacuolar membranes – they subsequently demonstrated that ExoU acted as a phospholipase [410]. Also, Rodriguez-Escudero *et al* found that expression of EPEC effector Map caused the formation of large unbudded yeast cells - this pointed to depolarisation of the actin cytoskeleton [405]. The effector was subsequently shown to mimic activated Cdc42, a GTPase involved in regulating the actin cytoskeleton. Another significant morphological change that may be observed is filamentous yeast growth. This indicates perturbation of the filamentous growth pathway – a MAPK pathway also controlled by Cdc42 [160] [422]. The morphology of live yeast cells expressing selected effector proteins was observed using DIC microscopy.

Yeast chitin localisation can also provide insight into effector function. Chitin is a linear polymer found in the yeast cell wall and is composed of β-1,4-linked N-acetyl glucosamine (GlcNAc) [423]. Although chitin is a minor constituent of the cell wall, a high density of chitin is found at the bud neck and septum where it provides stretching resistance during

budding to ensure cell integrity [423]. Deposition at these sites is tightly controlled (both temporally and spatially) by the cell according to cell cycle and site of bud emergence [423]. Chitin present in bud necks and scars can be stained using calcofluor white (a fluorophore that binds specifically to chitin) [424]. The location/morphology of bud necks/scars can be used as reporters of effector function i.e. abnormalities may indicate that the effector disrupts MAPK signaling pathways, the secretory pathway or disrupts the cytoskeleton – all of which are essential for chitin deposition. In this study, the localisation of chitin within the cell wall of live yeast expressing selected effectors was investigated.

5.1.5. Truncation of NleG8-2

NleG8-2 is 216 amino acids in length. A RPS-BLAST search using the conserved domain database shows that residues 18-206 of NleG8-2 correspond (E-value = 5 x 10^{-50}) to DUF1076 (pfam06416) – a domain/family comprising several bacterial proteins exclusive to *E. coli* and *Salmonella*, of unknown function. To localise the activity within the protein, truncated versions of NleG8-2 were constructed. DNA primers were designed to amplify 150, 360 and 618 nucleotides of the NleG8-2 gene so that expression would result in expression of 1 – 50, 1 – 120 and 1 – 206 amino acids of NleG8-2 respectively. The effect on yeast growth inhibition was then tested. Rodriguez-Escudero *et al* used a similar approach to study the contribution of the C-terminus of Map to Map-induced toxicity [405]. They showed that the 15 C-terminal amino acids of Map were involved in Map-mediated inhibition of yeast growth [405].

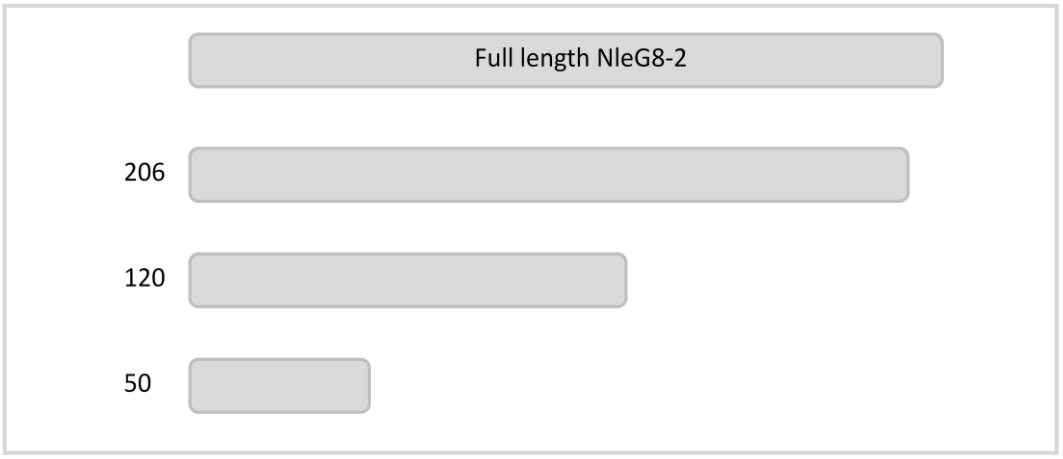

Figure 5.1: Truncated NleG8-2 proteins expressed in yeast.

5.1.6. Targeting residues of EspM2 and NleG8-2 for mutagenesis

Multiple alignments of novel effector proteins and their homologues show that there are conserved amino acid residues and motifs in a number of effector families. It was speculated that these conserved residues may be important for effector function and that substitution of the residues with alanine may disrupt effector function and attenuate effector toxicity. The importance of three conserved residues of EspM2 was previously investigated using a mammalian cell model (Chapter 4). This study concentrates on the highly conserved WxxxE motif found in IpgB-like effectors [159] [388] and four residues that are conserved in the NleG family of effectors.

All effectors of the IpgB family of effectors contain an invariant WxxxE motif [159] (see figure 5.2). Secondary structural analysis by Alto *et al* suggests that these effectors have a common protein fold and that each effector is composed of 6 to 8 helices [159]. Site-directed mutagenesis was used to mutate Trp70 and Glu74 to alanine. The effect of substitution on effector toxicity was assayed using the yeast growth assay.

Within NleG family members there are four conserved residues: Cys136, Asp162, His176 and Cys194 (see figure 5.3). Significantly, these residues are often found in catalytic active sites [425]. This has led to speculation that NleG T3SS effectors may function enzymatically [2]. Site-directed mutagenesis was used to mutate each residue (individually) to alanine. Effector toxicity was then investigated using the yeast growth assay. The localisation of mutated effectors in yeast was also investigated.

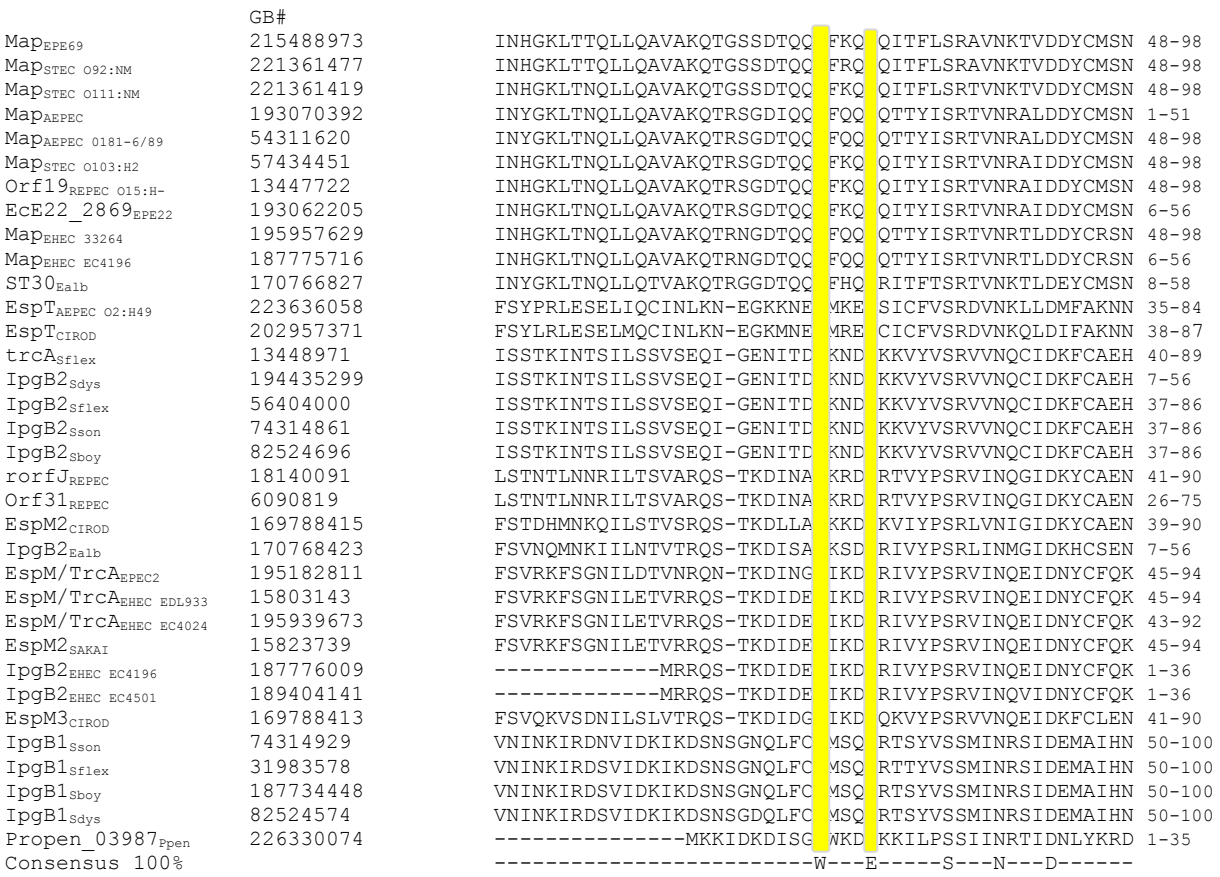

Figure 5.2: Alignment of IpgB family members. Homologues of EHEC O157:H7 Sakai EspM2 were identified by BLASTP. Conserved amino acids targeted for mutagenesis are highlighted in yellow. GenBank ID# is shown. Strain/species names are shown: CIROD (*C. rodentium*), REPEC (rabbit EPEC), Sson (*S. sonnei*), Sflex (*S. flexneri*), Sboy (*S. boydii*), Sdys (*S. dysenteriae*), Ppen (*Proteus penneri*), AEPEC (atypical EPEC E110019), EPEC2 (EPEC B171), EPE22 (EPEC22), EPE69 (EPEC 69), Ealb (*E. albertii*).

Figure 5.3: Alignment of NleG homologues. Conserved amino acid residues targeted for mutagenesis are highlighted in yellow. Figure adapted and used with permission from Tobe *et al* (2006) [2]. Homologues of *Citrobacter rodentium* NleG were identified using TBLASTN. Species/strain names are shown: CIROD (*C. rodentium*), EPE22 (EPEC E22), SAKAI (EHEC O157:H7 Sakai), EPE69 (EPEC 69), EPEC2 (EPEC B171), AEPEC (atypical EPEC E110019), SBONG (*S. bongori*).

5.2. Methods

5.2.1. DNA Cloning

PCR

NleG8-2 was amplified by PCR to contain no stop codon so that expression of a V5 and 6xHis (C-terminal) fusion was possible. Reaction mix and cycle used are shown in chapter 2. Primers used are shown in table 5.1.

PCR for truncated versions of ECs3486 NleG8-2

The reaction mix consisted of: 1 µl genomic DNA template (EHEC O157:H7 Sakai), 0.5 µl ExTaq DNA polymerase (Takara), 4 µl dNTP mix, 5 µl 5x ExTaq buffer (Takara), 10 µl forward primer (2 µM), 10 µl reverse primer (2 µM) and 19.5 µl dH_2O.

The reaction cycle was as follows:

95 °C 2 minutes
95 °C 30 seconds
Annealing temperature (50 – 60 °C) 30 seconds  Cycle x 30
72 °C 2 minutes
72 °C 5 minutes
4 °C until use

5.2.2. Site directed mutagenesis

Primer design

Mutagenic primers were designed using the primer design guidelines issued by Stratagene or by using the Quikchange Primer Design Program available at www.stratagene.com. Mutagenic primers used in this study are shown in table 5.1.

Mutant strand synthesis

Template plasmid DNA was obtained from a dam^+ *E. coli* strain to ensure selection for mutated plasmids following treatment with *Dpn* I.

EspM2 mutagenesis

The reaction mixture consisted of 5 µl of 10x reaction buffer, 5-50 ng dsDNA template (pENTR-SD-3485ns), 125 ng each mutagenic primer, 1 µl dNTP mix, 1 µl PfuUltra DNA polymerase (2.5 U/µl) and 50 µl dH_2O. The following reaction cycle was used:

95°C	30 seconds	
95°C	30 seconds	
55°C	1 minute	Cycle x 18
68°C	3 minutes	
4°C	until use	

NleG8-2 mutagenesis (performed by R. Younis)

The reaction mixture consisted of 3 µl 25 mM $MgSO_4$, 5 µl 2 mM dNTP, 125 ng each mutagenic primer, 5 – 50 ng dsDNA template (pENTR-SD-3486ns), 1 µl (1 U/µl) KOD hot start polymerase (Merck, USA), 5 µl 10x KOD reaction buffer, 31 µl H_2O and 1 µl DMSO.

Reaction cycle:

94°C 2 minutes
94°C 15 seconds
60°C 30 seconds
72°C 1 minute
72°C 5 minutes

Cycle x 18

Dpn I digestion

10 U Dpn I restriction enzyme was added to each reaction mix after amplification. The mixture was mixed by pipetting and then incubated for 1 hour at 37°C to digest the nonmutated, parental DNA. *Dpn* I treated DNA was then transformed into chemocompetent *E. coli* (strain XL-1 Blue) as outlined in chapter 2. Mutagenesis was confirmed by DNA sequencing as described in chapter 2.

Primer	Sequence (5'-3')	Mutation introduced
ECs3486.gwF	CACCATGCCAGTCATATTAAAT	N/A
ECs3486revNS	AATACTGTTTTGTTGAAGTGGG	N/A
Truncation primers		
ECs3486_50aaR	ACCATCCATAAACTGGATATAATG	N/A
ECs3486_120aaR	AAATGCATTCGAATTTATTCG	N/A
ECs3486_206aaR	TAATGCGTAAAAACATTG	N/A
Mutagenic primers		
NleG8-2		
New_QC3486_C136A	ACTTTTCCTGTACTGAGGAACATCTTAATGCTCCGATAACGCTACA	NleG8-2 C136A
New_QC3486_C136Ar	TGTAGCGTTATCGGAGCATTAAGATGTTCCTCAGTACAGGAAAAGT	NleG8-2 C136A
D162A_f_new	GAAATTCAGAAATATGTGCATTATATGCTCAGGAAGCATTGACTGA	NleG8-2 D162A
D162A_r_new	TCAGTCAATGCTTCCTGAGCATATAATGCACATATTTCTGAATTTC	NleG8-2 D162A
H176A_f_new	GCGTAACGCTCTCGCCCCCCTCAGCCGT	NleG8-2 H176A
H176A_r_new	ACGGCTGAGGGGGGCGAGAGCGTTACGC	NleG8-2 H176A
C194A_f_new	TTGCTCCAGAAATGATTATAAGCAAAGACAAGGCTCATTTTAATATAACA -AAACAATGTTTTT	NleG8-2 C194A
C194A_f_new	AAAAACATTGTTTTGTTATATTAAAATGAGCCTTGTCTTTGCTTATAATC -ATTTCTGGAGCAA	NleG8-2 C194A
EspM2		
3485.5afW	CAGAGTACTAAAGATATTGATGAG**GCG**ATAAAAGA	EspM2 W70A
3485.5arW	CGTTCATCTTTTATCGCCTCATCAATATCTTTAGTACTCTG	EspM2 W70A
3485.5bfE	GAGTGGATAAAAGAT**GCA**CGGATAGTATATCCCTC	EspM2 E74A
3485.5brE	GAGGGATATACTATGCCTGGATCTTTTATCCACTC	EspM2 E74A
Sequencing primer		
pDEST52f	CCTCTATACTTTAACGTC	N/A

Table 5.1: Primers used in this study.

5.2.3. TOPO cloning

Purified PCR product (NleG8-2) was cloned into pENTR-SD/D-TOPO (Invitrogen, UK) according to procedures outlined in chapter 2. Entry vectors produced earlier in the study (see chapter 3) were also used. Entry vectors used are listed in table 5.2.

5.2.4. LR recombination

In this study, fifteen effectors were expressed within yeast from the pYES-DEST52 Gateway vector (Invitrogen, UK). Expression vectors used in this study are shown in table 5.2. pYES DEST52 contains a URA3 auxotrophic marker for selection of yeast transformants and a *GAL1* promoter for high-level, galactose-inducible protein expression in *S. cerevisiae*. This promoter is induced in the presence of galactose [399], repressed in the presence of glucose [398] and not affected by raffinose. The carbon source added to the SC-Ura media therefore allows control over effector gene expression. The vector also has a 2μ origin for high copy replication in yeast and a pUC origin for high copy replication in *E. coli*. The vector contains a V5 epitope and 6xHis tag; recombination between entry vector and pYES-DEST52 allows expression of an effector- V5/6xHis fusion protein that can be easily detected using anti-V5 [400] or anti-His [401] antibodies (see figure 5.4).

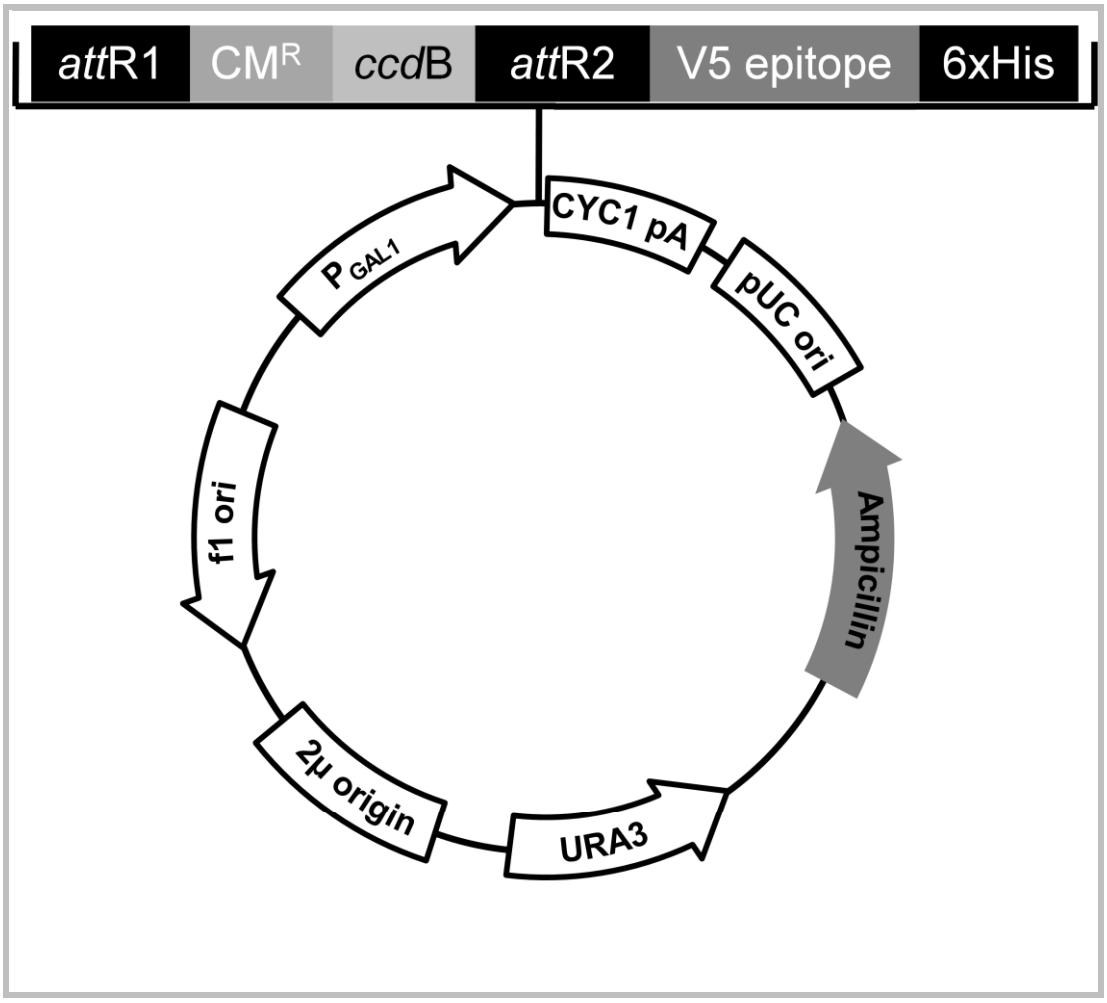

Figure 5.4: pYES DEST52.
Gateway destination vector for expression of V5/6x His fusion proteins in yeast.

5.2.5. Plasmid constructs

Effector	Sakai ID	Entry vector	Source	Destination vector	Source
EspY4	ECs4653	pSB10.22B	S. Beatson, this study	pYES DEST52 4653	This study
Map	ECs4562	SME14a	This study	pYES DEST52 Map	This study
NleB1	ECs3857	pENTR-SD-3857	This study	pYES DEST52 3857	This study
NleG8-2	ECs3486	pENTR-SD-3486nsfp3	This study	pYES DEST52 3486	This study
EspM2	ECs3485	pENTR-SD-3485ns	R.Younis, this study	pYES DEST52 3485	This study
EspJ	ECs2714	pENTR-SD-2714	This study	pYES DEST52 2714	This study
NleG2-3	ECs2156	pENTR-SD-2156	This study	pYES DEST52 2156	This study
NleG6-2	ECs2155	pSB3.31	S. Beatson, this study	pYES DEST52 2155	This study
EspM1	ECs1825	pENTR-SD-1825	This study	pYES DEST52 1825	This study
NleF	ECs1815	pSB10.10B	S. Beatson, this study	pYES DEST52 1815	This study
NleH	ECs1814	SME46b	This study	pYES DEST52 1814	This study
EspO1-1	ECs1567	pENTR-SD-1567	This study	pYES DEST52 1567	This study
EspX7	ECs1560	pENTR-SD-1560	This study	pYES DEST52 1560	This study
NleD	ECs0850	pR1.27a	This study	pYES DEST52 0850	This study
EspY1	ECs0061	pSB3.47a	S. Beatson, this study	pYES DEST52 0061	This study
Control vector (Gateway cassette removed)					
N/A	N/A	N/A	N/A	pYES DEST52e	This study

Table 5.2: Entry and destination vectors used in this study.

Effector	Entry vector	Source	Destination vector	Source
Mutated EspM2				
$EspM2_{W70A}$	pENTR-SD 3485 5a	This study	pDEST52 3485 5a	This study
$EspM2_{E74A}$	pENTR-SD 3485 5b	This study	pDEST52 3485 5bE	This study
Truncated NleG8-2				
$NleG8\text{-}2_{1\text{-}50}$	pENTR-SD-3486 50	This study	pDEST52 3486 50	This study
$NleG8\text{-}2_{1\text{-}120}$	pENTR-SD-3486 120	This study	pDEST52 3486 120	This study
$NleG8\text{-}2_{1\text{-}206}$	pENTR-SD-3486 206	This study	pDEST52 3486 206	This study
Mutated NleG8-2				
$NleG8\text{-}2_{C136A}$	pENTR-SD-C136A	R. Younis, this study	pDEST52 C136A2	This study
$NleG8\text{-}2_{D162A}$	pENTR-SD-D162A	R. Younis, this study	pDEST52 D162A	This study
$NleG8\text{-}2_{H176A}$	pENTR-SD-H176A	R. Younis, this study	pDEST52 H176A2	This study
$NleG8\text{-}2_{C194A}$	pENTR-SD-C194A	R. Younis, this study	pDEST52 C194A1	This study

Table 5.3: Vectors containing mutated/truncated effector genes used in this study.

5.2.6. High-efficiency Transformation of Yeast

Yeast strains used in this study are outlined in table 5.4

Strain	Genotype	Source
FY833	*MATα his3Δ ura3Δleu2Δ lys2Δ trp1Δ GAL2*	S. Dove, University of Birmingham
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	S. Dove, University of Birmingham

Table 5.4: Yeast strains used in this study.

Reporter yeast strains used in this study are outlined in table 5.5

Strain BY4741	RFP Localisation	Source
RFP-Sac6	Actin. Sac6p is an actin bundling protein homologous to fimbrin [426].	S. Dove, University of Birmingham
RFP-Sec66	ER membrane. Sec66 is a component of the Sec63p complex required for translocation of presecretory proteins into the ER [427].	S. Dove, University of Birmingham
RFP-Kip3	Microtubule. Kip3p is a kinesin microtubule motor required for normal spindle assembly and microtubule-cortical interactions [428].	S. Dove, University of Birmingham
RFP-Spc42	Spindle pole. Spc42 is a major component of the spindle pole body anchored within the nuclear membrane [429].	S. Dove, University of Birmingham

Table 5.5: Yeast reporter strains used in this study.

Buffers for yeast transformation

Polyethylene glycol (PEG; 50% w/v) was made by dissolving polyethylene glycol (MW 3350; Sigma) in dH_2O to make a 50% w/v solution. The solution was sterilised by filtration through a 0.22 μm filter (Millipore, UK).

Single-stranded carrier DNA (2 mg/ml) consisted of 200 mg of high-molecular weight DNA (Sigma D1626) dissolved in 100 ml of TE buffer (pH 8.0). The solution was mixed vigorously with a magnetic stirrer at 4°C o/n. Aliquots were stored at -20°C until needed.

Transformation of Yeast

5 ml of YPD or SC media was inoculated with the desired strain and incubated with shaking (200rpm) overnight at 30°C. Cells were pelleted at 3000g for 5 minutes and the supernatant was removed. Cells were resuspended in 2.5 ml of sterile H_2O and centrifuged again. H_2O was removed and cells were resuspended in 1.0 ml of 100 mM lithium acetate (LiAc). Cells were pelleted at 13000g for 5 seconds and the LiAc was removed. Cells were resuspended in 400 μl of 100 mM LiAc (cell density was approximately 2 x 10^9 cells/ml). 50μl samples of the cell suspension were added to sterile microfuge tubes. The cells were again pelleted by centrifugation at 13000rpm for 5 seconds and the LiAC was removed. The 'transformation mix' was then added (in the order listed): 240 μl of PEG (50% w/v), 36 μl of 1.0 M LiAc, 25 μl of boiled and chilled single-stranded carrier DNA (2.0 mg/ml), 50 μl of sterile H_2O and plasmid DNA (0.1-10 μg). The microfuge tube was then vortexed vigorously for approximately 1 minute until the cell pellet was completely mixed. Cells were incubated for 30 minutes at 30°C then heat shocked for 20-25 minutes at 42°C. Cells

were pelleted at 6000rpm using a microfuge and the transformation mix removed. Cell pellets were resuspended in 400 µl of sterile H_2O. 200µl aliquots were plated onto selective SC agar plates.

5.2.7. Yeast immunofluorescence

Based on protocol described by Pringle *et al* [430].

Solutions for yeast immunofluorescence

Phosphate buffer consisted of 40 mM KPO_4 (pH 6.5) plus 500 µM $MgCl_2$ in dH_2O. Sorbitol-phosphate buffer consisted of 40 mM KPO_4 (pH 6.5) plus 500 µM $MgCl_2$ plus 1.2 M sorbitol in dH_2O. Blocking solution consisted of 0.5% (w/v) BSA in 1 x phosphate buffered saline (PBS) (pH 7.4).

Zymolyase solution contained 10 mg zymolyase, a β-1,3-glucanase enzymatic preparation (AMS Biotechnology, UK), dissolved in sorbitol-containing phosphate buffer. The solution was mixed by vortexing frequently for 1 hour (incubated on ice between vortexing). The solution was then centrifuged at 13000 rpm for 10 minutes then frozen in liquid nitrogen and stored at -80°C.

Polylysine solution. High molecular weight polylysine (>300,000 MW; Sigma) was dissolved as a 1% stock solution in dH_2O and frozen in liquid nitrogen (stored at -80°C).

Slide preparation:

All solutions were spun at 13000 rpm for 10 minutes immediately before use to reduce dust and other particle contamination of the slides. The wells of Teflon-faced slides (Polysciences) were coated with 20 µl of a 0.1% polylysine solution and slides were incubated in a moist chamber at room temperature for 10 minutes. The wells were then washed five times with 20 µl dH_2O, dried and stored in a dust-free environment.

2 ml SC-Ura + glucose was inoculated with desired strain and incubated with shaking (200 rpm) overnight at 30°C. The overnight culture was then diluted 1:200 into fresh SC-Ura + glucose and grown at 30°C with shaking for 3 hours. Cells were then washed twice in dH_2O and resuspended in 5 ml SC-Ura + galactose. Cells were incubated for 4 hours at 30°C with shaking. 3.4 ml of 10% formalin was added and the cells incubated for a further 10 minutes at 30°C with shaking. Cells were centrifuged at 2000 rpm for 3 minutes and resuspended in 0.6 ml of phosphate buffer and 0.4 ml 10% formalin. Cells were incubated for 1 hour at 30°C. Cells were washed twice in the phosphate buffer (no formalin) and then again (gently) in the sorbitol phosphate buffer. Cells were resuspended in 0.5 ml sorbitol buffer and stored overnight at 4°C. Cells were treated for 15 minutes with 30 µl of 10 mg/ml Zymolyase (100T) at 37°C. Cells were washed once in sorbitol buffer and resuspended (very gently using low vortex speeds) in 200µl sorbitol buffer. Cells were placed on ice. 20 µl of the cell suspension was spotted onto each well of the prepared Teflon-faced slides and incubated at room temperature for 10 minutes. Liquid was aspirated from the wells and the slide was immersed in cold methanol for 6 minutes and then cold acetone for 30 seconds. Excess acetone was immediately removed from the slide and the slide allowed to dry quickly on a

warm surface. Cells were blocked by adding 20 µl blocking solution to each well. Slides were incubated in a moist chamber at room temperature for 1 hour. Blocking solution was then removed and 20 µl blocking solution containing FITC-conjugated anti-V5 antibody (Invitrogen) 1:200 was added to each well. Slides were incubated for 1 hour in a moist chamber at room temperature. The cells were then washed 4 times for 10 minutes with 20 µl/well of fresh blocking solution. Excess blocking solution was removed from the wells and 5 µl citifluor AF1 (Citifluor, UK) an anti-bleaching mounting solution was added. A square coverslip was then placed over 4 wells (no bubbles). Excess citifluor was removed and the coverslip sealed in place with nail polish. The slides were allowed to dry and were then either viewed immediately or stored at -20°C until use.

Fluorescence microscopy

Fixed and stained cells were viewed using a Nikon E600 microscope (with a Nikon 100x objective) fitted with an XF100-3 filter cube (Omega Optical, US). Images were captured with an ORCA digital camera (Hamamatsu, US). Images were processed using Simple PCI (Hamamatsu) software.

5.2.8. Calcofluor staining

Calcoflour (Sigma) was added to yeast cells growing in SC-Ura+glucose or SC-Ura+galactose (OD_{600} = 0.5) to a final concentration of 100 µg/ml. Cells were incubated for 5-10 minutes at 30°C, washed twice with dH_2O and observed with a DAPI-compatible filter set.

5.2.9. Yeast protein extraction

5ml SC-Ura+raffinose (2%) was inoculated with 10µl stationary phase FY833 (transformed with pDEST52-effector construct that had been grown in SC-Ura+glucose) and incubated overnight at 30°C 200rpm. 2.5ml of overnight culture (OD_{600} =1) was induced with 2% galactose for 2 hours 30°C 200rpm. 2.5 OD_{600} units of induced and non-induced cells were harvested by centrifugation 13000rpm 15 min. Supernatant was removed and pellets frozen at -80°C overnight. Pellets were resuspended in 100µl dH_2O. NaOH was added to a final concentration of 0.1M and the suspension vortexed to mix. The suspension was then incubated at room temperature for 5 min before being centrifuged at 13000rpm for 1 min. The supernatant was removed and the pellet resuspended in 50µl 2x SDS loading buffer. The proteins were boiled for 3 min and briefly centrifuged to remove cell wall debris. 6µl was then loaded onto a 12% SDS-PAGE gel for separation and analysis.

5.2.10. Western blotting

Protein was extracted from induced and non-induced yeast cells as described above. The proteins were separated by SDS-PAGE and electrophoretically transferred onto Hybond-P membranes as described in chapter 2. Membranes were probed with horse radish peroxidase (HRP) conjugated anti-V5 antibodies (at a dilution of 1:200) to detect effector-V5 fusions as described in chapter 2.

5.2.11. Growth retardation assay

Single colonies of transformed FY833 (carrying desired effectors in pYES DEST52) were used to inoculate 2ml SC-Ura+raffinose (2%) broth. These were then incubated at 30°C 200rpm overnight. The optical density OD_{600} of each culture was measured and normalised to OD_{600}=1. A 10-fold dilution series was prepared from the normalised cultures and 10µl of each dilution applied to both SC-Ura+glucose agar (non-inducing) and SC-Ura+galactose agar (inducing). Both plates were incubated at 30°C for 48 hours. Growth of yeast on inducing and non-inducing plates was then compared. To acquire images of plates, plates were placed onto a scanner and a piece of black card was placed on top to provide a dark background. Plates were then scanned and images were processed using Microsoft Powerpoint software.

5.3. Results

5.3.1. Confirmation of expression of effectors in yeast

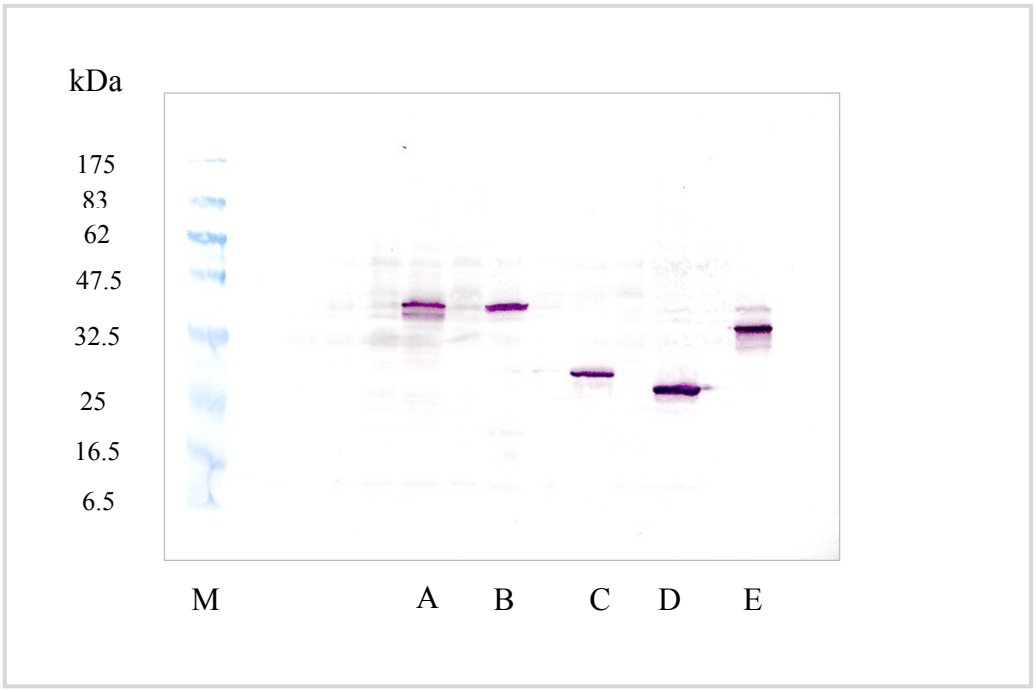

Figure 5.5: Western blot of yeast cell lysates from cells expressing (A) NleH1-2, (B) NleB1, (C) EspJ, (D) NleF, (E) EspY1. Proteins were visualised using HRP-conjugated anti-V5 antibody (Invitrogen). M = prestained protein marker (Sigma).

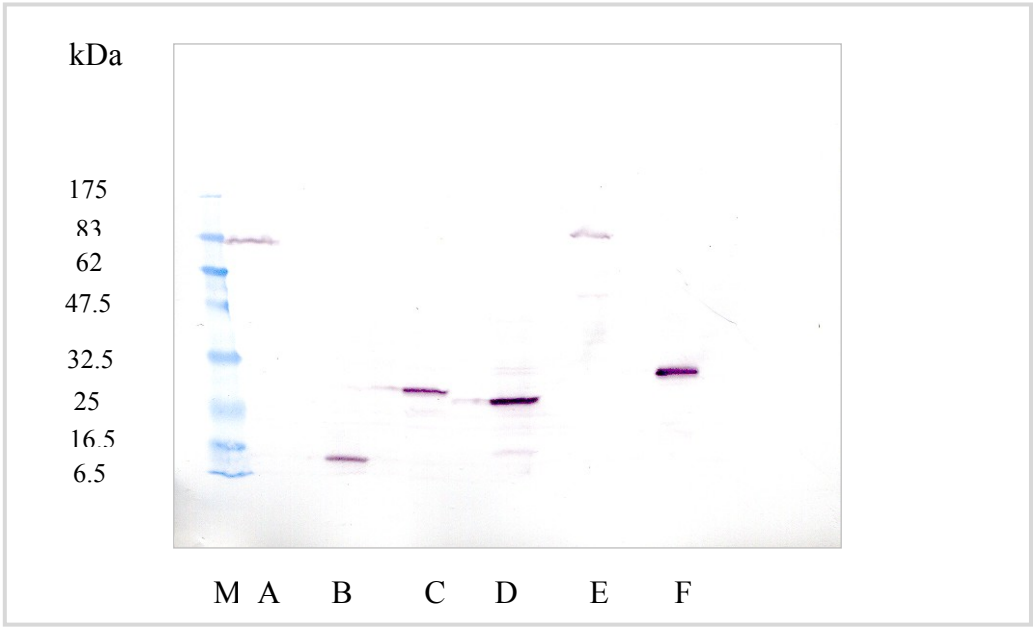

Figure 5.6: Western blot of yeast cell lysates from cells expressing (A) EspX7, (B) EspO1-1, (C) NleG6-2, (D) NleG2-3, (E) EspY4, (F) NleD. Proteins were visualised using HRP-conjugated anti-V5 antibody (Invitrogen). M = prestained protein marker (Sigma).

5.3.2. Yeast growth inhibition

Yeast strain FY833 was successfully transformed with Gateway destination vectors (pYES DEST52) carrying novel effector genes. Sixteen transformants were obtained. Expression of effector-V5 fusion proteins was controlled by the carbon source used in the growth media (galactose induced expression, glucose repressed expression). Growth of 10-fold serial dilutions of yeast culture on inducing and non-inducing media was compared. No growth inhibition was seen for yeast grown on non-inducing media i.e. yeast grew to comparable levels as yeast carrying empty vector pYES DEST52e. An exception to this was yeast carrying EspM2 which showed slight growth inhibition on non-inducing plates (figure 5.8). In contrast, severe growth inhibition was seen for yeast carrying EspM2 (ECs3485), NleG8-2 (ECs3486) and NleG2-3 (ECs2156) grown on inducing SC-Ura+galactose media. This shows that expression of these effectors is toxic to yeast.

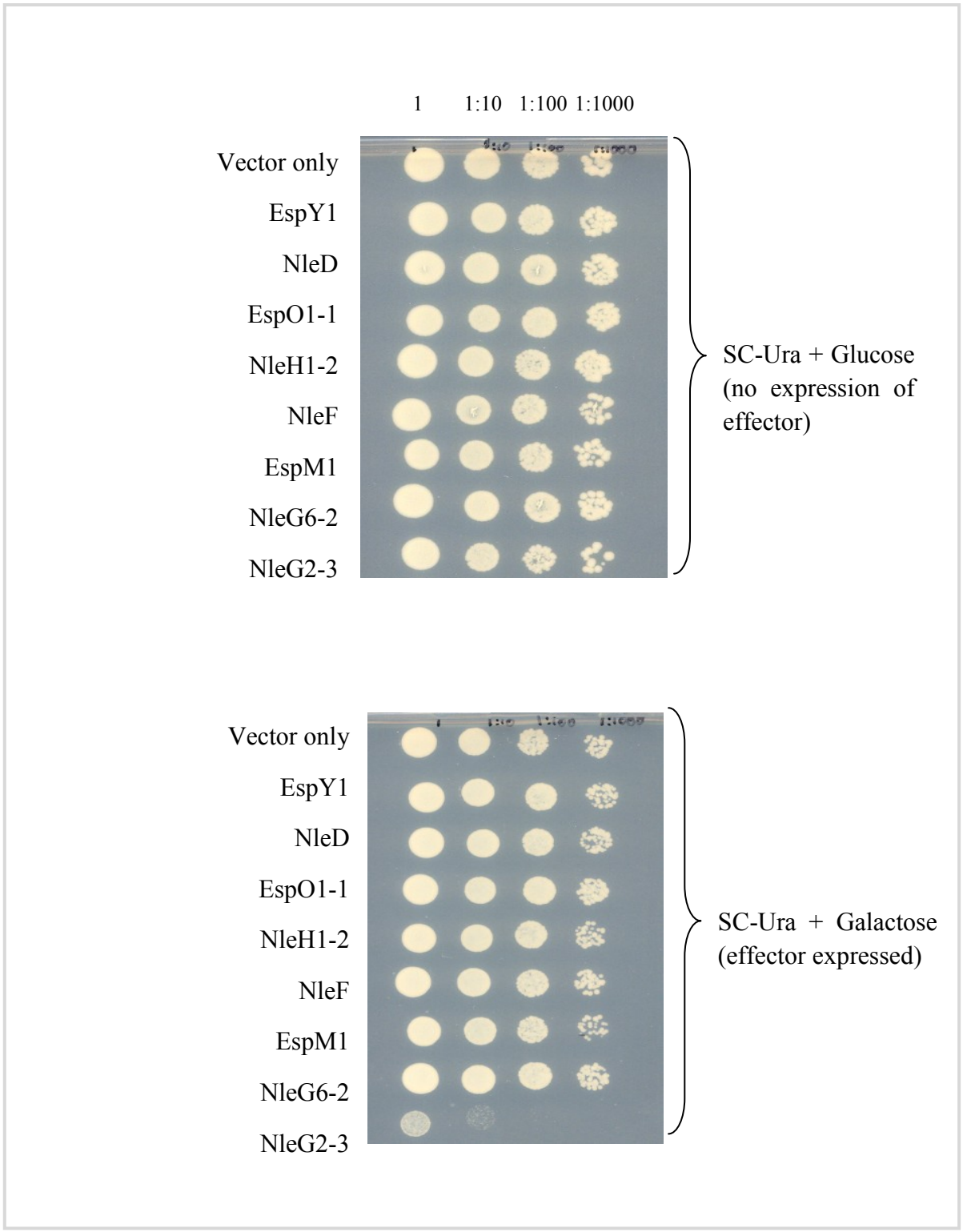

Figure 5.7: Yeast growth assay. 10-fold serial dilutions of saturated yeast culture (10 µl) were plated onto non-inducing (glucose) and inducing (galactose) SC-Ura media. Plates were incubated at 30°C for 48 hours. Yeast carrying pYES DEST52e vector were used as a negative control. Effectors carried by the yeast are indicated. Expression of NleG2-3 caused growth retardation on inducing media.

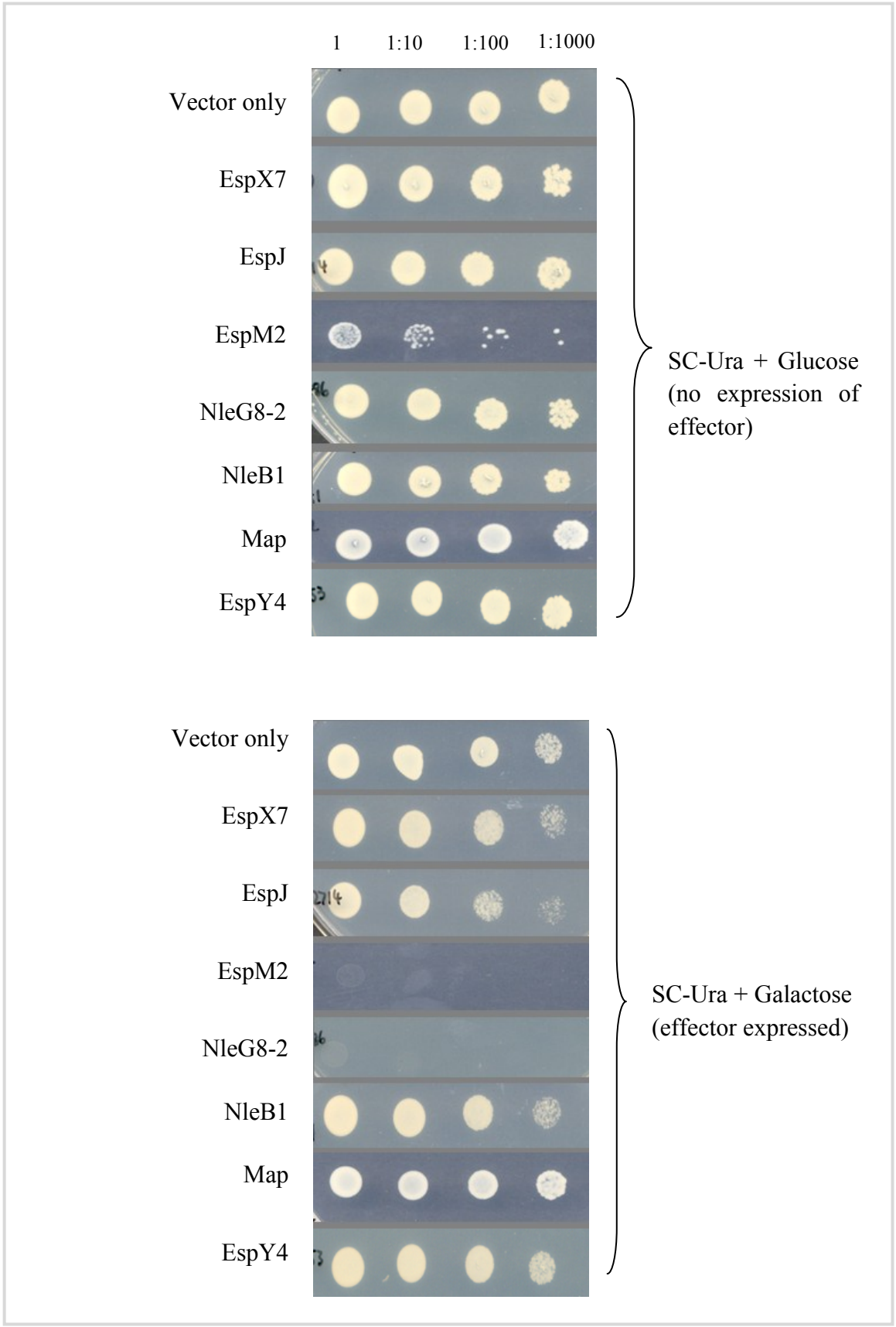

Figure 5.8: Yeast growth assay. 10-fold serial dilutions of saturated yeast culture (10 µl) were plated onto non-inducing (glucose) and inducing (galactose) SC-Ura media. Plates were incubated at 30°C for 48 hours. Yeast carrying pYES DEST52e vector were used as a negative control. Effectors carried by the yeast are indicated. Expression of EspM2 and NleG8-2 caused growth retardation on inducing media.

5.3.3. Amino acid substitution of the EspM2 WxxxE motif decreases the toxicity of EspM2

Yeast carrying EspM2 showed growth inhibition on both non-inducing and inducing media (figure 5.8), although growth inhibition was most severe on inducing media (no detectable growth). In contrast, yeast carrying mutated EspM2, in which the conserved tryptophan (W) and glutamic acid (E) residues of the WxxxE motif were substituted with alanine, did not show growth inhibition on non-inducing media (similar growth as the control). Yeast carrying $EspM2_{W70A}$ and $EspM2_{E74A}$ did show growth inhibition when grown on inducing media but inhibition was not as severe as that caused by expression of $EspM2_{wt}$ – growth inhibition was only visible at higher dilutions of plated culture (figure 5.9). Expression of mutated EspM2 was therefore not as toxic to yeast as expression of $EspM2_{wt}$.

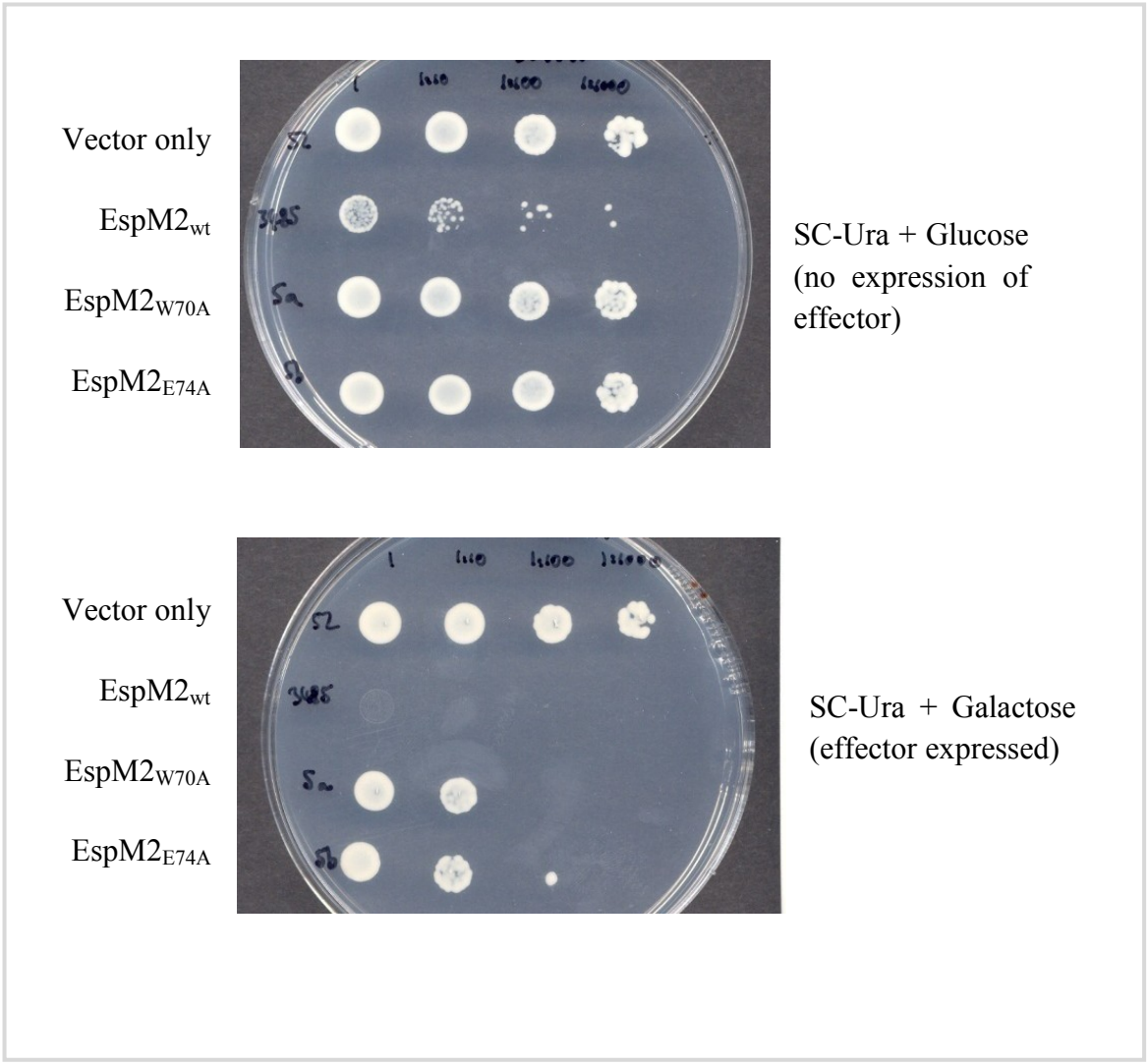

Figure 5.9: Mutant EspM2 yeast growth assay. 10-fold serial dilutions of saturated yeast culture (10 µl) were plated onto non-inducing (glucose) and inducing (galactose) SC-Ura media. Plates were incubated at 30°C for 48 hours. Yeast carrying pYES DEST52e vector were used as a negative control. $EspM2_{wt}$ causes growth retardation on both non-inducing and inducing plates. $EspM2_{W70A}$ and $EspM2_{E74A}$ cause less severe growth retardation than $EspM2_{wt}$ on both types of plate.

5.3.4. Truncation of NleG8-2

Expression of NleG8-2 was found to inhibit yeast growth (figure 5.8). In an attempt to determine sequence-activity relations for NleG8-2, truncated versions of NleG8-2 were tested using the yeast growth inhibition assay. Truncation of NleG8-2 to lengths of 1-50, 1-120 amino acids appeared to decrease toxicity of the effector in the yeast growth assay i.e. yeast carrying NleG8-$2_{1\text{-}50}$ and NleG8-$2_{1\text{-}120}$ showed similar levels of growth as the control when grown on inducing media. Expression of NleG8-$2_{1\text{-}206}$ caused growth inhibition similar to that caused by expression of wild type NleG8-2 (figure 5.10).

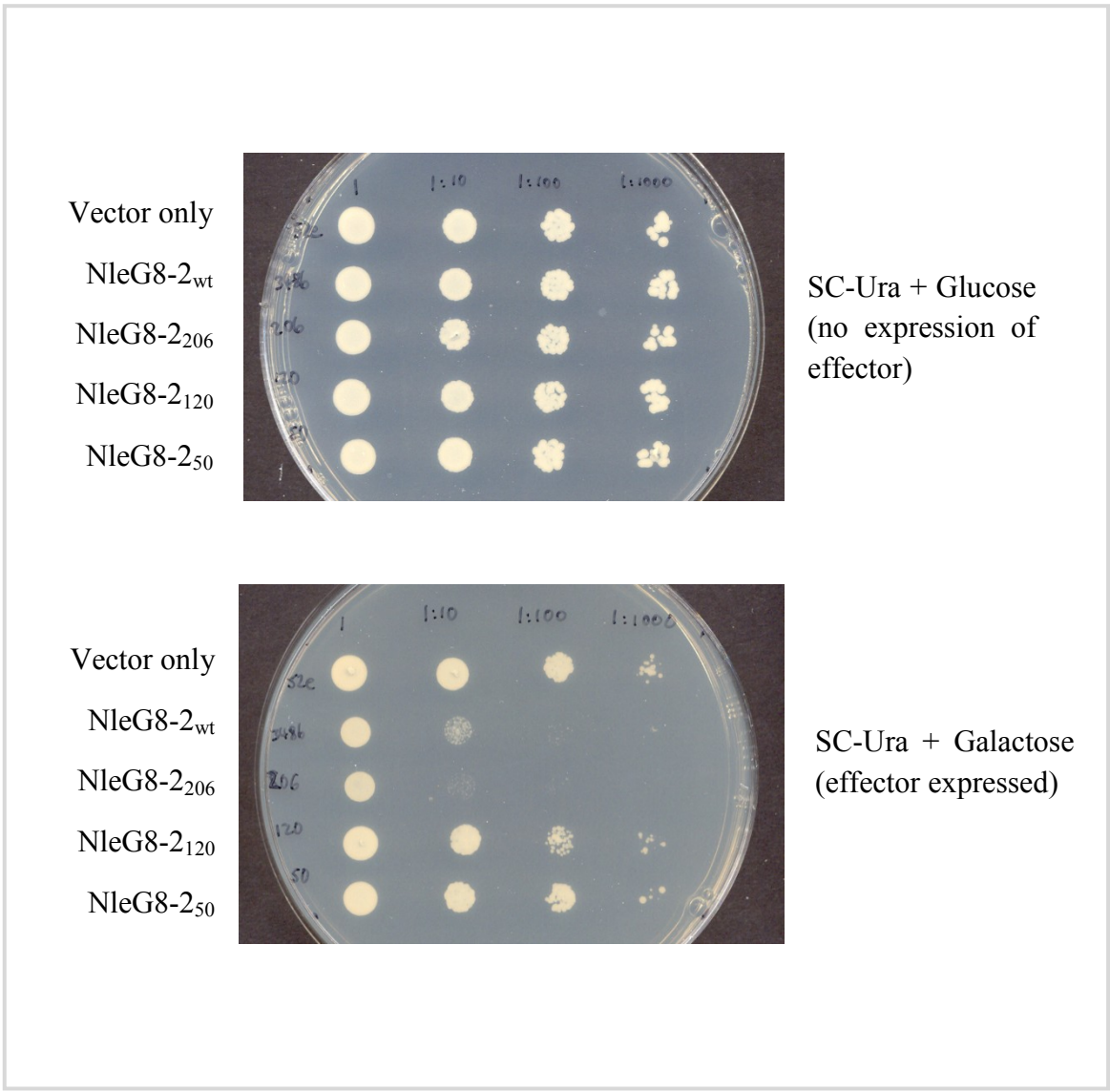

Figure 5.10: Truncated NleG8-2 yeast growth assay. 10-fold serial dilutions of saturated yeast culture (10 µl) were plated onto non-inducing (glucose) and inducing (galactose) SC-Ura media. Plates were incubated at 30°C for 48 hours. Yeast carrying pYES DEST52e vector were used as a negative control. Expression of NleG8-2_{wt} and NleG8-2_{206} caused growth retardation.

5.3.5. Mutant NleG8-2 growth assay

Further investigations into sequence-activity relations of NleG8-2 included yeast growth inhibition assays of yeast expressing mutated NleG8-2. Yeast carrying NleG8-2wt, NleG8-2_{C136A}, NleG8-2_{D162A}, NleG8-2_{H176A} and NleG8-2_{C194A} showed no growth inhibition on non-inducing plates (all showed similar growth to control). Growth inhibition was observed in yeast expressing NleG8-2_{wt} and NleG8-2_{C194A} on inducing plates – expression of NleG8-2_{C194A} caused similar growth inhibition as expression of NleG8-2_{wt}. No growth inhibition was observed in yeast expressing NleG8-2_{C136A}, NleG8-2_{D162A} or NleG8-2_{H176A}. Substitution of Cys136, Asp162 and His176 to alanine therefore decreased toxicity of NleG8-2 (figure 5.11). Western blotting confirmed that equal amounts of protein were expressed by all clones (figure 5.12). Therefore, differences in growth were not caused by differences in levels of protein expression.

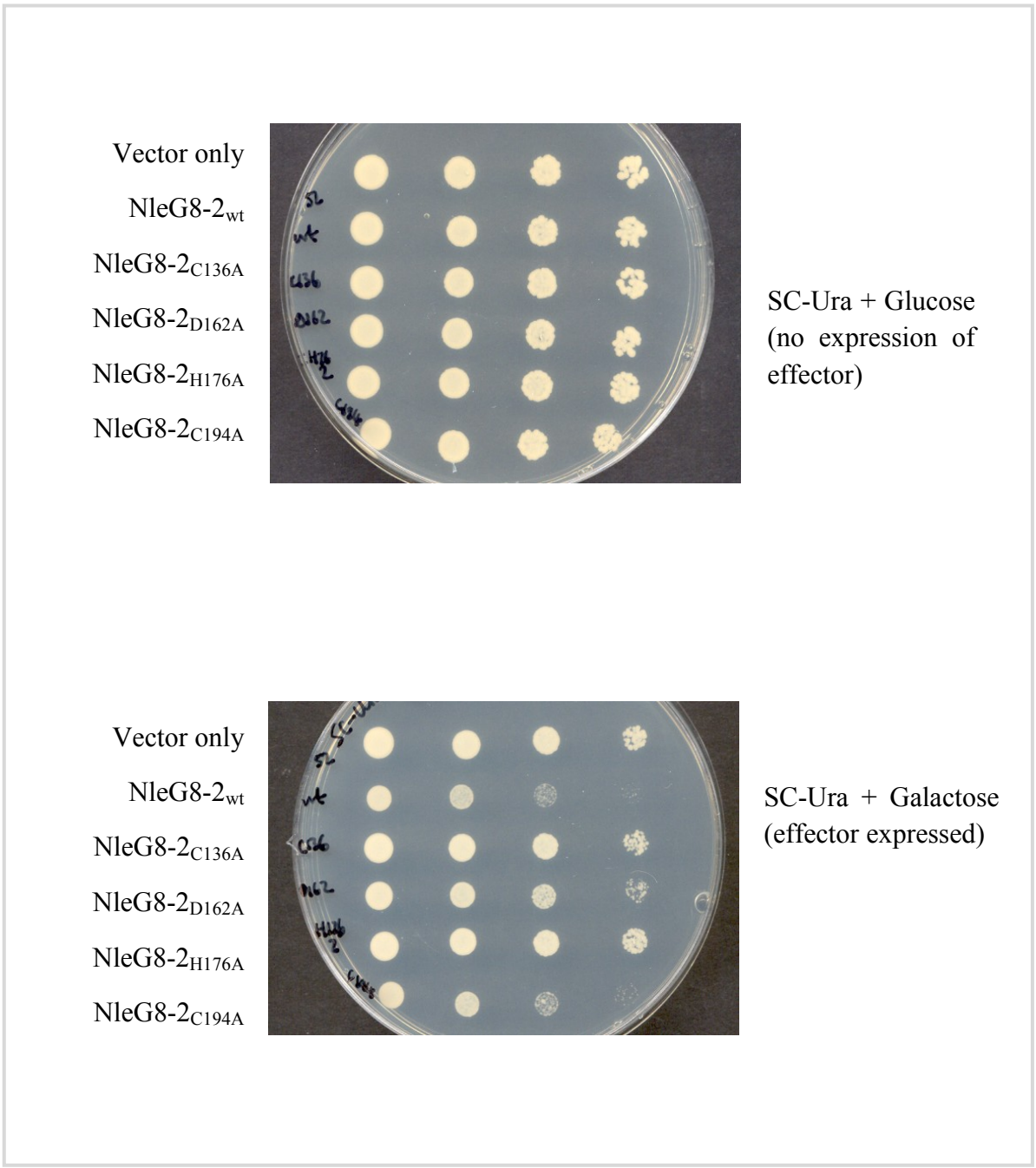

Figure 5.11: Mutant NleG8-2 yeast growth assay. 10-fold serial dilutions of saturated yeast culture (10 µl) were plated onto non-inducing (glucose) and inducing (galactose) SC-Ura media. Plates were incubated at 30°C for 48 hours. Yeast carrying pYES DEST52e vector were used as a negative control. Expression of NleG8-2_{wt} and NleG8-2_{C194A} caused growth retardation.

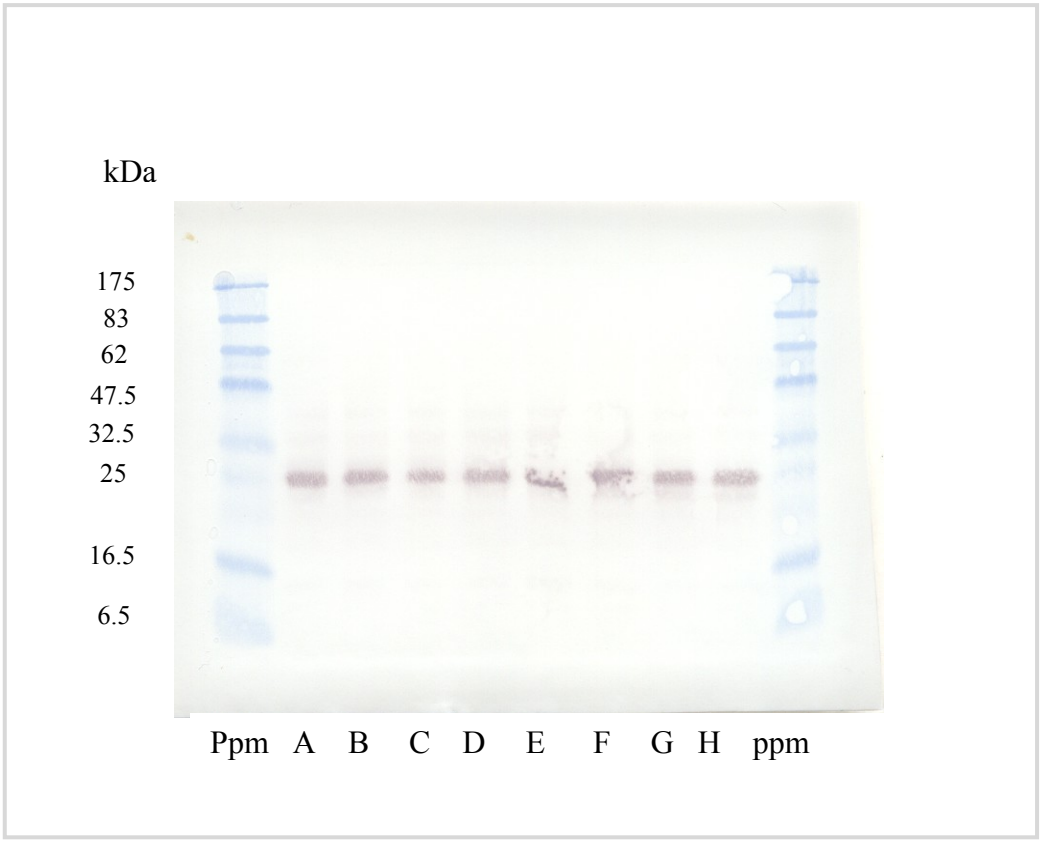

Figure 5.12: Western blot of NleG8-2. Proteins from yeast cell lysates of cells grown in SC-Ura+galactose media were separated by SDS-PAGE and analysed for NleG8-2 by western blot using HRP-conjugated anti-V5 antibody (Invitrogen). FY833 yeast strain expressing: (A) NleG8-2_{C136A}, (B) NleG8-2_{D162A}, (C) NleG8-2_{H176A}, (D) NleG8-2_{C194A}, (E) NleG8-2_{wt}. Yeast BY4741 reporter strains (F) Sac6, (G) Kip3, (H) Sec66 expressing NleG8-2_{wt}. Ppm – broad range prestained protein marker (Sigma), marker protein sizes are indicated (kDa).

5.3.6. Localisation of effectors in yeast

Yeast cells expressing V5-tagged effectors were fixed and stained with FITC conjugated anti-V5 antibody (Invitrogen). Images are shown in figure 5.13. Map-V5 was detected around the cell periphery in crescent-like structures whereas NleG8-2 was detected as punctate structures at the cell periphery. EspM2 appeared to localise to both the cell periphery (70% of cells) and throughout the cytoplasm (30% of cells). Tir showed strong staining of the yeast cell cytoplasm.

In an attempt to identify the subcellular localisation of NleG8-2, the effector was expressed in four separate yeast reporter strains. In these strains, RFP-tagged proteins; Kip3 (localises to microtubules), Spc42 (localises to the spindle pole body), Sec66 (localises to the ER membrane) and Sac6 (localises to actin) were used to identify cellular components. NleG8-2 did not co-localise with any of the RFP-tagged proteins and did not alter the localisation of these proteins (figures 5.14 and 5.15).

Mutation of conserved residues C136, D162, H176 and C194 did not appear to affect NleG8-2 localisation within yeast cells – the effector localised to punctate areas of the cell periphery (figure 5.16).

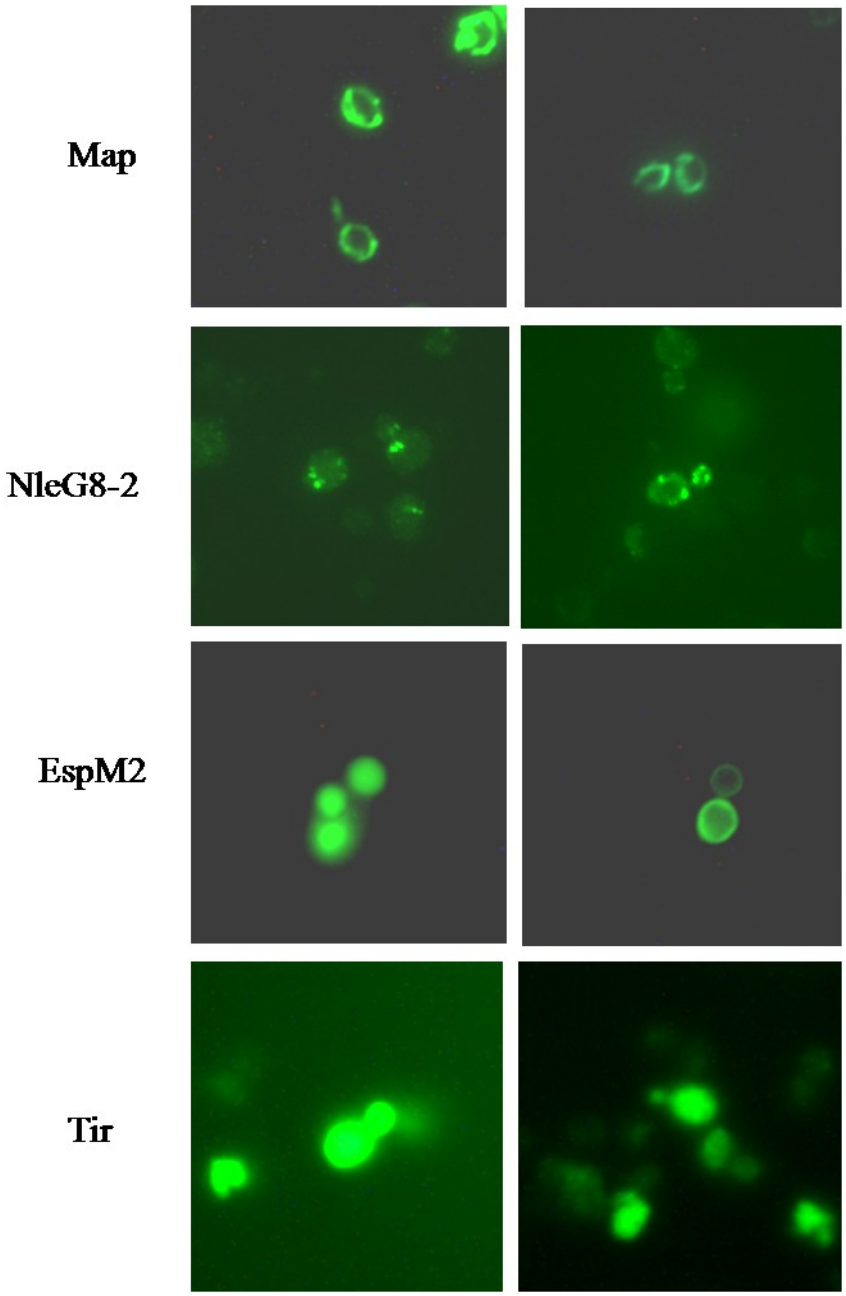

Figure 5.13: Localisation of effectors in yeast. Effector-V5 fusions were visualised in fixed yeast cells using FITC-conjugated anti-V5 antibody and fluorescence microscopy.

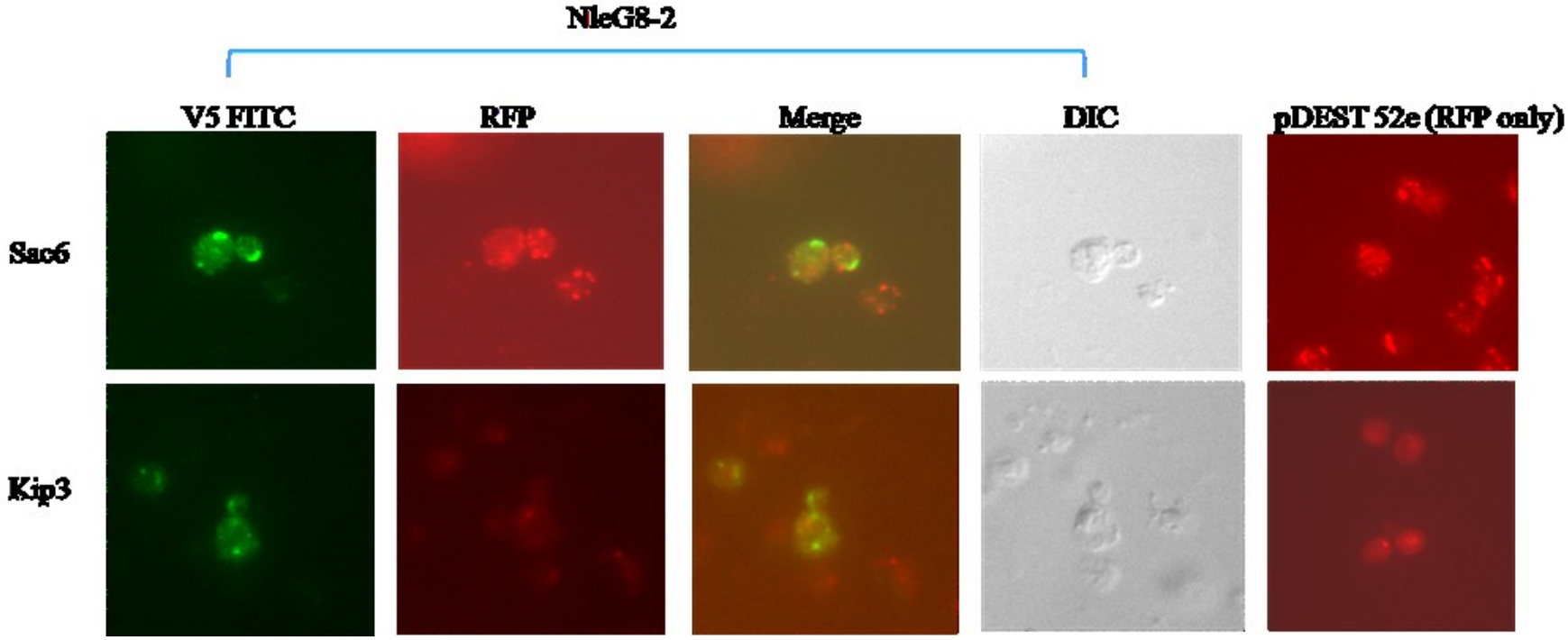

Figure 5.14: Localisation of NleG8-2 in yeast reporter strains. FITC-conjugated anti-V5 antibody was used to stain fixed yeast cells to show localisation of NleG8-2-V5 fusions (V5 FITC). RFP shows fluorescence of RFP-tagged protein constitutively expressed by reporter strain i.e. Sac6 (actin), Kip3 (microtubules). Merged image shows both FITC and RFP fluorescence. DIC image of stained cell is also shown. Localisation of RFP-tagged proteins expressed by yeast reporter strains in cells carrying control vector pYES DEST52e is also shown.

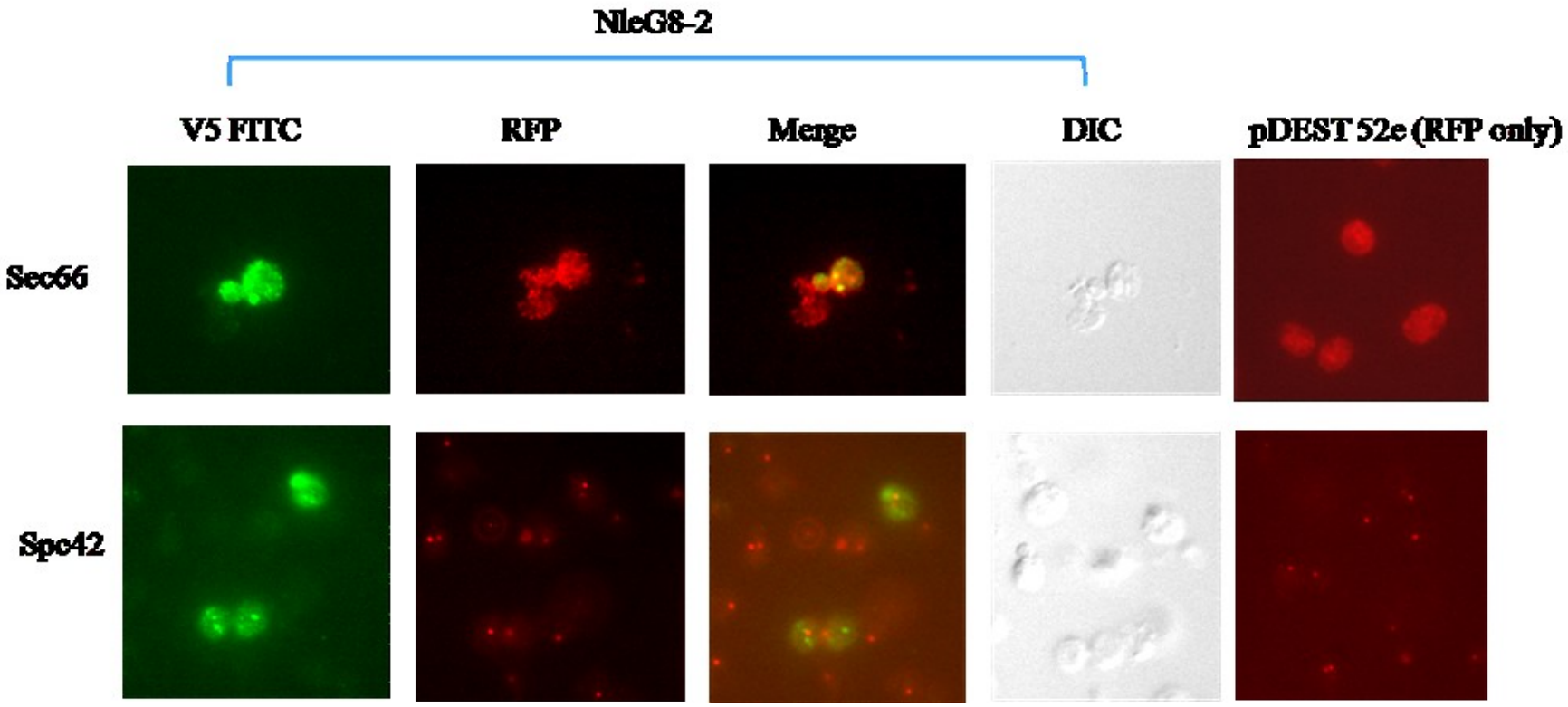

Figure 5.15: Localisation of NleG8-2 in yeast reporter strains. FITC-conjugated anti-V5 antibody was used to stain fixed yeast cells to show localisation of NleG8-2-V5 fusions (V5 FITC). RFP shows fluorescence of RFP-tagged protein constitutively expressed by reporter strain i.e. Sec66 (ER), Spc42 (spindle pole body). Merge shows both FITC and RFP fluorescence. DIC image of stained cell. Localisation of RFP-tagged proteins expressed by yeast reporter strains in cells carrying control vector pYES DEST52e is also shown.

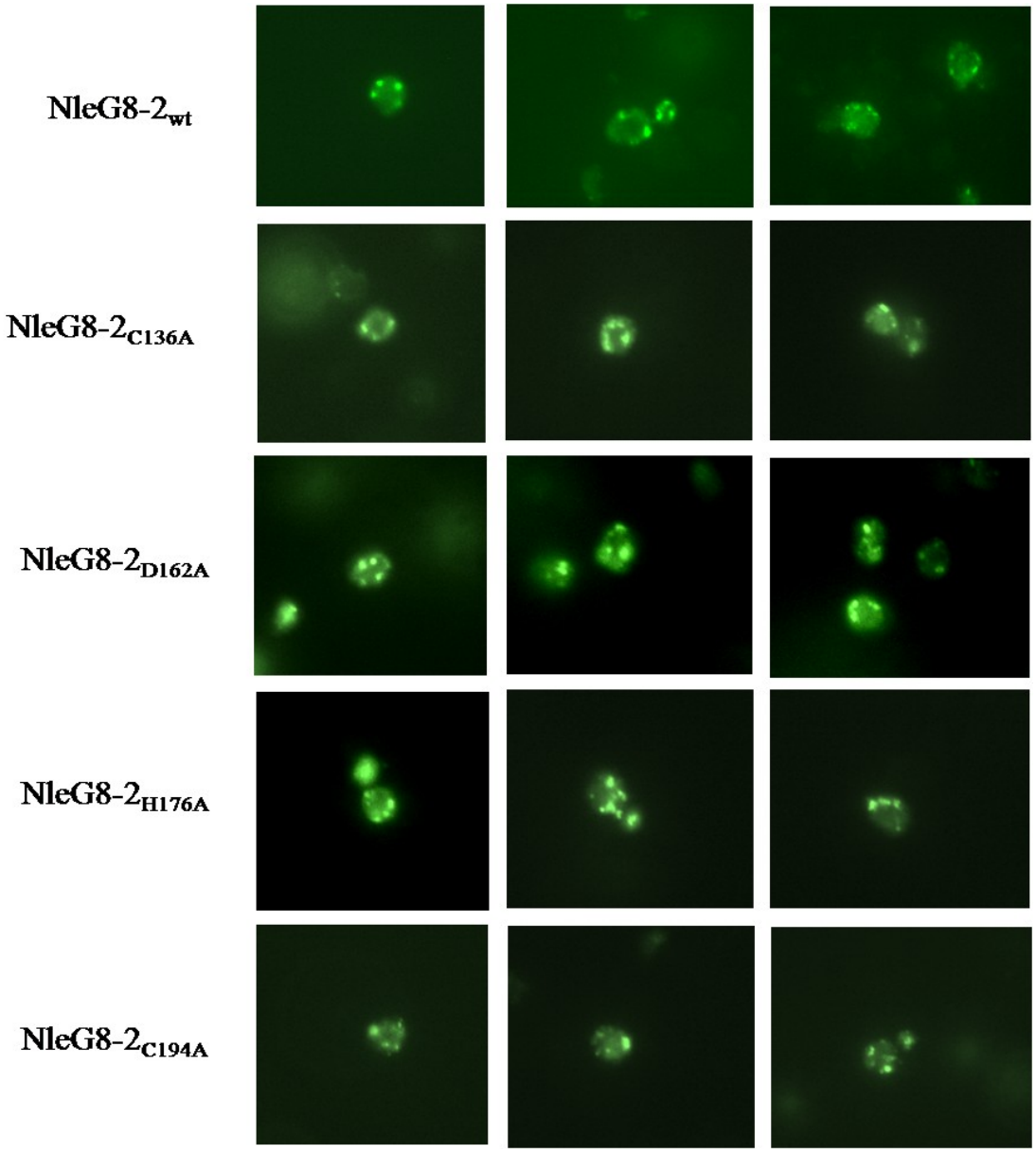

Figure 5.16: Localisation of NleG8-2 and mutated NleG8-2 in yeast cells. Effector-V5 fusions were detected in fixed yeast cells using FITC conjugated anti-V5 antibody (Invitrogen).

5.3.7. Yeast cell morphology and chitin localisation

Live yeast cells expressing selected effectors or carrying pYES DEST52e control vector were viewed using DIC microscopy. Cells expressing Map and NleG8-2 showed normal morphology and budding patterns whereas approximately 25% of cells expressing NleG2-3 were elongated and had unconstricted bud necks (figure 5.18). Cells expressing EspM2 also showed morphological changes including elongated cells and unusual bud necks (figures 5.17) although the changes were less severe than those changes caused by NleG2-3.

The chitin-specific fluorophore calcofluor white was used to stain chitin in the cell walls of live yeast cells. Chitin was localised to bud scars only in cells carrying pYES DEST52e control vector and cells expressing Map, NleG8-2 and EspM2. However, in cells expressing NleG2-3 abnormal cell morphology and budding patterns were accompanied by unusual chitin localisation (figure 5.18). Chitin was more diffusely localised within the yeast cell wall around the unconstricted bud necks.

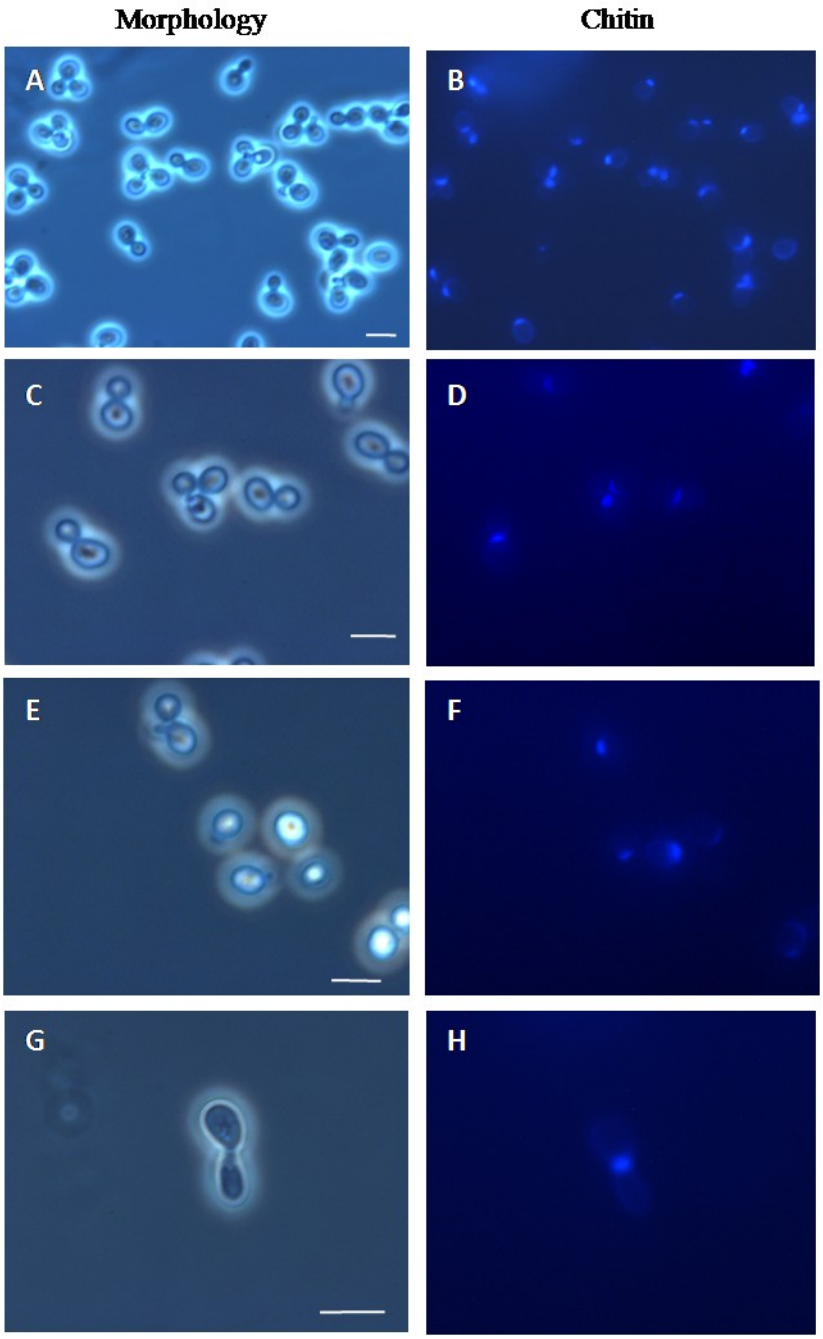

Figure 5.17: Yeast cell morphology and chitin localisation of cells expressing EHEC O157:H7 T3SS effectors. Cells were grown in SC-Ura+galactose for 4 hours to induce expression of effectors. Cells were viewed using DIC microscopy, chitin was visualised using a DAPI filter. Vector only (A, B); Map (C, D); NleG8-2 (E, F); EspM2 (G, H). Scale bar represents 5 μm.

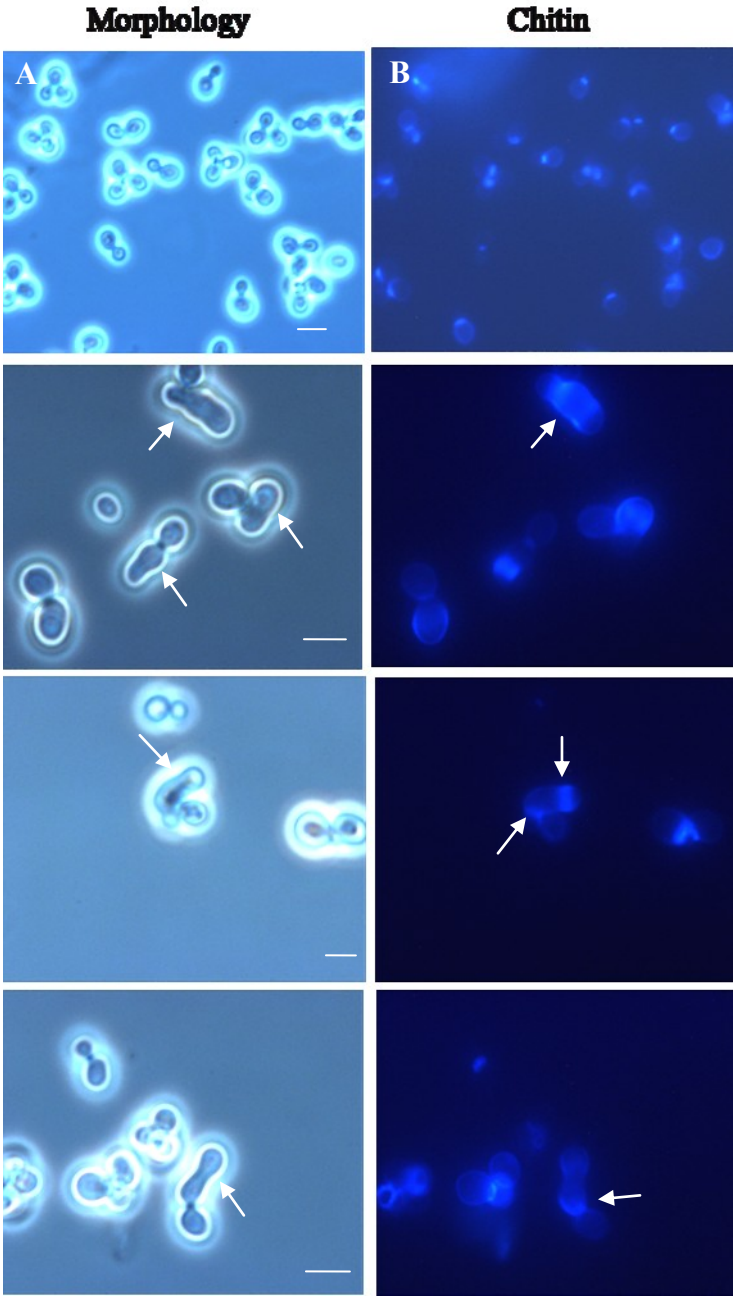

Figure 5.18: Morphology and chitin localisation of yeast cells expressing NleG2-3. Cells were grown in SC-Ura+galactose for 4 hours to induce expression of NleG2-3. Cells were viewed using DIC microscopy, chitin was visualised using a DAPI filter. Abnormalities in morphology/chitin localisation are indicated. Images A and B show cells containing vector only. Scale bar represents 5 µm.

5.4. Discussion

5.4.1. EspM2, NleG8-2 and NleG2-3 cause yeast growth inhibition

Yeast strain FY833 was transformed with pYES DEST52 containing selected EHEC O157:H7 T3SS effectors. The effect of effector expression on yeast growth was then tested by comparing growth of yeast on inducing and non-inducing media. As a control, yeast carrying (non-recombined) pYES DEST52e were used to show that growth inhibition was due to effector expression (yeast carrying this vector grew similarly on inducing and non-inducing media). All effector proteins were expressed at similar levels by yeast grown in inducing media.

Yeast expressing EspM2, NleG8-2 and NleG2-3 showed growth retardation suggesting that these effectors are toxic. It was difficult to obtain yeast transformants for EspM2 and colonies of transformants obtained were small. This problem was probably due to the toxicity of the effector – Alto *et al* could not recover yeast transformants for the EspM2 homologue, IpgB2 [159]. Interestingly, expression of EspM2 in yeast was so toxic that growth inhibition was seen in cells grown on glucose media whereby expression of the effector is repressed. This effect may be caused by leaky expression of effector from the *GAL1* promoter as was found by Slagowski *et al* (2008) [378] and Stirling *et al* (2006) [411]. Future studies of EspM2 expression in yeast may benefit from using an alternative, tightly controlled promoter such as *tetO* [417] [431].

Relatively few effectors were seen to affect yeast growth. It is possible that a number of effectors target cellular processes that are not normally rate-limiting for growth. In order to identify these effectors, the growth assay could be improved by the use of 'stressors' such as caffeine in the growth media. Such 'stressors' are added to the media at doses that do not affect the normal growth of yeast but do increase sensitivity to effectors [414] e.g. *Shigella* effector OspB only affects yeast growth when caffeine is added to the growth media [378]. Conditional sensitivity in the presence of stressors (known to affect certain cell processes) may give clues as to effector function [336]. For example, greater sensitivity to an effector in the presence of nocodazole (a drug that inhibits microtubule polymerisation [432]) may suggest that the effector targets the microtubule network [433].

Usually, protein tags and fusion to GFP does not interfere with effector function [378]. Surprisingly however, Map-V5 did not affect yeast growth, chitin localisation or cell morphology when tested in this study. This was unexpected as expression of Map has been shown by Rodriguez Escudero *et al* and Alto *et al* to cause the accumulation of large unbudded cells and growth retardation [405] [159]. However, in these studies Map was expressed with an N-terminal tag (GST or GFP). Interestingly, in accordance with results of this study, Rodriguez Escudero *et al* found that expression of C-terminally tagged Map (Map-GFP) did not cause severe growth inhibition even though the effector was still targeted to mitochondria [405]. These results suggest that addition of a C-terminal tag masks the effect of a toxic domain present at the C-terminus of Map – the toxicity of Map has subsequently been shown to depend on the last three amino acids of the protein, a TRL motif [159] [388]. In contrast to findings that tags/reporter proteins may decrease effector toxicity, Slagowski *et al* found that a small number of effectors were more toxic when

expressed as GFP fusions e.g. IpgB1 only caused growth inhibition when expressed as a GFP fusion [378]. The effect of expressing the effectors with a C-terminal V5/His tag must therefore be considered – the toxicity of novel effectors may have been masked/augmented by the C-terminal V5/His tag. To confirm the toxicity of novel effectors in yeast, each should be screened again using alternative tags/reporter proteins (e.g. myc, GFP). Effectors should also be tested using N-terminal tags. Currently pYES DEST52 is the only Gateway compatible yeast expression vector available, conversion of other yeast expression vectors to Gateway would be highly useful.

5.4.2. Localisation of effectors

Localisation of T3SS effectors within yeast can provide valuable insights into effector function. For example, the *Salmonella* effector SipA colocalised with actin when expressed in yeast [157]. SipA was subsequently shown to bundle actin filaments in the host cell which promotes formation of membrane ruffles and uptake of *Salmonella* [434]. In a study by Rodriguez-Escudero *et al,* Map-GFP localised to yeast mitochondria. Here, Map-V5 showed a similar localisation pattern to Map-GFP. However, mitochondrial markers (e.g. DAPI, Mitotracker) should be used to confirm that Map-V5 is also targeted to mitochondria. NleG8-2 was localised to punctate regions near the cell periphery and did not appear to co-localise with or disrupt the localisation of any of the RFP-tagged cellular proteins produced by any reporter strain. Other reporter strains/cell dyes specific for the Golgi, secretory vesicles and vacuole should be used to see if NleG8-2 is targeted to these cellular compartments. In a study by Li *et al* EPEC NleG localised to both the cytoplasmic and

membrane fraction of transfected HeLa cells and was not involved in the formation of the pedestal [435].

Unfortunately, the low level of fluorescence of RFP and rapid photobleaching of RFP when exposed to UV light meant that images obtained were of a poor quality and somewhat difficult to interpret. Confocal microscopy could have improved this and would have allowed visualisation of greater detail of subcellular structures and localisation of effectors. Localisation studies could also be improved through the use of antibody markers and dyes for specific compartments e.g. Mitotracker (Invitrogen) for mitochondria. Also, the use of a fluorescent reporter protein e.g. GFP would improve upon indirect immunofluorescence as effectors could be visualised within live cells. Procedures involved in preparing cells for staining for immunofluorescence (i.e. digestion of the cell wall with Zymolyase) were difficult and may have introduced artifacts. For example, over digestion made cells too fragile whereas under-digestion did not allow sufficient antibody into the cell. Digestion of the cell wall may also have resulted in cell distortion.

The results of this study and other studies into the localisation of T3SS effectors in mammalian and yeast cells suggests that factors other than effector sequence/structure are involved in determining the localisation of effectors within the host cell. These factors may include the context of effector delivery, the presence of other effectors and host responses to the effector [364].

5.4.3. Morphology and chitin localisation

Interestingly, NleG2-3 caused abnormal growth of yeast cells. The elongation of cells and disruption of budding may indicate that this effector perturbs the MAPK signaling pathway controlling filamentous growth [436]. The use of DAPI staining would have been useful to visualise nuclei within the cells to determine whether there was a loss of co-ordination between morphogenetic and nuclear events in the cell.

Patterns of chitin localisation in cells expressing NleG2-3 are unusual and it appears that the cells have been unable to form a tight ring of chitin at the bud neck. This may indicate that localisation of septin (essential for promoting cell wall synthesis at the bud neck through the recruitment of chitin synthases [423]) has been disrupted. In a study by Rodriguez-Escudero *et al,* a similar phenotype was observed in cells expressing EspF – expression of septin-GFP fusion proteins confirmed that septin was mislocalised in cells expressing EspF. Use of a reporter stain in which a septin-RFP protein is expressed may help to elucidate the mechanism by which NleG2-3 causes aberrant chitin localisation and cell morphology.

To investigate whether NleG2-3 affects MAPK signal cascades involved in morphogenesis and cell integrity, specific antibodies could be used to detect phosphorylation (activation) of components of the signal cascade. This was attempted using antibodies against the phosphorylated domain of p42/44 MAPK but neither phosphorylated or unphosphorylated proteins could be detected.

5.4.4. Mutant EspM2

EspM effectors contain a conserved WxxxE motif [159]. In order to determine whether these residues were required for EspM2 function, Trp70 and Glu74 were mutated to alanine by site directed mutagenesis. Interestingly, yeast grown on media containing galactose (expression of effector induced) showed reduced growth comparable with that caused by expression of wild type EspM2. However, yeast grown on glucose media (expression of effector is repressed) grew better than yeast carrying wild type NleG8-2. These results suggest that the toxicity of EspM2 was reduced but not abolished when W70 and E74 were mutated to alanine. This contrasts with results of Alto *et al* who showed that IpgB2 yeast toxicity was abolished when the WxxxE motif was mutated to alanine [159]. Further characterisation of EspM2 by Arbeloa *et al* has shown that the WxxxE motif is required for the function of EspM2 and other effectors (e.g. EspT) belonging to the IpgB family [388]. Results of this study suggest that the WxxxE motif is required for inhibition of yeast growth and may be important in the function of EspM2 but that other sequences/functions may mediate the severe growth retardation caused by EspM2.

5.4.5. Mutant NleG8-2

Results of growth assays of yeast expressing truncated versions of NleG8-2 suggested that the C-terminus, specifically amino acids 120-216 were important for effector function. It was found that within this region lay four residues; Cys136, Asp162, His176 and Cys194 that were conserved in all NleG family members. Residues C136, D162, H176 and C194 were mutated to alanine by site directed mutagenesis. Expression of NleG8-2_{C136A}, NleG8-2_{D162A}, NleG8-2_{H176A} abolished inhibition of yeast growth associated with expression of

wild type NleG8-2. Expression of NleG8-2_{C194A} caused similar levels of yeast growth inhibition as expression of wild type NleG8-2. It is therefore likely that residues Cys136, Asp162 and His176 are important for the function of NleG8-2. Mutation of these residues of NleG8-2 did not appear to affect the localisation of NleG8-2–V5 within the yeast cell. These residues are therefore unlikely to form part of a targeting motif or be involved in effector targeting within the host cell.

Interestingly, cysteine, aspartic acid and histidine are amino acids often found in the catalytic triad of cysteine proteases [425]. A number of T3SS effectors from animal and plant pathogens have been shown to act as cysteine proteases including *Yersinia* and *Pseudomonas* effectors YopT and HopPtoN [371] [437]. YopT cleaves Rho GTPases (releasing them from the host cell membrane) to cause actin cytoskeletal disruption [371]. The proteolytic activity of these effectors is dependent on invariant C/H/D residues found in all YopT family members. It is possible that NleG8-2 functions as a cysteine protease with Cys136, Asp162, and His176 forming the catalytic triad. In order to confirm this, proteolytic activity of NleG8-2 must be demonstrated. Purification of an NleG8-2–His fusion protein was attempted but was unsuccessful; therefore the effector could not be assayed for protease activity *in vitro* in this study. We have shown that NleG8-2 has a toxic phenotype when expressed in yeast. Members of the NleG family of effectors have not been well characterised or focused upon, unlike EspM2 and other IpgB-like effectors that have been characterised by Arbeloa *et al* [388] and Alto *et al* [159]. I therefore decided to concentrate on characterising and investigating NleG8-2 further using yeast genetic-based approaches – this work is presented in chapter 6.

CHAPTER SIX

Analysis of NleG8-2 function using a microarray and a yeast gene high copy suppression screen

6.1. Introduction

In addition to using yeast physiology to study candidate EHEC O157:H7 T3SS effectors, I decided to use yeast genetics to study effectors in more detail. This chapter describes the use of DNA microarrays and a high-copy suppression (HCS) screen to study NleG8-2.

The yeast genome was sequenced in 1996; so far more than 75% of yeast CDSs have a known or predicted function [438]. A variety of databases allow easy access to this information [439]. There are many tools and resources available for genome-wide and small scale experiments using yeast as a model e.g. comprehensive yeast DNA [440, 441] and protein [442] microarrays. Other powerful resources include isogenic strain collections where each strain carries a CDS that is either:

- deleted.
- fused to other proteins [443] e.g. fusions to green fluorescent protein (GFP) allow for localisation studies [421].
- fused to a tandem affinity tag (useful for co-immunoprecipitation and expression studies [444]).
- fused to the GAL4-binding domain – can be used in two-hybrid assays to investigate protein interactions [445].
- over-expressed (see below) [446] [447].

6.1.1. Over-expression and gene-deletion screening

There are two tools in particular that have been used to study type-III secretion and T3SS effector function: isogenic strain collections in which each CDS is deleted and plasmid-based libraries that allow over-expression of individual genes [159] [163] [336]. Such strain collections and libraries allow the identification of proteins that (when over-expressed or absent) confer resistance to effectors and restore yeast growth [446] [448]. The protein or pathway targeted by the effector can then be inferred (see below).

Synthetic genetic array (SGA) is a gene-deletion based approach that gives insights into the functions and protein-protein interactions of proteins encoded by mutated yeast genes [449] [450]. SGAs have been adapted to pathogenic genetic arrays (PGAs) to screen for mutants that are resistant to the effects of T3SS effectors. In this approach, effector genes (under the control of an inducible promoter) are introduced into a set of yeast deletion strains – each strain is deleted for a non-essential CDS. Strains that are hypersensitive to the effects of the T3SS effector or alternatively those strains that are resistant to the effector (growth restored) can then be isolated. The mutated gene of each strain can be identified to give clues as to the target of the T3SS effector. For example, Alto *et al* successfully used a PGA in addition to DNA microarrays to investigate the *Shigella* effector IpgB2 (the expression of which was found to be toxic to wild type yeast) [159]. The IpgB2 gene was integrated into the genome of over 5000 viable gene-deletion mutant yeast strains. Results showed that only strains carrying deletions in genes encoding components of the cell wall integrity (CWI) Rho1p/MAPK pathway were able to grow [159]. Therefore, it was predicted that IpgB2

targets the CWI MAPK signalling pathway – IpgB2 was subsequently shown to act as a Rho mimic [159] [388].

Over-expression screening has also been used to investigate protein function and was first used by Kasahara *et al* (1994) to identify a yeast gene (HKR1) responsible for resistance to HM-1 killer toxin, a *Hansenula mrakii* toxin that inhibits glucan synthesis [451]. The main use of this type of screening has been screening for drug resistance to facilitate identification of drug targets [452]. The principle of this approach is straightforward: over-expression of a gene results in increased protein product. If the over-expressed protein is the target of the drug, the yeast cell should be resistant to the drug. The gene being over-expressed can then be isolated and identified. Over-expression screening can also be used to study deleterious T3SS effectors. Shao *et al* found that yeast Cdc42 (a Rho GTPase) was a multicopy suppressor for the *Yersinia* effector YopT [371]. YopT was subsequently found to disrupt Rho GTPase localisation [371].

The study described here used a plasmid library-based approach (summarised in figure 6.1) to investigate the eukaryotic target of NleG8-2. Yeast strain FY833 carrying the NleG8-2 expression vector (or empty vector as negative control) was transformed with a genomic DNA library constructed on a high copy number vector (pYE13). Both NleG8-2 and the genes carried by the library plasmid were expressed when the yeast was grown on media containing galactose. Yeast that were able to grow under these conditions i.e. resistant to the effects of NleG8-2, were then selected. The library plasmid DNA was isolated from the resistant transformant and the over-expressed gene/s identified. The principle of this

approach denotes that the proteins encoded by these genes are likely to be targets of the effector or involved in resistance to the effector. It was proposed that this approach, used in conjunction with a DNA microarray, would give valuable clues as to the target/function of NleG8-2 (as has been achieved for other effectors [159] [163]).

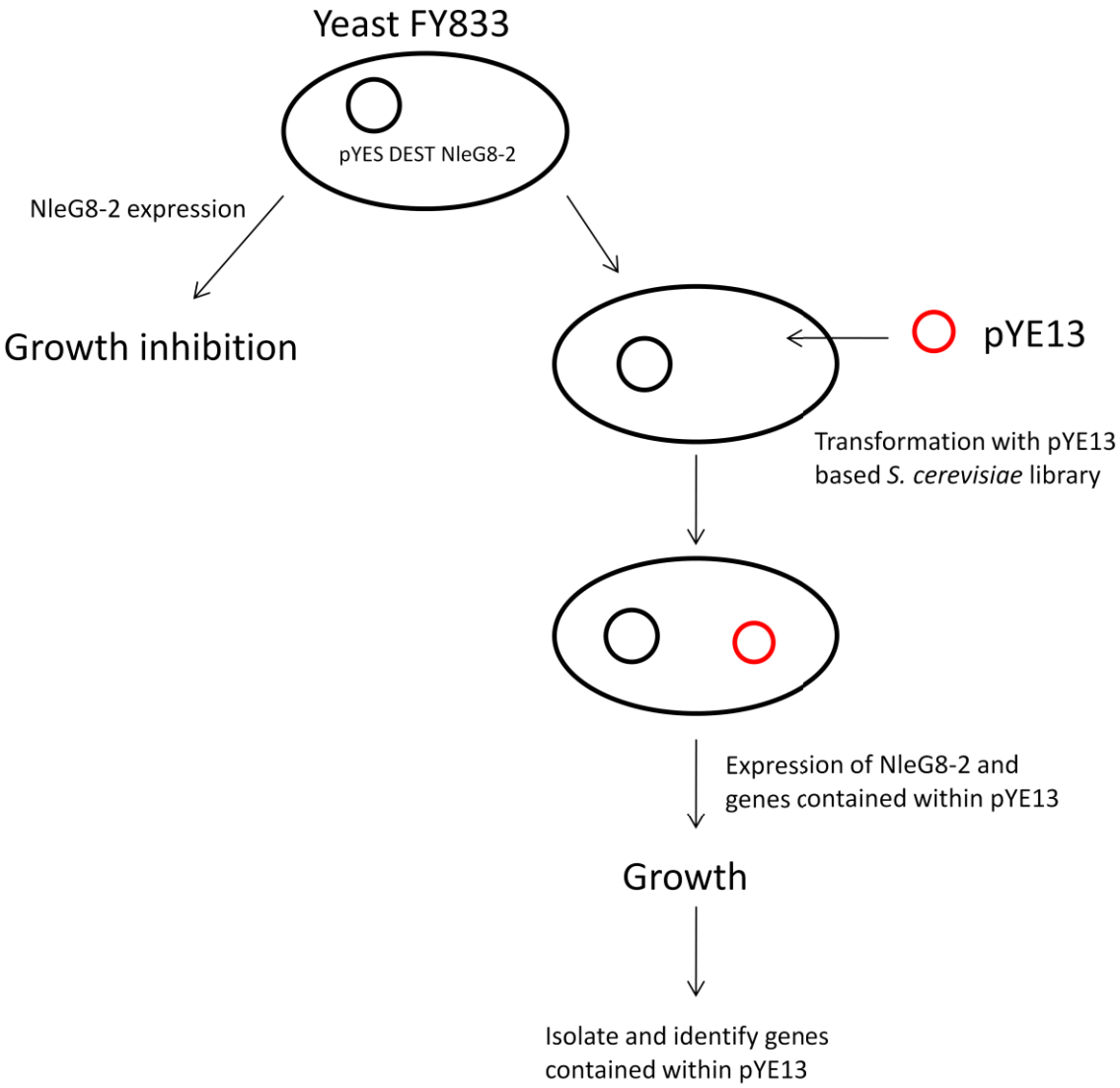

Figure 6.1: Summary of high copy suppression screen. Yeast transformed with pYES DEST NleG8-2 show growth retardation when grown on galactose media due to expression of NleG8-2. Yeast are then transformed with pYE13 genomic library. Both NleG8-2 and genes contained within the pYE13 insert are expressed when yeast is grown on galactose media. Yeast able to grow on galactose media (i.e. resistant to NleG8-2) are selected, the pYE13 clone isolated and the genes contained within the pYE13 insert identified.

6.1.2. DNA Microarrays

DNA microarrays have revolutionised genetic investigations as they are able to measure the expression of thousands of genes in parallel [453]. Microarrays are commonly used to identify genes that are differentially expressed in one set of cells relative to another e.g. wild-type versus mutant strains [454] [455], treated versus untreated cells [456] [457] or diseased versus healthy tissue [458] [459].

Microarrays have been used to study T3SS [3] and EHEC O157:H7 [460]. Transcriptional analysis of *eivF* and *etrA* mutant strains of EHEC O157:H7 using whole genome microarrays (based on the EHEC O157:H7 genome) revealed that LEE genes were up-regulated compared to the parent EHEC O157:H7 Sakai strain [3]. This showed that EivF and EtrA, proteins from a second T3SS locus, ETT2, influence transcription of the LEE [3]. Microarrays have also been used to investigate bacterial and host cell responses to bacterial attachment. Recent analysis of the transcriptomes of EHEC O157:H7 and epithelial cells (HT-29) showed that over 300 HT-29 genes (including immune regulatory genes such as IL-8 and interferon-related genes) and over 900 EHEC O157:H7 genes (including an activator of the LEE1 promoter, tryptophanase [322]) were differentially regulated during bacterial attachment [461]. In another study, transcriptome analysis of attaching EHEC found upregulation of a gene encoding a protease that mediates inhibition of the IFN signal transduction cascade [462]. Identification of up-regulated genes such as these may provide novel targets for strategies aimed at interrupting the infection process [462].

Yeast genome microarrays have previously been used to investigate the function of T3SS effectors [159] [163]. The transcriptional profile of yeast expressing an effector may give insights into effector function i.e. genes may be up/down regulated in response to effectors which may in turn give clues as to the pathways targeted by the effector. Alto *et al* used yeast DNA microarrays to analyse the transcriptome of yeast cells expressing IpgB2 [159]. They found that IpgB2 affected the expression of 351 transcripts and that the overall transcriptional profile of yeast expressing IpgB2 correlated with that of a cell over-expressing activated Rho1p. This showed that IpgB2 stimulates small G-protein signalling in a similar way to GTP-bound Rho1p [159]. In order to gain an insight into the function of NleG8-2, the transcriptome of yeast expressing NleG8-2 was compared to the transcriptome of yeast expressing an empty vector to identify those genes that were up/down-regulated in response to NleG8-2.

6.1.3. Microarray methodology

This study used GeneChip Yeast Genome 2.0 arrays (Affymetrix, US). These microarrays are constructed by photolithography and contain 5744 probe (25-mer oligonucleotide) sets for 5841 of the 5845 genes present in the *Saccharomyces cerevisiae* genome. The microarray methodology used in this study is summarised in figure 6.2 and is as follows [463]:

1. RNA is harvested from yeast cells expressing NleG8-2 (or empty vector, negative control).
2. Reverse transcriptase is used to make cDNA from the RNA.

3. An *in vitro* transcription reaction is then used to create cRNA from the cDNA.
4. cRNA is biotinylated and fragmented.
5. Fragmented biotinylated cRNA is then allowed to hybridise to probes on the microarray.
6. Arrays are washed and stained with streptavidin-phycoethrythrin (SAPE).
7. The signal is amplified by washing the array with biotinylated anti-streptavidin (goat) antibody and staining with SAPE.
8. The microarray slide is scanned using fluorescence microscopy.

The intensity of phycoerythrin fluorescence (caused by SAPE binding to biotinylated cRNA attached to individual probes) is used as a measure of transcription of the gene (represented by the probe) under the conditions/treatment of the cell. In order to determine whether changes in gene expression are significant, data are statistically analysed (e.g. using *Student's t*-test) [464].

RNA hybridisation to the microarrays was performed and results were analysed at the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) at The University of Manchester, UK.

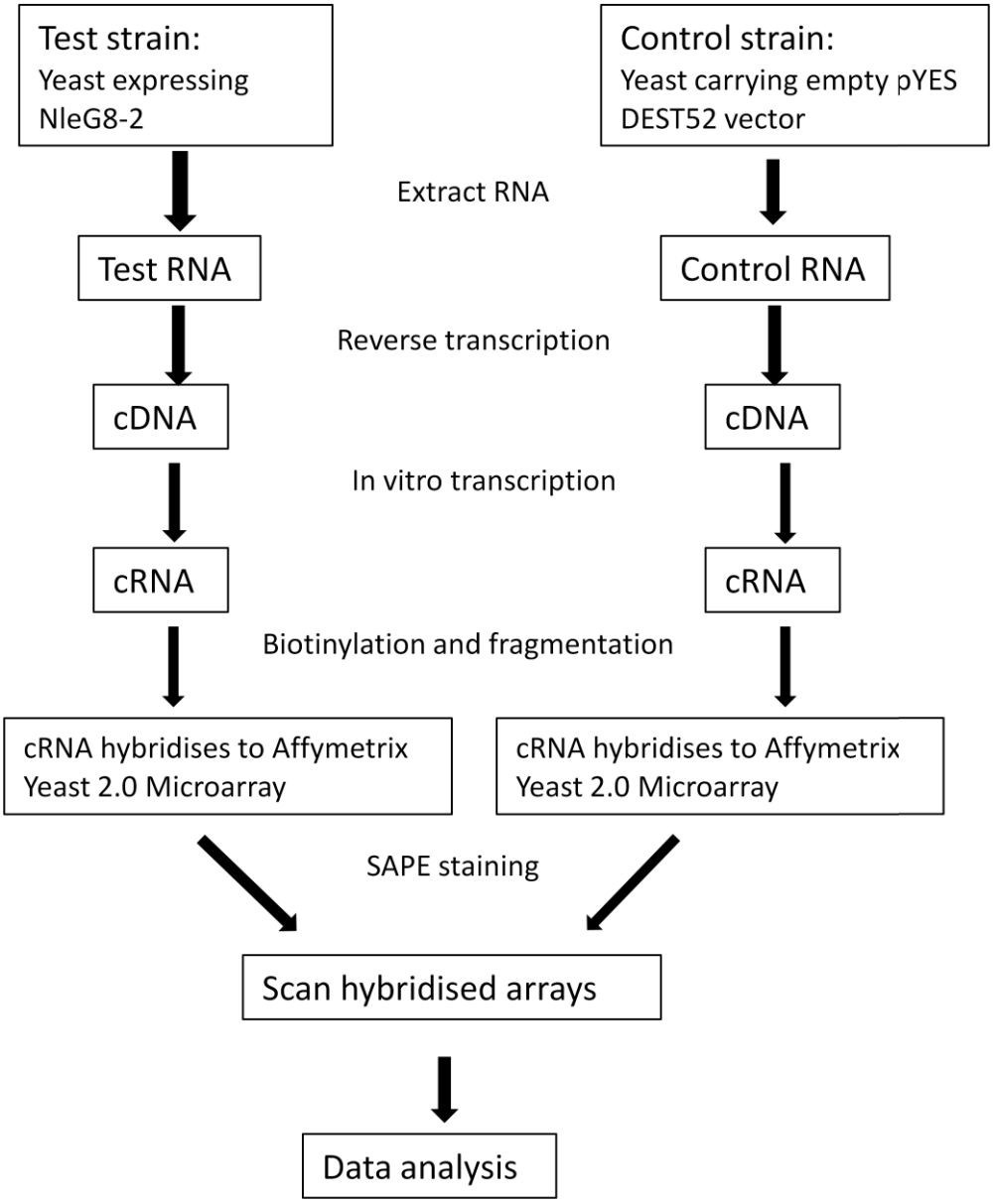

Figure 6.2: Affymetrix microarray methodology

6.2 Materials and Methods

6.2.1. Whole genome microarray

Harvesting yeast cells for RNA extraction

5 ml SC-Ura+glucose was inoculated with a single yeast colony (either FY833 pYES DEST52 or FY833 NleG8-2). Cells were incubated in a shaking (200rpm) water bath overnight at 30°C. 100 µl of stationary phase yeast culture was added to 200 ml fresh SC-Ura+glucose and incubated overnight at 30°C (200rpm). The log phase culture was washed twice in sterile dH_2O by centrifuging for 10 minutes at 12000rpm in a Beckman-Coulter centrifuge using a JA-20 rotor. Four sterile 50 ml Oak ridge centrifuge tubes (Nalgene) were used per culture. Cell pellets were resuspended in 1 ml SC-Ura and inoculated into 200 ml fresh SC-Ura+galactose (2%). Cells were incubated in 1 l conical flasks for 4 hours at 30°C and 200rpm. The cell density of each culture was measured. Cells were placed on ice and transferred to pre-cooled centrifuge tubes. Cells were centrifuged at 4000rpm (at 4°C) for 3 minutes. The supernatant was removed. The cell pellet was resuspended in 1 ml supernatant and slowly pipetted into liquid nitrogen using a Pasteur pipette so that spheres (5 mm) of frozen culture were formed. Spheres were collected into a pre-chilled 50 ml Falcon tube and stored at -80°C. RNA extraction, cRNA preparation, microarray hybridisation and data analysis was carried out by B. Rash and A. Hayes at the COGEME facility, University of Manchester.

RNA extraction (performed by B. Rash, University of Manchester)

A 5 ml Teflon vessel was pre-cooled in liquid nitrogen. The frozen cells and a 7 mm tungsten carbide bead were added to the vessel and the vessel shaken at 1200 rpm for 2 minutes in a Micro-Dismembrator to break the cells. 2 ml TRIzol (Invitrogen) was added to the frozen powder (cells) and the mixture split into two microcentrifuge tubes. The samples were homogenised by vortexing for 1 minute. The samples were kept at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. The samples were then centifuged at 12000 rpm for 10 minutes to remove polysaccharides and high molecular weight DNA. 0.4 ml chloroform was added to each sample and the samples shaken vigorously by hand for 15 seconds. The samples were left for 5 minutes at room temperature then centrifuged at 12000 rpm for 5 minutes. The aqueous phase (containing RNA) was transferred to a fresh microfuge tube and 0.5 ml isopropyl alcohol (IPA) was added to precipitate the RNA. Samples were kept at room temperature for 10 minutes. The RNA was then pelleted at 12000 rpm for 10 minutes. The supernatant was carefully removed and the RNA pellet was washed with 1 ml 70% ethanol by vortexing and centrifugation at 12000 rpm for 10 minutes. The pellet was allowed to air dry for 5 minutes. The RNA pellet was then dissolved in 500 µl DEPC-treated dH_2O. 500 µl LiCl buffer was added to the RNA sample and the RNA allowed to precipitate for 1 hour at -20°C. The pellet was washed twice more (as before) with 70% ethanol, air dried for 5 minutes and dissolved in 100 µl DEPC treated dH_2O. RNA was visualised and the quality of RNA checked by running 5 µl on a 1% agarose gel. RNA was quantified using an Agilent Bioanalyser. cDNA and biotinylated, fragmented cRNA were prepared following the manufacturer's instructions.

Hybridisation was also performed according to the manufacturer's instructions (www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).

Data Analysis

Arrays were scanned at the COGEME facility (University of Manchester, UK) using a GeneChip scanner – fluorescence of phycoerythrin was detected at 570 nm. GeneChip Operating software (GCOS) was used for initial analysis of microarray slides i.e. bad/absent/falsely identified spots were filtered out. Dr. A. Hayes (University of Manchester) then analysed the data generated by GCOS using Genespring GX (Agilent, US) software. The data were normalised to allow the comparison of three independent replicates performed for each condition. Genes showing significantly different levels of expression ($P<0.05$) in yeast expressing NleG8-2 were identified using a paired *t*-test. Genes identified by this test were then filtered using a multiple testing correction. This process attempts to decrease the number of false positives that may appear to be statistically significant when using P-values. The Benjamini-Hochberg false discovery rate was used in this study. The false discovery rate is the proportion of genes expected to occur by chance relative to the proportion of identified genes. The Benjamini-Hochberg FDR was applied to the normalised data with a significance cut-off of 0.75. The reliability of microarray results can also be improved by performing more replicates to decrease the number of false positives and false negatives identified.

6.2.2. Yeast high copy suppression (HCS) screen

Media

M9 minimal medium consisted of 200 ml M9 (2x) salts, 1.6 g/l yeast synthetic drop out media supplement without leucine (Sigma), 400 µl 0.1M $CaCl_2$, 2 ml glucose (40%), 400 µl 1M $MgSO_4$, supplemented with 100µg/ml ampicillin. For agar plates 200ml water agar (3%) was added.

Yeast plasmid based library preparation

Yeast genomic library AB320 in pYE13 was obtained from the American Type Culture Collection (ATCC). This library consists of 5 – 20kb genomic inserts in the shuttle vector pYE13 (a high copy number plasmid 25-80 copies/cell). The library was supplied in *E. coli* RR1 in a glycerol stock at a titre of 2.0 x 10^7 cells/ml. 10000 clones are needed to represent all genes in the library. I decided to collect at least three times this amount to make sure all clones were represented i.e. 30000 clones. Optimum plating density is 20000 colonies per plate therefore 1µl of the glycerol stock was diluted in 1 ml of LB broth and spread onto a large (20 cm x 20 cm) plate (total of 3 plates). Cells were allowed to grow overnight at 37°C. The next day, 25 ml LB broth (supplemented with ampicillin) was added to each plate and the plate rocked gently for 1 hour at room temperature to release colonies from the agar surface. Cell suspension from each plate was collected into a 1 l flask and 300 ml LB + Ampicillin was added. The cells were allowed to grow for a further 2 hours at 37°C 180rpm. Plasmid DNA was extracted from cells and precipitated using a Qiagen maxiprep kit

according to the manufacturer's instructions. DNA was resuspended in 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Strain	Genotype	Source
RR1 (recA+ HB101 *E. coli* derivative)	F⁻ supE44 *lac*Y1 *ara*-14 *gal*K2 *xyl*-5 *mtl*-1 *leu*B6 *pro*A2 Δ(*mcr*C-*mrr*) *rec*A+ *rps*L20 *thi*-1 λ-	American Type Culture Collection (ATCC)
KC8 *E. coli*	*hsd*R, *leu*B600 *trp*C9830 pyrF::Tn5 *his*B463 lac ΔX74 *str*A *gal*U,K	Clontech

Table 6.1: *E. coli* strains used in the propagation of pYE13

Yeast transformation with library

5 ml SC-Ura+glucose was inoculated with a single yeast colony. Cells were incubated in a shaking (200rpm) water bath overnight at 30°C. 500µl of this overnight culture was then used to inoculate 200 ml fresh SC-Ura+glucose. Cells were incubated overnight at 30°C (200rpm). Cells were centrifuged for 5 minutes at 3000 x g in 50 ml Falcon tubes and the supernatant removed. Cells were resuspended in a final volume of 25 ml 0.1M LiAc solution. Cells were again centrifuged at 3000 x g for 5 minutes and the supernatant was discarded. The following transformation mix was added (in the order listed): 2.4 ml 50% PEG 3350, 0.36 ml of 1.0M LiAc, 0.5 ml ssDNA and 0.25 ml sterile dH_2O (containing 20 µl DNA library). The cells and transformation mix were thoroughly mixed by vortexing for 2 minutes. 400 µl cell suspension was added to sterile microcentrifuge tubes and cells were heat shocked for 22 minutes at 42°C. Cells were centrifuged at 100 x g for 30 seconds and resuspended in a final volume of 20 ml SC-Ura-Leu. Cells were grown for 3 hours at 18°C.

A volume of cell suspension sufficient to obtain 2000 colonies per plate was plated onto SC-Ura-Leu+galactose in large 20 cm x 20 cm plates (Corning). Cells were incubated for 3 days at 25°C.

Plasmid rescue from yeast

400µl of glass beads (450 µm diameter) were added to a 1.5ml microcentrifuge tube. 200µl of extraction buffer was added to the beads. Yeast cells were then added to the tube: yeast cells were scraped from the patch on the selective plate using a 1ml pipette tip (cells coated 2mm of the tip) and added to the tube. 200µl of phenol/chloroform mix was added to the tube and cells were vortexed vigorously for 7 minutes. Tubes were centrifuged for 10 minutes at 13000rpm. 140µl of the supernatant was transferred to a sterile microcentrifuge tube and 500µl ethanol/NH_4Ac was added. Tubes were vortexed for 30 seconds. Tubes were then centrifuged for 15 minutes at 13000rpm at 4°C. The supernatant was removed and the pellets washed with 250µl 70% ethanol. Pellets were air dried for 10 minutes and resuspended in 10µl dH_2O.

Electroporation

Electroporation cuvettes (2 mm electrode gap) were cooled on ice for 30 minutes prior to electroporation. Electrocompetent *E. coli* strain KC8 (20 µl; Clontech) were added to the cuvette, followed by 2µl recovered plasmid. Cells were electroporated at 2.5kV. Immediately following electroporation, 200µl SOC medium was added to the cuvette. The cell suspension was transferred to a sterile microcentrifuge tube and incubated at 37°C for 1 hour. 150µl of the cell suspension was pipetted onto M9 – Leu agar plates and spread

evenly around the plate using a plastic spreader. Plates were incubated for 2 days at 37°C. Plasmids were obtained from transformed KC8 by standard miniprep procedures as detailed in chapter 2. DNA sequencing (using the primers listed in table 6.2) was used to obtain the sequences of the yeast genomic inserts of the plasmids.

Primer	**Sequence**
pYEp13seq-40	5′ CGCTACTTGGAGCCACTATCG 3′
pYEp13seq-60	5′ CCGCCGCCCAGTCCTGCTCG 3′
pYEp13seqrev	5′ GTGATGCCGGCCACGATGC 3′

Table 6.2: pYE13 DNA sequencing primers used in this study.

6.3. Results

6.3.1. Yeast library transformation

Transformation of FY833 pYES DEST-3486 with the pYE13 plasmid-based library was repeated 3 times. Yeast FY833 pYES DEST52e was transformed with the plasmid based library as a control. 20000 colonies per SC-Ura-Leu plate were obtained for the control transformation showing that transformation with the pYE13 plasmid library was successful. 21 colonies of FY833 pDEST-3486 yeast were able to grow on SC-Ura-Leu+galactose (i.e. suppression of growth inhibition). Each colony was picked. Of the 21 colonies picked from the original plates only 16 grew on SC-Ura-Leu+galactose when streaked onto fresh plates. It is likely that the remaining five colonies (that did not grow when restreaked) were anomalous, perhaps growing on top of transformation debris, not directly on galactose media therefore not expressing NleG8-2 at the same levels as other colonies. Recovery of the library plasmid was attempted for each of the 16 isolates. Ten library plasmids were successfully recovered. No plasmids could be recovered from the six remaining colonies indicating that resistance to the effector was probably due to a chromosomal mutation. KC8 *E. coli* were transformed with yeast cell extracts in order to amplify the library plasmid. Successful transformation of KC8 with the 10 recovered plasmids was seen with between 1 and 42 colonies obtained on each plate. One colony of transformed KC8 was picked from each plate. The plasmid was amplified and purified by miniprep. To confirm that resistance to the effects of NleG8-2 were dependent on the library plasmids, fresh yeast FY833 pYES DEST-3486 were transformed with each of the recovered plasmids. More than 1000 colonies were obtained per transformation when plated on SC-Ura-Leu+glucose and all strains grew as well as FY833 pYES DEST52e when restreaked onto SC-Ura-Leu+galactose.

6.3.2. Genes identified by high copy suppressor screen

Sequences of pYE13 plasmid inserts were obtained through DNA sequencing using primers listed in table 6.2. Sequences were identified using the BLAST facility on the *Saccharomyces* genome database (SGD) website (www.yeastgenome.org). Genes contained within the inserts are shown in table 6.3.

Transformant	Chromosome	Region	Genes
1	XVI	92482-96878	CIN2
2	VI	146481-150679	LOC1, DEG1
3	XIV	519318-562724	VAC7, MSG5, COX5A, COG5, YNL050C, SFB2, ALG11, SLM2, YNL046W, LAP2, YIP3, BOP3, COG6, YNL040W, BDP1, GPI15, IDH1, NCE103
4	XII	85387-87909	ISA1 (partial), TPO1
5	VII	719785-723935	SPT6 (partial), DAM1
6	X	207119-213767	CCT7, GZF3
10	III	27365-31919	KAR4, YCL056C
12	VIII	170201-174000	SLT2 (partial), RRM3, YHR032W (partial)
15	XVI	923239-929949	OPT2, YPR196W
16	XVI	52665-61750	THI21, YPL257W (partial)

Table 6.3: Genes present in yeast genomic inserts of pYE13 obtained from yeast pYE13 transformants resistant to NleG8-2.

6.3.3. Results from microarray

Statistical analysis of 3 biological replicates (normalised) showed that 33 yeast genes were significantly up/down regulated when NleG8-2 was expressed compared to empty vector pYES DEST52e. Table 6.4 shows those 20 genes that were significantly up regulated when NleG8-2 was expressed. Table 6.5 shows those 13 genes that were significantly down regulated when NleG8-2 was expressed.

Gene	NleG8-2 log_2 change in expression	Control log_2 change in expression	FDR corrected P value	Un-corrected P value
ATG14	0.24396355	-0.25174126	0.54066426	2.47E-04
ADP1	0.21325588	-0.18376286	0.7418809	0.0036259
LDB17	0.15847778	-0.18701713	0.69583017	0.0026743
SPG3	0.23451614	-0.20057519	0.624788	0.0014056
RPL2A	0.080376625	-0.09576479	0.7215294	0.0033673
VPS62	0.21762784	-0.2945741	0.6932907	0.0023974
PHB2	0.099705376	-0.12216377	0.5721855	5.51E-04
DOG2	0.068860054	-0.108840145	0.5721855	6.41E-04
HPF1	0.09053389	-0.11002779	0.5721855	0.0010252
MRPL8	0.14485581	-0.09985542	0.624788	0.00136
BET4	0.21451695	-0.39233527	0.6932907	0.0025639
VPS70	0.17858696	-0.18124802	0.6932907	0.0023418
MRP49	0.056836445	-0.07747873	0.5721855	8.15E-04
CWP2	0.030136744	-0.031445503	0.624788	0.0015776
TUL1	0.0804224	-0.07997767	0.18507534	2.06E-05
LOT6	0.06335894	-0.07263056	0.6932907	0.0022296
RPS31	0.063635506	-0.059439022	0.7418809	0.003666
CST9	0.063355125	-0.10863749	0.6932907	0.0026011
SLZ1	0.27074432	-0.22138388	0.5721855	9.79E-04
GDH1	0.14543247	-0.16374111	0.7215294	0.0032961

Table 6.4: Yeast genes that were upregulated in yeast expressing NleG8-2. FDR – false discovery rate.

Gene	NleG8-2 log_2 change in expression	Control log_2 change in expression	FDR corrected P value	Un-corrected P value
POP5	-0.07967726	0.057573635	0.54066426	2.47E-04
PHO11/PHO12	-0.090715885	0.12421814	0.5721855	7.02E-04
PRS4	-0.15346973	0.11938318	0.624788	0.0061008
CCT6	-0.06419722	0.1024119	0.7215294	0.003043
NPY1	-0.053647995	0.026236853	0.18507534	3.39E-05
POP3	-0.18177192	0.30887666	0.5721855	6.28E-04
AAH1	-0.31523672	0.46882185	0.645003	0.0017707
NCS2	-0.22807248	0.46882185	0.7215294	0.0032698
YIP3	-0.098649345	0.100541115	0.5721855	4.93E-04
PRS5	-0.12467575	0.2036918	0.7418809	0.0035366
RPB8	-0.17324512	0.10171763	0.6932907	0.0022402
ELP4	-0.09937986	0.161686262	0.5721855	5.28E-04
ARO7	-0.14434178	0.2225523	0.624788	0.001466

Table 6.5: Yeast genes that were down regulated in yeast expressing NleG8-2. FDR – false discovery rate.

6.3.4. Functions of the genes identified

Table 6.6 shows the functions of the proteins encoded by the genes identified by transcriptome analysis and the high copy suppressor screen. Data for protein function was obtained from gene annotation in SGD. The genes are grouped according to their product's role in the yeast cell e.g. proteins localised to or necessary for the correct functioning of the mitochondria are grouped together under 'Mitochondria'. Evidence for each gene product is shown next to gene name i.e. up - up regulated in microarray, down - down regulated in microarray. If the protein is encoded by a high copy suppressor encoded within pYE13, the plasmid isolate is shown: 'S3' – suppressor from transformant 3.

Gene	Function
Secretory pathway/cell wall	
COG5/COG6 (S3)	Components of the conserved oligomeric Golgi complex, a cytosolic tethering complex that functions in protein trafficking to mediate fusion of transport vesicles to Golgi compartments.
YIP3 (S3, down)	Protein localized to COPII vesicles, proposed to be involved in ER to Golgi transport; interacts with members of the Rab GTPase family and Yip1p; also interacts with Rtn1p.
SFB2 (S3)	Probable component of COPII coated vesicles that binds to Sec23p; homologous to Sec24p; involved in ER to Golgi transport and in autophagy.
BET4 (up)	Subunit of Type II geranylgeranyltransferase required for vesicular transport between the ER and Golgi; provides a membrane attachment moiety to Rab-like proteins Ypt1p and Sec4p.
TUL1 (up)	Golgi-localized RING-finger ubiquitin ligase (E3), involved in ubiquitinating and sorting membrane proteins that contain polar transmembrane domains to multivesicular bodies for delivery to the vacuole.
ATG14 (up)	Autophagy-specific subunit of phosphatidylinositol 3-kinase complex I (with Vps34/15/30p); Atg14p targets complex I to the phagophore assembly site (PAS); required for localizing additional ATG proteins to the PAS.
GPI15 (S3)	Protein involved in the synthesis of N-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI), an intermediate in the synthesis of glycosylphosphatidylinositol (GPI) anchors.
CWP2 (up)	Covalently linked cell wall mannoprotein, major constituent of the cell wall; plays a role in stabilizing the cell wall; involved in low pH resistance; precursor is GPI-anchored.
ALG11 (S3)	Alpha-1,2-mannosyltransferase, involved in asparagine-linked glycosylation in the ER.
PHO11 (down)	One of three repressible acid phosphatases, a glycoprotein that is transported to the cell surface by the secretory pathway; induced by phosphate starvation and coordinately regulated by PHO4 and PHO2.
VPS62 (up)	Vacuolar protein sorting (VPS) protein required for cytoplasm to vacuole targeting of proteins.
VPS70 (up)	Protein of unknown function involved in vacuolar protein sorting.
VAC7 (S3)	Integral vacuolar membrane protein involved in vacuole inheritance and morphology; activates Fab1p kinase activity under basal conditions and also after hyperosmotic shock.
RNA/DNA	
LOC1 (S2)	Nuclear protein involved in asymmetric localisation of ASH1 mRNA; binds dsRNA in vitro; constituent of 66S pre-ribosomal particles.
DEG1 (S2)	Non-essential tRNA pseudouridine synthase, important for translation efficiency and cell growth.
BDP1 (S3)	Essential subunit of RNA polymerase III transcription factor (TFIIIB).
SPT6 (S5)	Transcriptional RNA pol II subunit.
RRM3 (S12)	DNA helicase for DNA replication and Ty transposition. Related to Pif1p.
RPL2A (up)	Protein component of the large (60S) ribosomal subunit.
RPS31 (up)	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin; ubiquitin may facilitate assembly of the ribosomal protein into ribosomes; interacts genetically with translation factor eIF2B.

Gene	Function
RNA/DNA (cont.)	
RPS31 (up)	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin; ubiquitin may facilitate assembly of the ribosomal protein into ribosomes; interacts genetically with translation factor eIF2B.
POP3/POP5 (down)	Subunits of both RNase MRP, which cleaves pre-rRNA, and nuclear RNase P, which cleaves tRNA precursors to generate mature 5' ends.
NCS2 (down)	Protein required for thiolation of the uridine at the wobble position of Lys(UUU) and Glu(UUC) tRNAs; has a role in urmylation and in invasive and pseudohyphal growth.
RPB8 (down)	RNA polymerase subunit ABC14.5, common to RNA polymerases I, II, and III.
ELP4 (down)	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA; required for Elongator structural integrity.
Cytoskeleton	
CIN2 (S1)	GTPase activating protein (GAP) for Cin4p; tubulin folding factor C for β-tubulin (Tub2p) folding.
DAM1 (S5)	Kinetochore protein involved in microtubule binding.
CCT6 (down)	Part of the TCP1/CCT complex that folds actin and tubulin.
CCT7 (S6)	Part of the TCP1/CCT complex that folds actin and tubulin.
Signalling	
MSG5 (S3)	Dual-specificity protein phosphatase; required for maintenance of a low level of signaling through the cell integrity pathway, adaptive response to pheromone; regulates and is regulated by Slt2p; dephosphorylates Fus3p.
SLM2 (S3)	Phosphoinositide PI4,5P(2) binding protein, forms a complex with Slm1p; acts downstream of Mss4p in a pathway regulating actin cytoskeleton organization in response to stress; phosphorylated by the TORC2 complex.
SLT2 (S12)	Serine threonine MAP kinase. Cell integrity pathway signalling.
PRS4/PRS5 (down)	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase, synthesizes PRPP, which is required for nucleotide, histidine, and tryptophan biosynthesis. Has a role in remodelling of the cell wall and cell integrity signalling.
YHR032W (S12)	Unknown protein (putative substrate of cAMP-dependent protein kinase (PKA).
CST9 (up)	SUMO E3 ligase; required for synaptonemal complex formation; localizes to synapsis initiation sites on meiotic chromosomes; potential Cdc28p substrate.
Mitochondria	
COX5A (S3)	Subunit Va of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain; predominantly expressed during aerobic growth.
IDH1 (S3)	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle.
ISA1 (S4)	Mitochondrial matrix protein involved in iron-sulphur cluster biosynthesis.
PHB2 (up)	Subunit of the prohibitin complex (Phb1p-Phb2p), a 1.2 MDa ring-shaped inner mitochondrial membrane chaperone that stabilizes newly synthesized proteins; determinant of replicative life span; involved in mitochondrial segregation.
MRPL8 (up)	Mitochondrial ribosomal protein of the large subunit.
MRP49 (up)	Mitochondrial ribosomal protein of the large subunit.

Gene	Function
Transporters/permease	
ADP (up)	Putative ATP-dependent permease of the ABC transporter family of proteins.
OPT2 (S15)	Oligopeptide transporter.
TPO1 (S4)	Polyamine transporter.
Other	
LAP2 (S3)	Leucyl aminopeptidase yscIV (leukotriene A4 hydrolase) with epoxide hydrolase activity, metalloenzyme containing one zinc atom.
BOP3 (S3)	Protein of unknown function, potential Cdc28p substrate; overproduction confers resistance to methylmercury.
NCE103 (S3)	Carbonic anhydrase; involved in non-classical protein export pathway.
GZF3 (S6)	Transcription factor for nitrogen catabolite repression.
KAR4 (S10)	Transcription factor required for karyogamy and meiosis.
YPR196W (S15)	Maltose activator.
THI21 (S16)	Encodes protein involved in thiamine biosynthesis.
YPL257W (S16)	Unknown protein; may have 3 TMDs and may associate with heat shock protein.
LDB17 (up)	Protein of unknown function.
SPG3 (up)	Protein required for survival at high temperature during stationary phase.
LOT6 (up)	FMN-dependent NAD(P)H:quinone reductase, may be involved in quinone detoxification; expression elevated at low temperature; sequesters the Cin5p transcription factor in the cytoplasm in complex with the proteasome under reducing conditions.
DOG2 (up)	2-deoxyglucose-6-phosphate phosphatase, member of a family of low molecular weight phosphatases, similar to Dog1p, induced by oxidative and osmotic stress, confers 2-deoxyglucose resistance when overexpressed.
SLZ1 (up)	Sporulation-specific protein with a leucine zipper motif.
GDH1 (up)	NADP(+)-dependent glutamate dehydrogenase, synthesizes glutamate from ammonia and alpha-ketoglutarate; expression regulated by nitrogen and carbon sources.
NPY1 (down)	NADH diphosphatase, hydrolyzes the pyrophosphate linkage in NADH and related nucleotides.
AAH1 (down)	Adenine deaminase; involved in purine salvage; transcriptionally regulated by nutrient levels and growth phase.
ARO7 (down)	Chorismate mutase, involved in aromatic amino acid biosynthesis.

Table 6.6: Genes identified in the HCS screen and microarray. Up: up-regulated in cells expressing NleG8-2; down: down-regulated in cells expressing NleG8-2. Potential suppressors (S) are shown with the number of the pYE13 transformant e.g. S3: suppressor from transformant 3.

6.4. Discussion

A high copy plasmid suppressor (HCS) screen was performed. Toxicity of NleG8-2 expression was rescued by high copy number suppressors from a *S. cerevisiae* pYE13-based library. Plasmids (pYE13) were isolated from colonies able to grow when NleG8-2 was expressed. Isolated plasmids were retested for their ability to suppress the growth inhibition caused by NleG8-2 by plasmid purification and retransformation. Table 6.3 shows the genomic inserts of each recovered pYE13 plasmid and lists the genes (i.e. potential suppressors) present in the inserts. Transcriptome analysis of yeast expressing NleG8-2 and yeast expressing empty vector pYES DEST52 showed that 33 genes were significantly up/down regulated when NleG8-2 was expressed. Tables 6.4 and 6.5 show those genes that were significantly up/down regulated respectively. Table 6.6 lists suppressors and differentially regulated genes according to their function/localisation within the yeast cell.

6.4.1. The yeast secretory pathway

A large number of the genes identified by the HCS screen and microarray appear to be involved in the secretory pathway and cell wall biosynthesis. A further 18 gene products are involved in protein modification, trafficking and cell wall integrity (CWI) signalling. Cell surface expansion and cell wall biosynthesis/remodelling are key processes in growth, division, mating and the response to environmental changes in budding yeast [423]. The secretory pathway is integral to these processes as cell wall components and plasma membrane proteins involved in cell wall remodelling are delivered to the plasma membrane by the secretory pathway. Disruption of the secretory pathway hinders cell wall biosynthesis

and reduces cell wall integrity [423]. Yeast cells detect changes in cell wall biosynthesis and integrity using sensor proteins such as Mid2 and Wsc. These proteins transmit signals via kinase cascades to activate transcription factors such as Rlm1 thus inducing a transcriptional response e.g. to promote cell wall remodelling [465]. It is possible that NleG8-2 affects the secretory pathway which in turn disrupts cell wall biosynthesis/integrity and triggers CWI signalling. Interestingly, in a study by Grose *et al*, the Ugp1 overexpression phenotype (i.e. sensitivity to cell wall integrity stress conditions) [466] was suppressed by a similar complement of genes as found in this study. This indicates that NleG8-2 may cause cell wall stress.

A basic graphical representation of the yeast secretory pathway, its interactions with cell wall synthesis and CWI MAPK signalling pathways is shown in figure 6.3. The localisation of potential suppressors or up/down regulated gene products involved in this pathway is also shown.

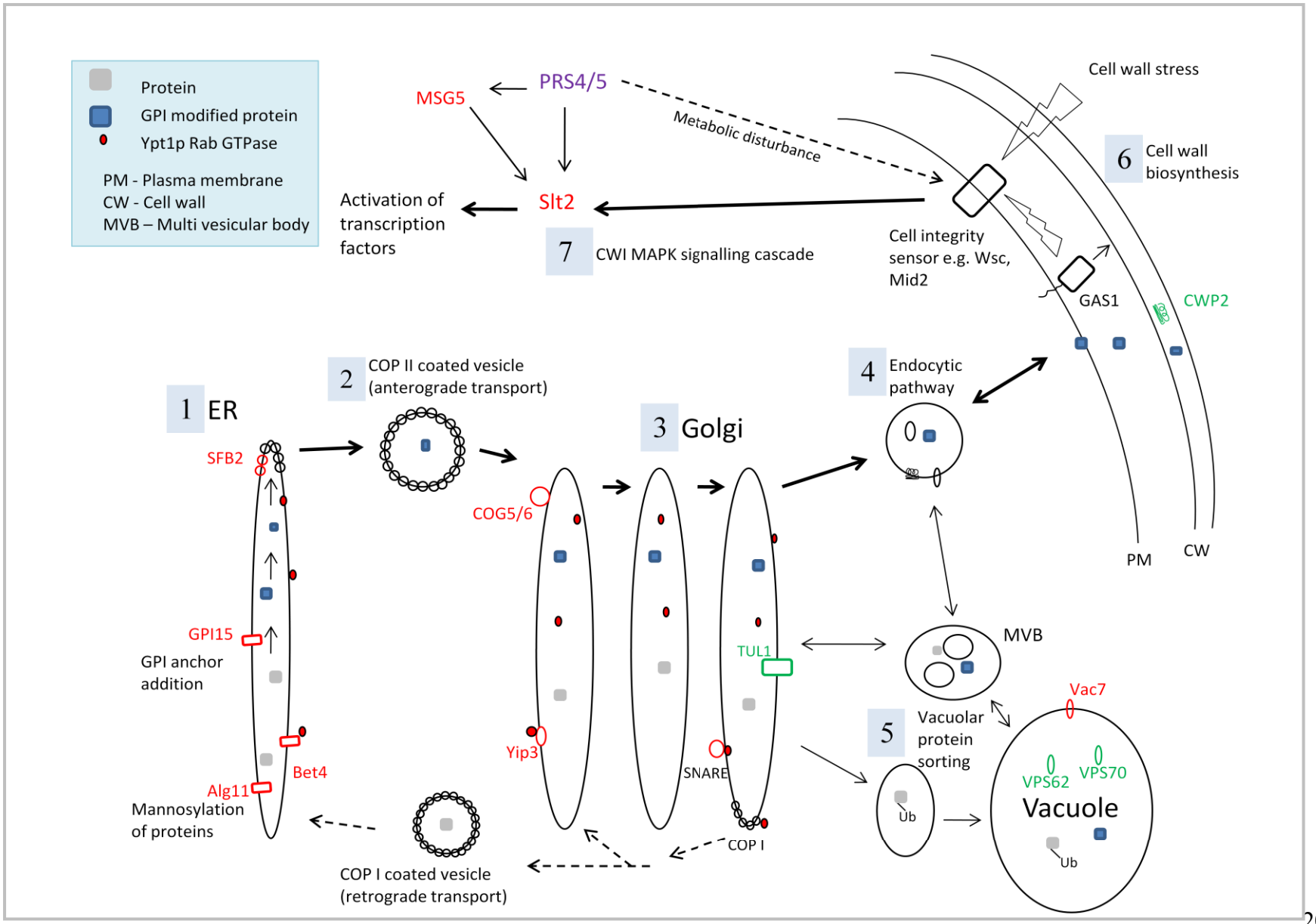

Figure 6.3: The yeast secretory pathway and its interactions with the cell wall. Potential suppressors identified in the HCS screen are shown in red lettering, up-regulated genes (microarray) in green and down-regulated genes (microarray) in purple. Ub - ubiquitin. Steps of the secretory pathway (indicated by numbers) are explained in the text.

The yeast secretory pathway and cell wall integrity:

1. ER. Proteins intended for secretion are translocated into the ER. Proteins may be modified e.g. glycosylphosphatidylinositol (GPI) modified or mannosylated [423] in the ER [467] by proteins such as GPI15 and ALG11. Proteins are selectively packaged into COPII coated vesicles for anterograde transport to the Golgi [468]. Selective packaging of proteins into COPII vesicles from the ER depends on multiple factors including components of the COPII coat and the Rab GTPase Ypt1p [469]. GPI-modified proteins depend on Ypt1p for selective packaging [469].

2. COPII vesicles travel to the Golgi where they are tethered to the Golgi membrane to promote membrane fusion. Accurate fusion requires the COPII coat, Rab GTPases, tethering factors and SNARE complexes [470].

3. Proteins are modified (e.g. glycosylated) as they move through the Golgi (anterograde transport) [471]. Golgi-resident/ER-resident proteins are transported back to the correct compartment in COPI coated vesicles (retrograde transport) [472].

4. Proteins intended for secretion are delivered to the plasma membrane in endocytic vesicles via early/late endosomes using the endocytic pathway [423].

5. Proteins may also be directed from the Golgi to the vacuole either directly (ALP pathway) or indirectly (CPY pathway) via intermediate multivesicular bodies (MVB) [473]. Proteins with polar residues in their transmembrane domains e.g. misfolded

proteins may be ubiquitinated (by Tul1) and transported to the vacuole for degradation [473].

6. Proteins are secreted. GPI-modified proteins are anchored in the plasma membrane by the GPI anchor [474]. Alternatively the GPI anchor may be cleaved to release the proteins into the periplasm/cell wall [475, 476]. Proteins such as Gas1p contribute to cell wall biosynthesis [477] [478]. Cell wall stress (caused by osmotic shock/biosynthesis disruption/oxidative stress etc.) is detected by cell wall sensor proteins such as Wsc [479]. Wsc sensors also detect metabolic disturbances via PRS4 and PRS5 [480].

7. Cell wall stress causes sensor proteins to trigger a MAPK signalling cascade via the Slt2 pathway to effect transcriptional changes via activation of transcription factors such as Swi4/Swi6 [481] and Rlm1 [482] [436].

6.4.2. ER to Golgi vesicle trafficking

As highlighted in figure 6.3, potential suppressors/up/down regulated genes from each stage of the secretory pathway/CWI signalling pathway were identified. Notably, five of these genes (SFB2, COG5, COG6, YIP3 and BET4) are involved in COPII-mediated vesicular transport between the ER and Golgi:

SFB2 (suppressor)

SFB2 is a homologue of Sec24, a component of the COPII coat [483]. The COPII coat is composed of numerous Sec complexes plus a GTPase [484] and is required for selective packaging of proteins into transport vesicles destined for the Golgi [485]. SFB2 is specifically required for secretion of the plasma membrane protein Gas1p, an essential enzyme involved in cell wall synthesis [486].

COG5 and COG6 (suppressors)

Both COG5 and COG6 form part of the evolutionarily conserved oligomeric Golgi complex (COG) [487] which acts to tether Golgi/vesicle membranes to promote membrane fusion. Cytosolic COG interacts with the Golgi membrane via Rab GTPases Ypt1p and Ypt6p [488] [489], SNARE proteins and the COPI coat [471].

YIP3 (potential suppressor and down-regulated gene)

YIP3 is a Rab-GDI displacement factor (GDF) that forms complexes with Ypt1p [490]. Rab GTPases are continually distributed to different parts of the cell as a consequence of their role in directing vesicular transport. Therefore, an important process within the cell is Rab recycling, whereby inactive Rabs (Rab-GDI) are retrieved and delivered back to their resident organelle e.g. Golgi. Rab-GDI displacement factors are involved in reloading Rabs back onto their target membranes [490]. YIP3 ensures that Ypt1p is delivered back to the ER membrane to promote COPII budding [490]. YIP3 is prenylated to facilitate membrane attachment. Interestingly, overproduction usually inhibits growth and causes the ER to proliferate (this is thought to be due to deficient COPII budding) [490].

BET4 (upregulated)

BET4 is part of a geranylgeranyltransferase that catalyses the prenylation of Rab-like GTPases, Ypt1p and Sec4p [491]. Prenylation of Rabs enables them to attach to vesicular/organelle membranes and direct movement of vesicles within the cell [492].

Interestingly, all of the products of the suppressor genes mentioned above interact with or are necessary for the correct functioning/localisation of Ypt1p. This suggests that NleG8-2 may affect the Rab GTPase (and associated vesicular targeting). In addition to the functions described above, Ypt1p is required for selection of GPI-anchored proteins into COPII vesicles [469]. GPI15, an enzyme involved in the synthesis of GPI anchors was also identified as a potential suppressor of NleG8-2. GPI is a complex glycoplipid that can anchor cell surface proteins to membranes [493]. A large number of cell wall proteins are GPI-modified [494].

These proteins would most likely be affected by disruption of Ypt1p as they depend on Ypt1p for selection into COPII vesicles and vesicle-mediated transport to the plasma membrane [495]. Mislocalisation of GPI-modified cell wall proteins in this way would likely diminish the integrity of the cell wall (as has been shown to occur in yeast with a non-functioning secretory pathway [496]). We might therefore expect to see evidence of disruption of the cell wall and associated MAPK signalling pathways in cells expressing NleG8-2.

6.4.3. Disruption of the secretory pathway affects the yeast cell wall and CWI signalling

Numerous suppressors and differentially regulated genes are involved in cell wall synthesis and CWI signalling (see below). Significantly, a number of these have been shown to be able to compensate for cell wall defects caused by impaired delivery of membrane and cell wall precursors.

6.4.4. Cell wall biosynthesis

CWP2 was upregulated in cells expressing NleG8-2. CWP2 is a major cell wall mannoprotein and is GPI-modified. CWP2 is transported to the plasma membrane via the secretory pathway and has been shown to act as a multicopy suppressor for cells in which the bud neck is weakened by impaired delivery of membrane and cell wall precursors [496]. Increased CWP2 may compensate for the loss of cell wall strength caused by NleG8-2-mediated disruption of the secretory pathway. ALG11, a mannosyltransferase essential for N-linked glycosylation of proteins [497] was identified in the HCS screen. The outer cell wall is composed of highly

glycosylated mannoproteins [498] thus ALG11 may provide mannoproteins for the outer cell wall layer to improve cell wall strength. Also, N-glycosylation of Mid2p is required for Mid2p to perceive changes in the cell wall [499] – ALG11 might therefore increase the ability of the cell to sense cell wall stress.

6.4.5. CWI signalling

The CWI pathway is involved in cell wall remodelling. Slt2 is the MAP kinase of this route and activates the transcription factor Rlm1. CWI signalling can be triggered by; heat stress, hypo-osmotic shock, oxidative stress, cell wall stressing agents and impairment of cell wall biosynthesis [500]. Delocalisation of signalling components can also activate the CWI pathway [501]. A number of components of the CWI signalling pathway were identified as suppressors (MSG5 and Slt2) or were up/down regulated in the microarray (PRS4 and PRS5). PRS4/5 signals through MSG5 and Slt2 in the CWI MAPK signalling pathway [480]. A role for Slt2 in assembly of the chitin ring at the bud neck has recently been demonstrated by Gomez *et al*. [502]. *Slt2* was shown to affect septin localisation at the bud neck causing bud neck fragility. Notably, overexpression of CCT7 (another potential suppressor of NleG8-2) was shown to reduce bud neck fragility in an *Slt2* mutant [502]. It is possible that CCT7 may act as a suppressor of NleG8-2 by compensating for mislocalised membrane proteins that are involved in cell wall/chitin synthesis.

The genes identified in this study suggest that CWI MAPK signalling in cells expressing NleG8-2 is triggered by the cell integrity sensor Wsc. This plasma membrane protein

responds to cell wall stress, PRS4/5 and a plasma membrane protein, Gas1p (although the nature of the signals received and mechanism of signal transduction are poorly understood [423]). Gas1p is a 1-3 β-glucan synthase and is involved in construction of the cell wall. Interestingly, Gas1p is GPI-modified and as such relies on Ypt1p for sorting into COPII vesicles. Gas1p is also directed to COPII vesicles by SFB2. SFB2 and GPI15 (potential suppressors) are integral to the trafficking and localisation of Gas1p to the plasma membrane. It seems likely therefore that Gas1p is mislocalised in cells expressing NleG8-2 leading to cell wall stress (sensed by Wsc/Mid2 causing CWI signalling). Overexpression of SFB2 may compensate for loss of Ypt1p-mediated sorting of Gas1p into COPII vesicles whereas GPI15 may increase the amount of Gas1p-GPI in the cell (thereby increasing the probability of Gas1p reaching the plasma membrane). Gas1p was not however, identified in the screens therefore we may only speculate that activation of the CWI pathway is by this route. Slt2 and MSG5 may act as suppressors of NleG8-2 by increasing signalling through the CWI pathway thereby preparing the cell for cell wall stress caused by aberrant protein trafficking.

6.4.6. Disruption of vacuolar protein sorting

The yeast vacuole(s) is similar to the mammalian lysosome and has three main functions: protein degradation, ion and metabolite storage and detoxification [473]. Multiple vesicular pathways transport proteins to and from the vacuole. There are a number of genes encoding vacuolar proteins that have been identified by the screens. These include VPS62, VPS70 and Vac7. The functions of VPS62 and VPS70 are poorly understood although it is presumed that they are involved in regulation of vacuole fusion and fission as mutants demonstrate unusual vacuole morphology [503]. Vac7p is involved in generating phosphoinositides (e.g.

PtdIns[3,5]P_2) on vacuolar membranes. Phosphoinositide signalling is crucial for vesicle trafficking and fusion between vacuolar membranes [473]. VPS62, VPS70 and Vac7 point to a role for NleG8-2 in regulating/disruption of vacuole fusion/fission. Fusion of vesicles and small vacuoles to the main vacuole, like ER and Golgi-derived vesicles, requires SNARE proteins, tethering factors and Rab GTPases whereas fission requires COPI and COPII coat proteins. It is therefore highly likely that vacuolar function and/or morphology would be affected if NleG8-2 disrupted vesicular transport by affecting Rab GTPase or COPII function. Another upregulated gene product involved in Golgi-vacuole protein trafficking is ATG14. ATG14 associates with VPS34 a protein required for regulation of retromer function i.e. helps to retrieve late Golgi proteins from the endosome [503]. ATG14, VPS62, VPS70 and Vac7 may compensate for NleG8-2-mediated disruption of vacuolar protein sorting.

6.4.7. NleG8-2 does not appear to target the cytoskeleton or mitochondria

Surprisingly, only four cytoskeleton-related genes were identified by the HCS screen or microarray. These were: Cin2, Dam, CCT6 and CCT7. Interestingly all are involved in binding/folding tubulin. CCT6 and CCT7 form part of the Cct double ring chaperonin complex in yeast which helps to fold actin and tubulin [504]. Cin2 is involved in activating tubulin folding whereas Dam1 is involved in chromosome segregation by binding chromatids to the kinetochore. The Golgi is usually found at the same site as the centrosome (the major microtubule organising centre) and organisation of Golgi cisternal stacks depends on microtubules [505]. However, disruption of microtubules has only a minor effect on protein trafficking [506]. NleG8-2 appears to have a significant effect on protein trafficking therefore it is unlikely that NleG8-2 disrupts microtubules. CCT7, as mentioned above is a suppressor

of yeast growth defects associated with mislocalised cell wall proteins and weak cell walls [502].

Surprisingly, no actin related genes were identified in this study. T3SS often target the actin cytoskeleton and associated Rho GTPases [507] [508] [356]. We may have expected to see some perturbation of the actin cytoskeleton/function as a result of CWI signalling. However, experimental evidence suggests that the CWI pathway drives polarisation of the actin cytoskeleton and is not involved in actin depolymerisation [501]. Therefore any depolymerisation caused by cell wall stress would be transient as CWI signalling would restore localisation [465]. Actin-related genes would not be expected to act as suppressors of NleG8-2 or be up/down regulated in cells expressing NleG8-2.

A small number of mitochondria-related genes were identified in the HCS screen and microarray including COX5A, ISA1, PHB2, IDH1, MRPL8 and MRP49. These genes encode a variety of proteins involved in unrelated processes e.g. ribosomal subunits and respiratory enzymes. It does not appear therefore that NleG8-2 targets mitochondria or mitochondrial processes. Correct mitochondrial function is dependent on proper functioning of the secretory pathway [509] therefore the mitochondrial-related suppressors may rescue mitochondrial dysfunction caused by a perturbed secretory pathway.

Other genes identified by the microarray and HCS screen encode a variety of enzymes, transcription factors and unknown proteins. Their significance in resistance to NleG8-2

mediated growth retardation is unclear. They likely represent non-specific genes that would also enhance growth in the control or genes that have been isolated by being encoded next to a suppressor. This highlights the need for each potential suppressor to be tested individually.

6.4.8. Effect of NleG8-2 on the host cell

Overall the genes identified by the yeast genomic screens appear to point to a role for NleG8-2 in regulating protein trafficking within the host cell. In yeast, it seems that NleG8-2 disrupts protein selection into secretory vesicles and intracellular vesicular transport (most likely ER to Golgi protein transport). We predict that disruption of vesicular transport leads to a decrease in the delivery of enzymes such as Gas1p that are involved in cell wall synthesis to the plasma membrane. Delivery of cell wall precursors (glucans, chitin etc) may also be reduced. This affects cell wall integrity leading to activation of the CWI MAPK signalling pathway. Transcriptional changes are thus induced by activated transcription factors which results in growth inhibition.

The effect on the yeast cell wall is likely to be a side-effect of NleG8-2 disruption of the secretory pathway. In mammalian cells, disruption of protein trafficking and secretion by NleG8-2 might lead to subversion of the cell or the immune response i.e. by preventing GPI-anchored proteins such as cell surface receptors, cell adhesion molecules, complement regulatory proteins and hydrolases reaching the cell surface [510]. Alternatively, disruption of secretion may prevent ingestion of bacteria by predatory protozoa or prevent fusion of deleterious vesicles with EHEC-containing vacuoles within bacterivorous cells. Notably,

NleA was shown by Kim *et al* (2007) to bind Sec24 and disrupt COPII coat function thereby reducing the efficiency of cellular secretion [511]. This shows that disruption of COPII function and disruption of the host cell secretory pathway may contribute to EHEC O157:H7 virulence. It would be interesting to dissect the relationship between NleG8-2 and NleA to determine whether they act cooperatively (or are reciprocally redundant) to perturb the secretory pathway of host cells to contribute to cellular subversion.

6.4.9. Possible molecular mechanism of NleG8-2

As mentioned in the introduction to this thesis, T3SS effector proteins often target/mimic host cell GTPases [507]. Many T3SS effectors target Rho-like GTPases involved in regulating the host cell cytoskeleton, although a number of effectors have been found to target Rab GTPases (see below). Rab GTPases are at the forefront of vesicular trafficking within the cell and as such, represent a probable T3SS effector target [512]. For example, *Pseudomonas aeruginosa* effector ExoS possesses ADP-ribosyltransferase activity which results in inhibition of Rab activity; this interferes with clathrin-coated vesicle maturation [512]. *Pseudomonas syringae* effector AvrPto interacts with two Rab proteins to decrease extracellular vesicle trafficking and secretion of plant defence proteins [513]. Many intracellular T3S organisms target Rab GTPases using T3SS effectors to control which types of (i.e. beneficial/deleterious) vesicles can fuse with the pathogen-containing compartment. For example, *Chlamydia spp* have been shown to recruit Rabs to facilitate fusion of Golgi-derived host vesicles with the inclusion membrane [514]. Similarly, *Salmonella* containing vacuoles (SCVs) mature in a Rab7-dependent manner but avoid lysosomal fusion [515].

As discussed in chapter 5, NleG8-2 contains 3 invariant residues that are similar to the C/H/D catalytic triad found in cysteine proteases. We found that yeast growth inhibition caused by NleG8-2 was dependent on these residues (see chapter 5). It is possible therefore that NleG8-2 may function as a novel form of cysteine protease. Other T3SS effectors that have cysteine protease activity include SseL, a *Salmonella* effector that acts as a deubiquitinase to kill macrophages [155] and the *Yersinia* effector YopT which disrupts the cytoskeleton. Interestingly, YopT causes cytoskeletal changes by disrupting the localisation of Rho-like GTPases by removing their prenyl modification [371]. Removal of prenyl groups from the Rho GTPases is dependent on the cysteine protease catalytic triad (C/H/D residues) [371]. Prenyl groups are essential for small GTPase targeting and localisation within eukaryotic cells as they allow the GTPase to attach to membranes [492].

We could speculate that NleG8-2 has a similar function to YopT i.e. that cysteine protease activity removes the prenyl moiety from GTPases, except that NleG8-2 targets Rab GTPases (most likely Ypt1p) to disrupt vesicle trafficking within the host cell. This hypothesis is supported by the types of suppressors identified by the HCS screen - many potential suppressors were involved in the secretory pathway, particularly processes controlled by Ypt1p. A mechanism for the suppressor activity of the four Ypt1p interacting proteins would be as follows: Bet4 may increase the prenylation of Ypt1p thereby compensating for the loss of prenyl groups caused by NleG8-2. Overexpression of COG subunits may increase the probability of prenylated Ypt1p interacting with COG thereby increasing the number of successful Golgi-vesicle fusion events. Overexpression of YIP3 may increase Rab/Ypt1p recycling and ensure that any remaining prenylated Rabs are available for directing vesicular transport/protein secretion. Ypt1p was not identified in either the microarray or HCS screen

therefore its identification as the target of NleG8-2 is far from certain. An alternative target may be YIP3 which is also prenylated. Disruption of YIP3 function might cause similar effects on the yeast cell as loss of Ypt1p function. More research (as described in chapter 7) is required to investigate the function of NleG8-2 and the possibility that this effector acts as a cysteine protease to de-prenylate Rab GTPases/Rab accessory proteins.

6.4.10. Validation of HCS screen results

In order to confirm which of the genes identified through the HCS screen are involved in suppression of NleG8-2-mediated growth inhibition there are a number of issues that must be resolved. Firstly, the level of expression of NleG8-2 must be quantified in each suppressor strain to show that the level of NleG8-2 expression is the same as FY833-NleG8-2. This will ensure that no suppressors were isolated simply because they diminished expression of NleG8-2. Also, most HCS plasmids contained multiple genes. Therefore each gene must be tested individually in FY833-NleG8-2 to find the gene responsible for suppression of growth inhibition. For example, at present it is impossible to say which gene present in pYE13 from transformant 3 is the true suppressor. Unfortunately there was insufficient time in this study to complete this aspect of the assay. In addition, the suppressor activity of each suppressor gene identified should also be quantified as the efficacy of a suppressor may give clues as to the target of NleG8-2. Another issue that must be resolved is the use of the GAL1 promoter for NleG8-2 expression. Growth on galactose is known to stress cells [516] and alters the synthesis of UDP-glucose (a substrate for cell wall polymers and glycoproteins). It is therefore possible that the genes involved in cell wall and glycoprotein synthesis may be involved in a general response to growth on galactose i.e. these suppressors may act by

relieving the stress caused by galactose and not NleG8-2. In order to determine if this is the case, NleG8-2 could be expressed from an alternative expression vector containing a different promoter (e.g. *tet*O promoter).

Further studies could also make use of stress-inducing conditions e.g. caffeine, high temperature to determine if yeast cells expressing NleG8-2 are more sensitive to the effects of the effector under these conditions and also to see if the suppressor activity of a given gene is lost under these conditions. For example, changes in suppressor activity in the presence of Brefeldin A (a drug that disrupts the Golgi [517]) might indicate that the suppressor functions by relieving stress on the Golgi caused by NleG8-2. There are numerous methods that could be used to detect secretory stress. A block in membrane trafficking from the ER causes a slowing of phospholipid synthesis in the ER and can be detected by increased triacylglycerol synthesis using fluorescence-activated cell sorting (FACS) [518].

6.4.11. Validation of microarray results and issues raised

The Affymetrix microarray is, like other types of microarray, subject to limitations. Statistical settings and filters may lead to different conclusions as changes in gene expression may appear to be significant (or not) based on the test/P-value used. To ensure the statistical validity of the microarray results several steps were taken during the course of the experiment to reduce discrepancies. Three biological replicates of yeast expressing NleG8-2 and yeast expressing pYES-DEST52e (empty vector control) were performed; yeast cells were harvested at the same growth rate and yeast were frozen immediately after harvest to prevent

alterations in gene expression due to changes in environmental conditions. However, the possibility of differences in gene expression due to expression of an exogenous protein i.e. non-specific effects, should be considered. The variance in transcription for all probes should also be considered in order to assess whether the differentially regulated genes as listed in tables 6.4 and 6.5 were the only genes to show transcriptional changes when NleG8-2 was expressed. Unfortunately, graphical data showing the variance in transcription across all probes was not available. Microarray results could be validated further by performing more replicates. Also, performing a microarray on mammalian enterocytes infected with EPEC expressing NleG8-2 and enterocytes infected with an EHECΔNleG8-2 mutant may provide further evidence for the role of NleG8-2 in EHEC O157:H7 infection (although redundancy between NleG effectors as discussed in chapter 1 may affect interpretation of results).

6.4.12. Evaluation of the techniques used

The two approaches, microarray and high copy suppression screen complemented each other. Results from the HCS screen validate results from the microarray. For example, YIP3 was identified in the HCS screen and was seen to be down-regulated using the microarray. This is evidence to suggest that NleG8-2 affects YIP3 or a pathway requiring YIP3. Results from this study imply that NleG8-2 affects vesicular trafficking in the host cell, possibly through disruption of Rab GTPase membrane targeting. Future investigations into the effects of NleG8-2 should therefore concentrate on characterising the ER, Golgi and the secretory pathway in mammalian/yeast cells expressing NleG8-2.

CHAPTER SEVEN

Discussion

7.1. Discussion

7.1.1. Summary of findings

Type III secretion is an important virulence factor of EHEC O157:H7. T3SS effectors encoded within the LEE have been well characterised. However, many effectors that are encoded outside the LEE are only just beginning to be investigated. The work presented in this thesis investigated several novel EHEC O157:H7 Sakai T3SS effectors that had been predicted *in silico* by PSI-BLAST homology searches.

Work began by investigating the translocation of putative effectors through the LEE encoded T3SS. Evidence that candidate effectors were secreted via type III secretion was provided by a β-lactamase translocation assay. The results from this assay, plus several other secretion/translocation assays performed by collaborators, confirmed that 39 candidate effectors were T3SS effectors. This exciting result confirms that the repertoire of T3SS effectors in EHEC O157:H7 is much larger than previously imagined.

Investigations into the localisation of effectors in mammalian cells revealed that novel effectors localise to various cellular compartments and organelles. Observation of the actin cytoskeleton in cells expressing novel effectors revealed that the EspM2 effector caused the formation of actin stress fibres in mammalian cells.

A yeast cell model was also employed to investigate the effects of selected effectors on cell growth, shape and physiology. Expression of a small number of effectors was toxic to yeast and inhibited yeast growth. Effects of effector expression also included redistribution of chitin in yeast cell walls and unusual cell morphology. Mutagenesis of EspM2 revealed that the conserved WxxxE motif is required for effector toxicity but that other regions and residues of the proteins may also be important.

Finally, work focused on the effector NleG8-2. Transcriptomics and a high copy suppression screen were used to investigate disruption of yeast cellular physiology. Results from both screens were promising and showed that NleG8-2 might target vesicular trafficking within the cell. Dissection of sequence-activity relations for NleG8-2 was also attempted, revealing that yeast growth inhibition was dependent on three conserved C/H/D residues. This finding supports the hypothesis that NleG8-2 functions enzymatically. This is the first time that an NleG family member has been shown to have an effect on host cells. In conclusion, the results summarised above satisfy the aims of the thesis listed in chapter one.

7.1.2. Re-evaluation of thesis

In re-evaluating this thesis, a number of issues are raised. For example, during transfection experiments it may have been productive to try other cationic lipid-based transfection reagents to improve transfection efficiency and reliability. Despite the problems associated with transfection, mammalian cells appear to be better than yeast for localisation studies as they are much larger - thus enabling subcellular structures to be visualized more easily.

Overall, the yeast cell model was useful for initial screening of effector toxicity – the yeast were easy to handle and the system allowed effectors to be screened simultaneously. However, more insight into effector function would have been gained through the use of 'stressors' in yeast growth assays as discussed in chapter 5. More insight into cellular localisation of effectors in yeast may also have been gained through the use of additional reporter strains and confocal microscopy, unfortunately these were not available during this study.

7.1.3. Further work arising from this thesis

Results from this thesis provide many avenues for further work. A pressing issue is to provide a definitive function/target of NleG8-2. Firstly, as discussed in chapter six, genes identified from the high copy suppression screen must be investigated for suppressor activity individually to define which are true suppressors. Secondly, the secretory system of yeast expressing NleG8-2 should be investigated to determine whether vesicular trafficking is disrupted. Although, no definitive function for NleG has been found in this study, the expansion of the NleG family in EHEC O157:H7 and the presence of numerous functional NleG genes in EHEC O157:H7 suggests that this effector is a significant player in EHEC O157:H7 virulence or colonisation. In order to determine the role of NleG in EHEC pathogenicity or colonisation, NleG could be knocked out and the effects of EHEC O157:H7 ΔNleG investigated using a bovine infection model, bIVOC or human IVOC. Alternatively EHEC NleG genes could be introduced into EPEC (possesses one NleG) and the effects on EPEC pathogenicity investigated.

Further questions regarding NleG8-2 include:

- What is the structure of NleG8-2?
- With which eukaryotic proteins does NleG8-2 interact? Purification of NleG8-2 was attempted during this study but was unsuccessful. Further work using alternative expression vectors may enable pull down/co immunoprecipitation studies to be performed and eukaryotic target proteins to be identified.
- How are the conserved Cys_{136}, Asp_{162} and His_{176} residues involved in effector function – is NleG8-2 a cysteine protease?
- Is NleG8-2 necessary for colonisation of humans or cattle?

In addition to questions regarding NleG8-2, there remain further questions regarding EHEC T3SS, effectors and pathogenicity in general:

- What are the functions of the remaining novel effectors?
- How much redundancy is there between the T3SS effectors of EHEC O157:H7?
- Are effectors involved in colonisation of cattle/humans or are they involved in bacteria-protozoa interactions e.g. do they offer defence against predation?
- Do effectors act in combination with other effectors – is the combination of effectors more important than each individual effector (as has been proposed by Kenny *et al* [357])?
- Does the combination of effectors define host specificity as shown for *P. syringae* [356]?

Interestingly, recent work by Asadulghani *et al* [294] demonstrated that although many Sakai prophages, that carry effectors in their passenger compartments, are defective (with the exception of NleA and Stx harbouring phage), many were able to excise themselves from the host genome, replicate, package phage DNA and be released from O157:H7 cells. Transfer of these apparently defective prophage to other *E. coli* was also demonstrated, probably due to interactions between defective prophage resulting in formation of functional phage [294]. These findings have exciting implications for T3SS effector research and our understanding of EHEC O157:H7 evolution. The use of next generation DNA sequencing technology and the increase in the number of sequenced bacterial genomes will undoubtedly reveal the existence of other T3SS effectors and provide insight into the vast metagenome to which EHEC O157:H7 phage-encoded effectors belong. Type-III secretion systems and their effectors will provide stimulus for exciting research for years to come. The mid-throughput approaches to effector confirmation, localisation and functional characterisation described in this thesis provide a useful framework for investigating novel T3SS effector repertoires.

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