Mechanisms of Action of the *Pseudomonas aeruginosa* Type III Secreted Toxins

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative bacterium found ubiquitously in soil and water. It is an opportunistic pathogen that is capable of causing disease in susceptible humans, for example patients suffering from cystic fibrosis, burns or the immunocompromised. *P. aeruginosa* produces a large number of virulence factors that enable it to colonise and infect a wide range of tissue types. Among these virulence factors are the toxins secreted by the type III secretion (TTS) system. TTS systems are found exclusively in Gram-negative bacteria and they consist of a needle like structure that injects toxins from the bacteria directly into the cytoplasm of a eukaryotic target cell. *P. aeruginosa* encodes a TTS system that translocates four known toxins into eukaryotic cells; Exoenzyme (Exo) S, ExoT, ExoU and ExoY. ExoS and ExoT contain two catalytic domains, an N-terminal GTPase activating protein (GAP) domain and a C-terminal ADP-ribosyltransferase (ADPRT) domain. ExoU exhibits phospholipase activity and ExoY is an adenylate cyclase. All the TTS toxins from *P. aeruginosa* require a eukaryotic cofactor for activation. The ADPRT domains of ExoS and ExoT require a member of the 14-3-3 protein family, while the eukaryotic cofactors for ExoU and ExoY remain unknown.

In this study we sought to further elucidate the mechanisms of action of the pseudomonal TTS toxins ExoS, ExoU and ExoY. Initially we used *Saccharomyces cerevisiae* as a model in which to study ExoS. We demonstrated that ExoS is so toxic to *S. cerevisiae* that the galactose-inducible *GAL1* promoter system produced enough ExoS under glucose-repressing conditions to prevent transformation of an ExoS expressing construct. We therefore utilised a tetracycline-regulated activator-repressor dual system to provide tight control of ExoS expression and demonstrated that ExoS was indeed highly toxic to yeast. Both the GAP and ADPRT domains of ExoS were cytotoxic to *S. cerevisiae* but the ADPRT domain was responsible for the extreme potency of this toxin. We demonstrated that the ADPRT domain of ExoS disrupted the actin cytoskeleton by causing large aggregates of densely stained cortical actin patches, thick disorganised actin cables and a general loss in actin polarity. The actin disruption phenotype was similar to that of a yeast mutant that expresses mutant actin that is unable to disassociate and therefore forms very stable actin fibres. Thus, the ADPRT domain of ExoS may act by stabilising filamentous actin in *S. cerevisiae*. ExoS also

caused an increase in the number of mating projections formed after treatment with the α-factor mating pheromone and inhibited normal bud formation after release from α-factor induced cell cycle arrest. Finally, using the *S. cerevisiae* system we showed that the ExoS ADPRT domain inhibits DNA synthesis following release from pheremone-induced growth arrest.

After establishing that yeast were sensitive to the toxic effects of ExoS, ExoU and ExoY we used a *S. cerevisiae* deletion library to screen for mutants able to grow in the presence of these TTS toxins. Most of the deletion mutants initially identified as being resistant to ExoS, ExoU or ExoY turned out to be false positives and probably arose due to a mutation in the toxin gene. Our screen did identify a number of yeast mutants that were unable to transcribe the toxin genes, for example deletion of either *GAL3* or *GAL4* rendered *S. cerevisiae* resistant to the *exoU* and *exoY* containing plasmids, as these yeast mutants were unable to utilise galactose to induce expression from the *GAL1* promoter. We also identified a yeast mutant with a deletion in the YGR064W/*SPT4* locus that was able to grow under inducing conditions for ExoS, ExoU and ExoY expression. We determined by complementation analysis that deletion of the *SPT4* gene enabled *S. cerevisiae* to grow. Spt4 is an RNA polymerase II transcription elongation factor required for transcription of long or GC-rich DNA sequences. Thus, we hypothesise that the *SPT4* yeast deletion mutant is resistant to the *exoS*, *exoU* and *exoY* constructs as it is unable to transcribe these GC-rich genes. Our failure to identify any *S. cerevisiae* deletion mutants that were capable of producing the pseudomonal TTS toxins but resistant to their effects suggests that no single, non-essential, non-redundant gene is required for their cytotoxic activity. Thus, the unknown eukaryotic cofactors for ExoU and ExoY cannot be non-essential, non-redundant proteins.

In the final part of this study we used a mammalian epithelial cell line to examine the modification, localisation and toxicity of ExoU. We discovered that ExoU was diubiquitinated at lysine residue 178 through a lysine 63 ubiquitin linkage. We demonstrated that a region at the C-terminus of ExoU between amino acids 679 and 683 was required for this modification and that the tryptophan residue at position 681 was required for wild type levels of diubiquitination. We demonstrated that ExoU localises to the plasma membrane of eukaryotic cells and that the same C-terminal region required for diubiquitination was also required for plasma membrane localisation. The

diubiquitinated form of ExoU was found exclusively in the membrane-enriched particulate cellular fraction and mutation of the diubiquitinated lysine residue 178 did not abolish plasma membrane localisation, suggesting that diubiquitination was a consequence not a cause of plasma membrane localisation. We also demonstrated that the C-terminal region between amino acids 679 and 683 was required for toxicity and phospholipase activity of ExoU. The tryptophan 681 residue was required for wild type levels of phospholipase activity but its mutation did not alter the toxicity of ExoU towards eukaryotic cells. We demonstrated that ubiquitination does not alter the toxicity or phospholipase activity of ExoU but does result in a small increase in degradation of the toxin. We also showed that the eukaryotic co-factor required for ExoU phospholipase activity fractionates with the membrane-enriched particulate cellular fraction. Our results suggest that the C-terminus of ExoU is required to target the toxin to the plasma membrane where its eukaryotic cofactor resides. Once at the membrane, ExoU can act as a phospholipase and is diubiquitinated. The consequences of diubiquitination are unclear but they may target the modified toxin to the endocytic pathway.

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PUBLICATIONS

Papers

1. **Stirling, F.R.** and Evans, T.J. Effects of the Type III Secreted Pseudomonal Toxin ExoS in the Yeast Saccharomyces cerevisiae. *Submitted.*

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Abstracts

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ABBREVIATIONS

3-oxo-C12-HSL	N-3-oxododecanoyl-homoserine lactone
ABP	actin-binding protein
ADP	adenosine diphosphate
ADPRT	ADP-ribosyltransferase
AHL	N-acyl homoserine lactone
AI	autoinducer
AIDS	acquired immunodeficiency syndrome
AMP	adenosine monophosphate
Arp	actin-related protein
ATP	adenosine triphopshate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
C4-HSL	N-butanoyl-homoserine lactone
cAMP	cyclic AMP
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units
CHO	Chinese hamster ovary
$cPLA_2$	cytosolic phospholipase A2
Crk	CT10 regulator of kinase
DAPI	4',6-diamidino-2-phenylindole
DDW	double distilled water
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNTB	5,5'-dithio-bis-(2-Nitrobenzoic Acid)
dNTP	deoxynucleotide triphosphate
Dub	deubiquitin
EDTA	ethylenediaminetetraacetic acid
EF	edema factor
EF-2	elongation factor-2
EGFP	enhanced green flourescent protein
EHEC	enterohemorrhagic E. coli

EPEC	enteropathogenic E. coli
ERM	Ezrin/Radixin/Moesin
Exo	exoenzyme
FAK	focal adhesion kinase
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HeLa	human negroid cervix epitheloid carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IKKb	inhibitor-kappa B kinase b
IL	interleukin
INT	tetrazolium salt
$iPLA_2$	calcium-independent phospholipase A2
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	Luria Bertani
LDH	lactate dehydrogenase
LEE	locus of enterocyte effacement
Ler	LEE encoded regulator
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MKK	MAPK kinase
MLD	membrane localisation domain
mRNA	messenger RNA
MVB	mutivesicular body
NAD+	nicotinamide adenine dinucleotide
NF	nuclear factor
NGS	normal goat serum
NO	nitric oxide

Nramp1	natural resistance-associated macrophage protein-1
N-WASP	neuronal Wiskott-Aldrich syndrome protein
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI(3)P	phopshatidylinositol-3-phosphate
PI(4)P	phopshatidylinositol-4-phosphate
PI(5)P	phopshatidylinositol-5-phosphate
$PI(3,4)P_2$	phopshatidylinositol-3,4-bisphosphate
$PI(3,5)P_2$	phopshatidylinositol-3,5-bisphosphate
$PI(4,5)P_2$	phopshatidylinositol-4,5-bisphosphate
$PI(3,4,5)P_3$	phopshatidylinositol-3,4,5-triphosphate
Plc	phospholipase C
PQS	*P. aeruginosa* quinolone signal
PtdIns	phosphatidylinositol
PTL	pancreatic triglyceride lipase
PTPase	protein tyrosine phosphatase
rExoU	recombinant ExoU
RNA	ribonucleic acid
Rpm	revolutions per minute
SD	synthetic dropout
SDS	sodium dodecyl sulfate
SH	Src homology
SpcU	specific Pseudomonas chaperone for ExoU
SPI	Salmonella pathogenicity island
$sPLA_2$	secreted phospholipase A2
SUMO	small ubiquitin-related modifier
TBS	Tris-buffered saline
TE	Tris-EDTA
Tir	translocated intimin receptor
TTS	type III secretion
YPD	yeast peptone dextrose

CHAPTER 1: GENERAL INTRODUCTION

1.1. Introduction

Pseudomonas aeruginosa is a ubiquitous environmental bacterium that is able to cause life-threatening diseases in compromised individuals. This opportunistic pathogen produces a wide-range of virulence factors that enables it to infect, grow and cause disease within susceptible hosts. One of the main virulence determinants of *P. aeruginosa* is its type III secretion (TTS) system that allows the injection of bacterial toxins directly into the cytoplasm of eukaryotic host cells. *P. aeruginosa* translocates at least four toxins through its TTS system: Exoenzyme (Exo) S, ExoT, ExoU and ExoY.

The work presented in this thesis explores the molecular mechanism of action of the *P. aeruginosa* TTS toxins, specifically ExoS and ExoU. This chapter provides an overview of the diseases caused by *P. aeruginosa* and the virulence factors it produces. Particular attention is paid to the TTS system and the toxins secreted by this system both in general and in *P. aeruginosa*. The aim of this chapter is to emphasise the key role TTS toxins play in pseudomonal infection and thus place into context the importance of my investigation into their action. Also, by reviewing what is currently known about the exotoxins *of P. aeruginosa*, the contribution of my results into understanding their mechanism of action can be assessed.

1.2. *Pseudomonas aeruginosa*

P. aeruginosa is the major pathogenic bacterium belonging to the family Pseudomonadaceae. The name *aeruginosa* arises from the green-blue colony colour of many clinical isolates. This colour is caused by the production of a blue pigment, pyocyanin, and a yellow-brown pigment, pyoverdin. *P. aeruginosa* is a Gram-negative rod-shaped bacterium with a length of 1-3 mm and a width of 0.5-1 mm (Fig. 1.1). It is highly motile due to the presence of a single polar flagellum (Fig. 1.1, A) and it also possesses many surface pili (Fig. 1.1, B). *P. aeruginosa* grows best in aerobic conditions but is also able to grow anaerobically if there is nitrate present to act as a terminal electron acceptor. This organism is able to metabolise a wide range of carbon sources and can survive in environments with minimal nutritional components. The

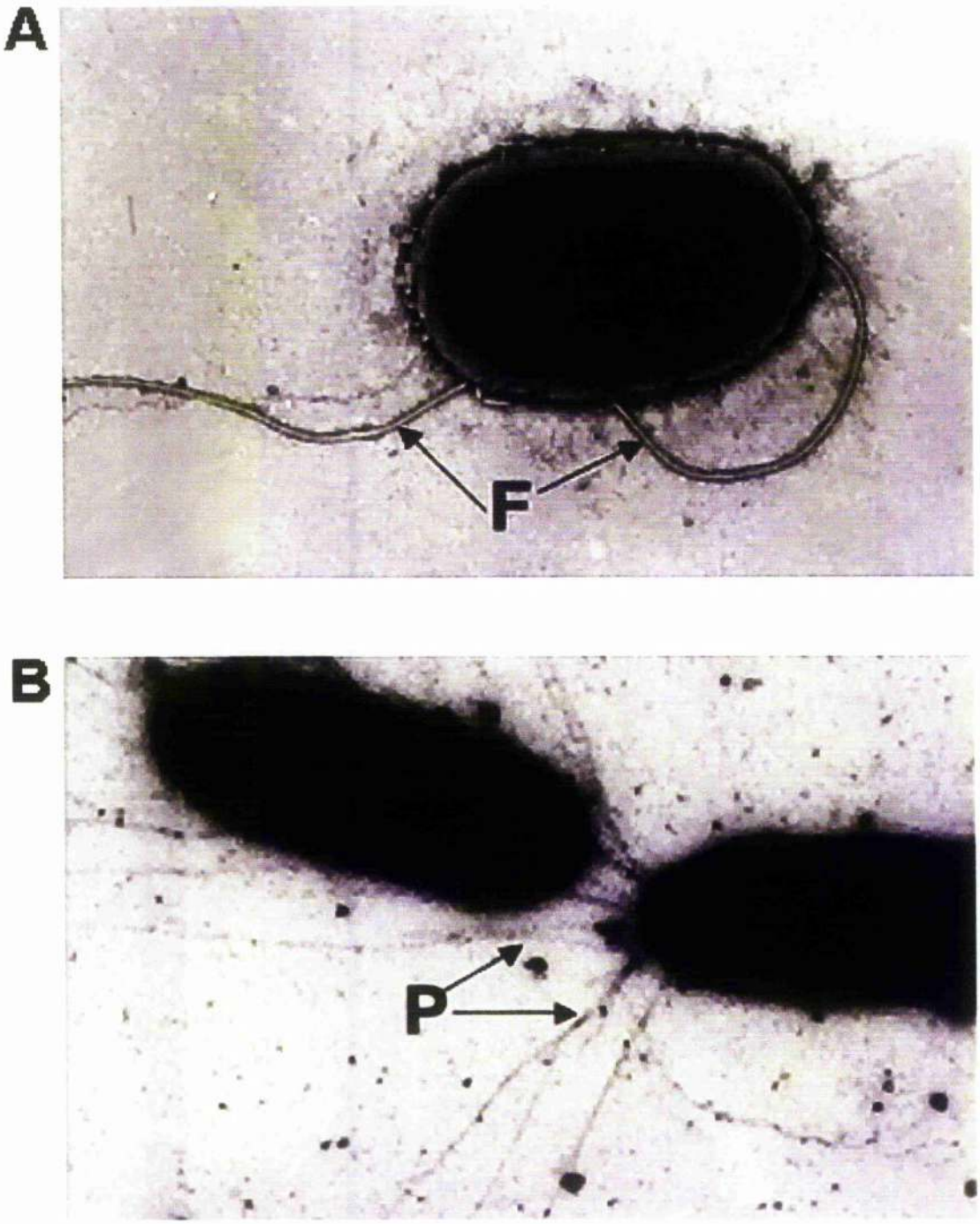


Fig. 1.1. Electron micrographs of *P. aeruginosa*.

Electron micrographs of *P. aeruginosa* showing (**A**) the single polar flagellum (F) and (**B**) the many polar pili (P). Taken from [1].

metabolic versatility of *P. aeruginosa*, combined with its tolerance to temperatures of up to 50°C, enables this bacterium to survive in many different niches. *P. aeruginosa* is found ubiquitously in soil and water and is able to colonise plants, animals and humans. It has been reported that up to 7% of healthy humans carry *P. aeruginosa* in the throat, nasal mucosa, or on the skin and up to 24% of stool samples contain this organism [2]. The prevalence of *P. aeruginosa* in the environment places this organism in an ideal position to infect susceptible humans when the opportunity arises.

1.3. *Pseudomonas aeruginosa* infections

Healthy individuals are usually highly resistant to *P. aeruginosa* infections and this opportunistic pathogen requires a compromise in the host's health status to establish an infection. *P. aeruginosa* can exploit breaches in the host defence, such as damaged skin or mucosal surfaces, to establish infection at virtually any site within the human body. Individuals most at risk from *P. aeruginosa* infections are burns victims, cystic fibrosis patients and the immunocompromised. *P. aeruginosa* is also a major cause of nosocomial infections and can cause ulcerative keratitis in contact-lens wearers. In this section of my introduction I will describe the conditions that predispose to pseudomonal infections and consider why these conditions lead to disease susceptibility.

1.3.1. Burns

Skin and mucosal surfaces act as anatomical barriers to infection and provide the first innate immune component encountered by a pathogen. When skin is badly burned, this barrier is destroyed and *P. aeruginosa* is able to infect the moist underlying tissue. *P. aeruginosa* grows rapidly in the dead or poorly perfused tissue and can reach numbers exceeding 10^5 organisms per gram of tissue [3]. In addition to destroying the surrounding tissue, *P. aeruginosa* can seed the blood at levels that overwhelm the host's innate immunity and cause sepsis. Evidence of the susceptibility of burned skin to *P. aeruginosa* infection is provided by the burned mouse model of infection. Whereas, 10^6-10^7 colony forming units (CFU) of *P. aeruginosa* were required to cause systemic dissemination and lethality in healthy mice, as few as 10 organisms were required for a comparable effect in mice with burned skin [4].

1.3.2. Cystic fibrosis

Cystic fibrosis results from a mutation of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR is a cyclic adenosine monophosphate (cAMP)-regulated chloride channel and patients homozygous for mutant *cftr* have severe defects in cAMP-mediated chloride permeability of epithelial cells. This defect in chloride permeability results in an imbalance in the fluid and electrolyte composition of epithelial cell secretions, which leads to accumulation of sticky dehydrated mucus in lung airways, pancreatic ducts and in the male sex ducts [5]. Over 90% of cystic fibrosis patients die as a result of pulmonary disease and *P. aeruginosa* is often the major pathogen contributing to this [6]. By adolescence, up to 85% of cystic fibrosis patients are infected by this organism and the aggressive inflammatory response it elicits is strongly implicated in progressive lung damage and subsequent death.

There are a number of hypotheses that seek to explain why cystic fibrosis patients are hypersensitive to *P. aeruginosa* infections (Fig. 1.2) [7]. The low volume hypothesis (Fig. 1.2, 1) [8] postulates that mutant CFTR leads to hyperabsorption of sodium and water by epithelial cells in the airways. The resultant decrease in volume of the airway surface liquid prevents the cilia beating efficiently and impairs mechanical mucociliary clearance. *P. aeruginosa* is therefore not cleared from the lungs and can cause infection. The high-salt theory (Fig. 1.2, 2) [9] speculates that mutant CFTR leads to increased levels of chloride and sodium in the airway surface liquid. The high salt levels inhibit the salt-sensitive antibacterial defence proteins, β-defensins, lysozyme and lactoferrin, and therefore prevent destruction of *P. aeruginosa*. Another hypothesis (Fig. 1.2, 3) [10] is based on the observation that epithelial cells in the lungs of cystic fibrosis patients express higher levels of asialo GM1. Asialo GM1 is reported to be a receptor for *P. aeruginosa*, therefore allowing the pathogen to bind to cells and withstand host clearance. A further theory (Fig. 1.2, 4) [11] proposes that in contrast to normal CFTR, mutant CFTR is unable to bind to lipopolysaccharide (LPS) on the surface of *P. aeruginosa* and promote bacterial internalisation. Epithelial cells that internalise bacteria can desquamate into the lumen and be cleared. Therefore inhibition of bacterial internalisation in cystic fibrosis lungs may lead to an inhibition of bacterial clearance. *P. aeruginosa* binding to CFTR also leads to the nuclear factor (NF)-κB-

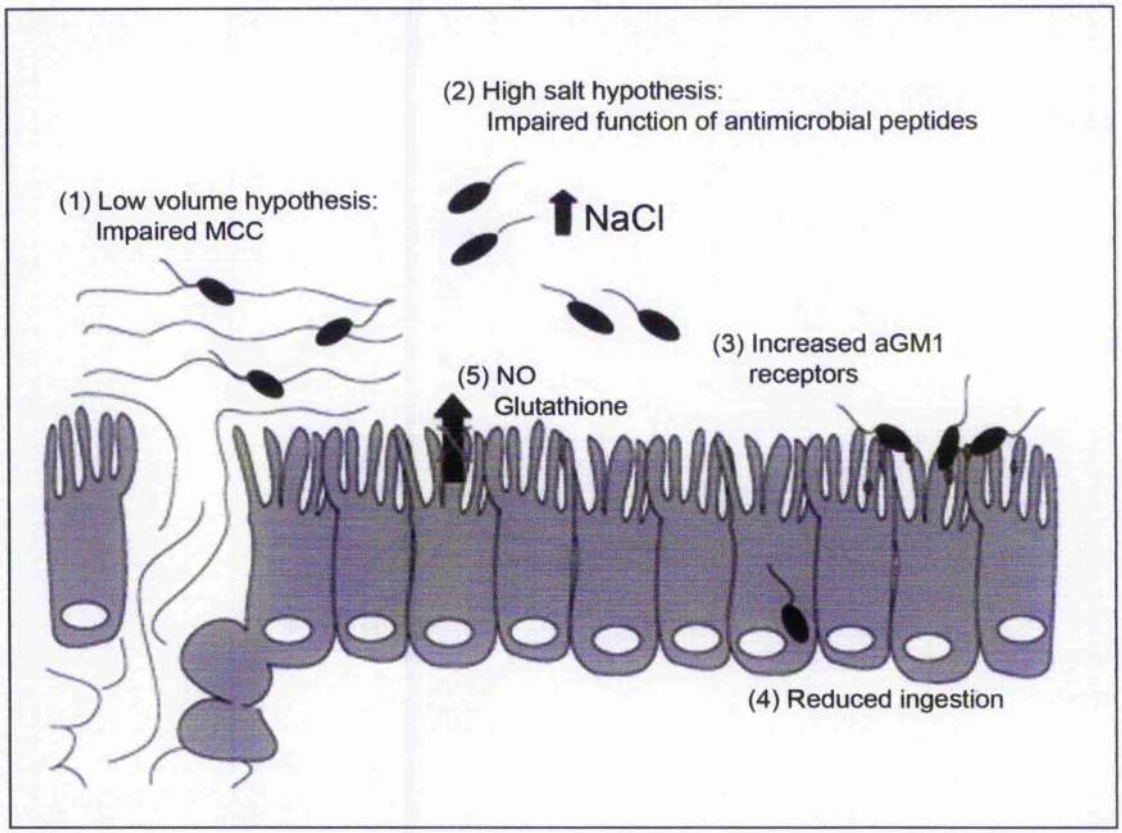


Fig. 1.2. Possible causes of the hypersensitivity of cystic fibrosis patients to *P. aeruginosa* infections.

A number of hypotheses have been proposed to explain the hypersensitivity of cystic fibrosis patients to *P. aeruginosa* infections, these include: (1) the low volume hypothesis, where a decrease in airway surface liquid volume results in impaired mechanical mucociliary clearance (MCC), (2) the high salt hypothesis, where an increase in sodium and chloride levels in the airway surface liquid leads to the impaired function of antimicrobial peptides, (3) an increase in asialo GM1 (aGM1) receptors on cystic fibrosis epithelial cells, resulting in increased *P. aeruginosa* binding and decreased clearance (4) the reduced bacterial ingestion due to mutant CFTR, leading to reduced clearance and (5) a decrease in the defence molecules, nitric oxide (NO) and glutathione, due to a mutant CFTR. Taken from [7].

mediated transcriptional activation of genes involved in neutrophil recruitment [12]. Thus individuals with mutant CFTR may lack the ability to recruit neutrophils, which are major cellular mediators of resistance to *P. aeruginosa* infections. A final hypothesis (Fig. 1.2, 5) suggests that mutant CFTR leads to low levels of the defence molecules nitric oxide (NO) and glutathione in the lungs of cystic fibrosis patients. There is evidence that CFTR regulates the levels of inducible NO synthase [13]. Therefore mutant CFTR may result in decreased levels of NO. Also glutathione is normally secreted via normal CFTR so its levels are decreased in the cystic fibrosis airway [14].

1.3.3. Other causes of *Pseudomonas aeruginosa* infection

Immunocompromised patients are particularly at risk from pseudomonal infections. Neutrophils are the primary cellular mediators of resistance against *P. aeruginosa* infections, therefore neutropenia is a major risk factor for infection [15]. Acquired immune deficiency syndrome (AIDS) patients and the immunosuppressed are also susceptible to nosocomial or community acquired *P. aeruginosa* infections [16].

P. aeruginosa is a major cause of hospital-acquired infections with data from the National Nosocomial Infections Surveillance System between 1992 and 1997 showing that this organism causes about 3% of blood-stream infections; 21% of pneumonias (the most frequent nosocomial pathogen isolated from the lungs); 13% of eye, ear, nose and throat infections (third most common); and 5% of cardiovascular infections [17]. Nosocomial infections are often associated with indwelling devices such as intravenous or urinary catheters and endotracheal tubes. Pseudomonal infections caused by intravenous catheters and surgical wound can lead to septicemia, indwelling urinary catheters can result in urinary tract infections and endotracheal tubes can cause ventilator-associated pneumonia.

Trauma or surface injury to the cornea provides *P. aeruginosa* with the opportunity to cause eye infections such as ulcerative keratitis. Keratitis can lead to rapid opacification and sometimes perforation of the cornea and result in sight loss. Extended-wear contact lens wearers are particularly at risk from developing

pseudomonal eye infections [18]. The improper use of contact lenses can lead to small scratches on the cornea and *P. aeruginosa* can grow in the contact lens solutions.

1.4. Virulence factors

As previously discussed, *P. aeruginosa* is able to infect a number of different tissues and cause a variety of diseases in compromised hosts. This versatility is achieved by the wide range of virulence factors produced by this organism. The virulence factors enable *P. aeruginosa* to adhere to host tissue, avoid the host immune system and multiply. In this section of my introduction, I will describe the virulence factors produced by *P. aeruginosa* and discuss their contribution to pathogenesis. I will also consider the ability of this bacterium to control the expression of these factors in a spatial and temporal manner. One of the major virulence determinants of *P. aeruginosa* is the TTS system and the toxins it secretes. As this is the major focus of my research, I will discuss this in detail in a later section.

1.4.1. Pili

P. aeruginosa produces pili that are located at the poles of the bacterium (Fig. 1.1, B). The pili of *P. aeruginosa* are homopolymers formed from thousands of copies of the 15 kDa pilin monomer encoded by the *pilA* gene [19]. In addition to the *pilA* gene there are about 40 genetic loci that control pilin synthesis and pili production [20]. The transcription of *pilA* is regulated by the alternative ribonucleic acid (RNA) polymerase factor, σ^{54}, and by a two-component regulator system comprising the PilS and PilR proteins [21].

The role of pili as a pseudomonal virulence factor has been demonstrated in a number of animal models of infection where mutants unable to produce pili are less virulent than their isogenic pili-expressing counterparts. For example, pili were shown to be important in virulence in a mouse model of acute pulmonary infection [22] and burn wound infection [23]. However, in other settings, such as the corneal scratch-injury eye model, pili were not required for virulence [24].

The contribution of pili to virulence may be the result of their ability to act as adhesins and mediate binding to host cells. Although the ability of pili to bind host tissue *in vivo* has never been demonstrated, there is strong evidence for pili enhancing adherence to mammalian cells *in vitro* [25-28]. It has been proposed that the pili receptors on target host tissue are the glycolipids GM1 and asialo GM1 [29-32] and that binding is mediated through a disuphide loop in the C-terminal of the pilin monomer [33-35]. Surprisingly, x-ray crystallographic studies have revealed that the proposed receptor-binding disulphide loop is not exposed at the pilus tip but buried within the pilus [36, 37], thus bringing into doubt the ability of this domain to mediate cell adhesion. These conflicting results may be reconciled by speculating that contact between the host cell and the bacterium causes a structural change in the pilus leading to exposure of the disulphide loop.

In addition to mediating adherence to host tissue, the twitching ability of pili may also contribute to their role in virulence. Pili are able to retract and extend [38], allowing the bacteria to "walk" over infected surfaces [39]. This twitching mobility is important in the formation of *in vitro* biofilms on abiotic surfaces [40] and may also be important in the avoidance of host phagocytes. The proteins encoded by the *pilT* and *pilU* genes provide energy for type IV pilus retraction [41], therefore mutants in these genes can be used to study the affect of abrogating pilus twitching motility on virulence. Twitching mutants exhibit reduced cytotoxicity towards various types of epithelial cells *in vitro* [42] and show reduced virulence in mouse models of acute pneumonia [42] and corneal disease [43].

Pili may also contribute to virulence by helping *P. aeruginosa* survive *in vivo* due to their role in natural DNA uptake, autoaggregation of cells and the development of microbial communities.

1.4.2. Flagella

In addition to pili, *P. aeruginosa* also produce a single polar flagellum extending from their cell surface (Fig. 1.1, A). Flagella are complex structures with over 50 genes involved in their synthesis and function [44]. The key structural components of the flagella are FliC, the flagellin subunit protein, and FliD, a protein that caps the flagella.

In *P. aeruginosa* there are two major types of flagella, the a and b type, produced by allelic variants in the *fliC* and *fliD* genes that are coinherited [45]. Pseudomonal flagella are produced by most environmental and nosocomial isolates but bacteria isolated from chronically infected cystic fibrosis patients have usually lost the ability to produce this structure [46].

P. aeruginosa lacking flagella are almost avirulent in animal models of infection [47, 48] thus proving a role for this structure in bacterial virulence. It has also been demonstrated that antibodies and a vaccine raised against flagella are effective at protecting animals from infection [49-51].

Flagella, like pili, are thought to contribute to virulence in part by their ability to bind to host tissue. It has been demonstrated that although flagella bind to asialo GM1, GM1 and GD1a on epithelial cells, this is a rare event [48] and the binding of the FliD cap protein to a variety of neutral and acidic oligosaccharides in mucin is thought to be much more important [52, 53]. In some situations binding to mucin would promote the removal of bacteria from host tissue, for example by mechanical mucociliary clearance in the respiratory tract. However, if mechanical mucociliary clearance was reduced in the lungs during pulmonary infection or mucus was trapped under a contact lens, mucin binding may enhance infection.

The importance of flagella motility in virulence has been illustrated in a burned-mouse model of infection [54] where non-motile flagella mutants had reduced virulence compared to the wild type bacteria [54]. The flagella of *P. aeruginosa* are glycosylated [55, 56] and this posttranslational modification is also an important determinant of flagellar-mediated virulence [54]. It has been shown that flagella are highly immunogenic [48, 57] and although this property may stimulate the host defence, perhaps explaining why flagella appear to be selected against in chronic cystic fibrosis infection, it may also contribute to disease severity.

1.4.3. Lipopolysaccharide

LPS is a major component of the outer membrane of Gram-negative bacteria. There are two isoforms of LPS in *P. aeruginosa*, the smooth form found on most environmental

and nosocomial isolates and the rough form found on most isolates from chronically infected cystic fibrosis patients. Both forms consist of a hydrophobic lipid A component that anchors the molecule to the membrane, and a core oligosaccharide. Smooth LPS also contains a long polysaccharide O-chain composed of tri- or tetrasaccharide repeating units. The O-chains found on smooth LPS isolates vary in length from 5 – 100 kDa and only 20 – 30 % of the LPS molecules on each cell contain O-chains [58].

It has been reported that LPS acts as an adhesin by binding to GM1 and the galectin-3 protein on epithelial cell surfaces [31, 59]. The outer-core oligosaccharide of LPS also binds to CFTR leading to internalisation of the bacteria [60]. As discussed above, in the lungs of individuals with normal CFTR, this would result in clearing of the bacteria by infected epithelial cells on the airway surface desquamating into the lumen. However, in the setting of a damaged cornea, binding to normal CFTR enhances virulence as infected cells are not lost and may provide a protected environment for the bacteria [61].

The O-side-chains of smooth LPS play a role in resisting host defences, for example by preventing lysis by complement, and this appears to be important in acute pseudomonal infections [62]. *P. aeruginosa* from cystic fibrosis patients, however, are often serum sensitive and LPS rough suggesting that LPS-mediated serum resistance is not crucial for the organisms survival in the cystic fibrosis lung [63]. Also the loss of O-side-chains in chronically infected individuals may be advantageous as they can evade the strong host response against O-side-chain antigens.

1.4.4. Iron acquisition

P. aeruginosa, like all living organisms, requires iron for growth and survival. Iron is not freely available in aerobic environments because it exists in the oxidised, ferric form (Fe^{3+}), which at pH 7 is extremely insoluble (10^{-18} M). In addition, in mammalian hosts iron is sequestered in haemoglobin, transferrin and other iron binding proteins. Thus, bacteria have developed specific mechanisms for iron acquisition including the synthesis of ultra-high affinity compounds named siderophores that physically capture the iron from host proteins by virtue of their superior binding affinity.

P. aeruginosa produces two siderophores to acquire iron, pyoverdin and pyochelin. Pyoverdin and pyochelin are secreted from *P. aeruginosa* when iron concentrations are low and chelate iron from mammalian host proteins. The ferripyoverdin and ferripyochelin then bind to the FpvA and FptA membrane receptors respectively and are transported back into the microbe via a specific uptake carrier system [64, 65]. When the ferrisiderophore reaches the cytoplasm, the iron is reduced, the soluble ferrous iron (Fe^{2+}) is transferred to molecules where it is required, and the siderophore is recirculated. When iron concentrations are high, the master regulator Fur represses the transcription of proteins required for iron acquisition [66].

In addition to the siderophores produced by *P. aeruginiosa*, there is also evidence that this organism can utilise siderophores produced by other organisms to acquire iron [67]. The variety of mechanisms for iron acquisition means that an essential role in virulence for either pyoverdin or pyochelin has not been demonstrated. However the absolute requirement for iron renders these systems important in virulence.

In addition to its role in iron acquisition, ferripyochelin is also responsible for damaging cells. Ferripyochelin can catalyse hydroxyl radical formation that enhances oxidant-mediated injury to pulmonary artery endothelial cells and respiratory epithelial cells [68]. The superoxide and hydrogen peroxide generated by neutrophils can also be used by ferripyochelin to enhance endothelial cell damage [69].

1.4.5. Proteases

A number of proteases are produced by *P. aeruginosa* including LasA protease, elastase, alkaline protease and protease IV. These proteases have wide substrate specificities and are thought to promote pseudomonal infection and dissemination by destroying physical barriers and compromising host immune effectors. It has been shown that elastase degrades collagen and non-collagen proteins and disrupts the integrity of the host basement membrane. LasA and elastase appear to act synergistically to degrade elastin with LasA nicking elastin making it more sensitive to subsequent degradation by elastase [70]. Elastase inhibits monocyte chemotaxis, therefore inhibiting clearance of the bacteria from the site of infection and preventing subsequent presentation of bacterial antigens to the host immune system [71]. Also

LasA may protect *P. aeruginosa* from host defences by causing the shedding of the ectodomain of syndecan-1, which may interfere with the ingestion or killing capability of phagocytes or render antimicrobial peptides inactive [72, 73].

A number of other potential proteases have been identified by activity assays and homology searches. However, due to the presence of a wide range of proteases that possess overlapping functions, it is difficult to dissect their role in pathogenesis. For example elastase deficient *P. aeruginosa* are less virulent in some models of infection but not in others [74].

1.4.6. Exotoxin A

Most clinical isolates of *P. aeruginosa* produce exotoxin A. This 66 kDa protein is an adenosine diphosphate (ADP)-ribosyltransferase (ADPRT) toxin that transfers the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD^+) to the modified histidine residue, diphthamide, located at position 715 of elongation factor-2 (EF-2) [75, 76]. EF-2 is involved in moving the nascent growing peptides produced on ribosomes, and its ADP-ribosylation and subsequent inactivation by exotoxin A inhibits protein synthesis and results in cell death. Exotoxin A has three structural and functional domains that are successively involved in the intoxication process [77, 78]. Domain I binds to the α2-macroglobulin/low density lipoprotein receptor-related protein enabling internalisation of the toxin via receptor-mediated endocytosis [79]. Domain II then mediates translocation of the toxin across the endosomal membrane into the cytosol [80] where the catalytic domain III can act to ADP-ribosylate EF-2 .

Protein synthesis inhibition by toxins is not sufficient to mediate target cell lysis and decreasing the ADPRT activity of exotoxin A does not abolish the toxicity of this protein [81, 82]. Therefore in addition to ADP-ribosylating EF-2, additional mechanisms may be involved in exotoxin A-induced cytotoxicity. It has been proposed that exotoxin A also induces apoptotic cell death in some cell lines, for example in human mast cells, by a caspase-8 and caspase-3 dependent mechanism [83].

Exotoxin A synthesis is regulated by iron [66]. When iron levels are low, Fur relieves its repression on the transcriptional activator RegA, which is then free to activate expression of the exotoxin A gene, *toxA*.

The contribution of exotoxin A to pathogenesis was initially assessed by adding the purified toxin to corneas, which resulted in rapid (< 24 h) destruction of the corneal epithelial cells, chemotaxis of polymorphonuclear leucocytes to the site and corneal ulceration [84]. In some animal infection models, *P. aeruginosa* mutants deficient in exotoxin A production exhibit reduced virulence, but this is not always the case. Exotoxin A may contribute to virulence by causing tissue damage and diminishing the activity of phagocytes, but when deleted other toxins may complement for its destructive actions.

1.4.7. Pore-forming cytotoxin

A few species of *P. aeruginosa* produce a toxin from the genome of a temperate phage integrated into the bacterial chromosome. The toxin is translated as a 31.7 kDa procytotoxin that is processed by removal of the C-terminus during bacterial autolysis to an active 29 kDa cytotoxin [85]. The mature, water soluble, acidic toxin is able to induce cell death by forming pores in the plasma membranes of a wide variety of eukaryotic cells. This pseudomonal pore-forming toxin contains a high percentage of β-sheets, is very amphiphilic in nature and oligomerises into a functional pentamer [86].

1.4.8. Phospholipase C

P. aeruginosa produces two forms of phospholipase C (Plc): one is haemolytic, PlcHR, and the other, PlcN, is not [87]. PlcHR hydrolyses phosphatidylcholine and sphingomyelin and PlcN hydrolyses phoaphatidylcholine and phosphatidylserine [88].

PlcHR has been implicated in virulence in several animal models but no role for PlcN has yet been demonstrated [89-91]. There is evidence that PlcHR degrades pulmonary surfactant and suppresses neutrophil respiratory burst activity, therefore facilitating chronic persistent infection by P. *aeruginosa* [88]. PlcHR also acts as a potent inflammatory agent, which may result in increased tissue destruction. Both Plcs

recognise phospholipids found predominantly in eukaryotic (e.g. phosphatidylcholine and sphingomyelin) not prokaryotic membranes, thus potentially providing specificity for damage to host tissues [88].

1.4.9. Rhamnolipid

In addition to PlcHR, *P. aeruginosa* also secretes another hemolysin, the heat-stabile glycolipid, rhamnolipid. A role for rhamnolipid in virulence has been suggested by studies in *Dictostelium discoideum* [92]. Rhamnolipid has been implicated in the degradation of lipids and the disruption of cell membranes in addition to inhibiting mucociliary transport and disturbing airway epithelial ion transport [93-96]. It has been suggested that rhamnolipid acts to solubilise lung surfactant, thus making it more susceptible to degradation by other virulence factors, for example Plc [97]. Rhamnolipid synthesis is controlled by quorum sensing and has been shown to be important in biofilms [98].

1.4.10. Pyocyanin

Pyocyanin is a blue redox-active secondary metabolite that is produced by *P. aeruginosa*. Numerous *in vivo* studies have indicated a role for this compound in virulence [99]. In addition, the presence of detectable levels of pyocyanin in sputum from cystic fibrosis patients and ear secretions from those suffering from *P. aeruginosa*-mediated chronic otitis indicates production during infection [100].

In vitro studies illustrate that pyocyanin disrupts cell respiration, ciliary function, epidermal cell growth, prostacyclin release, calcium homeostasis and induces apoptosis in neutrophils [99]. Pyocyanin also contributes to the imbalance of protease-antiprotease activity, which is detected in the airways of patients with cystic fibrosis, by inhibiting the α1-protease inhibitor [101]. Pyocyanin produces reactive oxygen species such as hydrogen peroxide and superoxide, which damage cells, and pyocyanin also inhibits the activity of catalase produced by the host in an attempt to counteract the damaging reactive oxygen species [102, 103].

The importance of pyocyanin in virulence has been demonstrated in a number of non-mammalian and mammalian models. *P. aeruginosa* strains deficient in pyocyanin exhibit reduced virulence in *Caenorhabditis elegans*, plants, *Drosophila melanogaster* as well as in the burnt-mouse models and mouse models of acute and chronic lung infection [99].

1.4.11. Alginate

Almost all strains of *P. aeruginosa* carry the genes required for the synthesis of the extracellular polysaccharide alginate. Alginate is a negatively charged, linear copolymer of partially O-acetylated β-1,4-linked D-mannuronic acid and its C5 epimer, α-L-guluronic acid [104]. Isolates of *P. aeruginosa* from cystic fibrosis patients produce large quantities of alginate and are said to have a mucoid phenotype [105]. Most environmental and nosocomial strains do not produce significant amounts of alginate and are therefore considered nonmucoid. The nonmucoid strains usually contain the genes required for alginate synthesis and can express the polysaccharide at low levels when grown *in vitro* [106, 107].

Clinical evidence suggests that individuals suffering from cystic fibrosis are infected with a nonmucoid strain of *P. aeruginosa* that switches to alginate production during chronic infection [108]. It is thought that alginate is the major virulence factor relevant to pathogenesis in the cystic fibrosis lung setting due to its ability to protect the bacteria from the host defence systems. Alginate appears to scavenge free radicals and thus may protect *P. aeruginiosa* from the reactive oxygen species released by inflammatory cells recruited to the site of infection [109]. It is thought that the physical and chemical barrier afforded to the bacteria by alginate protects them from phagocytic clearance and defensins [110]. The O-acetyl components of alginate are crucial for this protection from phagocytic cells [111]. Alginate also prevents neutrophil chemotaxis and complement activation but appears to enhance neutrophil oxidative burst [112]. Although high concentrations of antibodies against alginate are found in the sera of chronically infected cystic fibrosis patients they appear to be deficient in mediating opsonic killing and thus do not protect the host [113].

The importance of alginate as a virulence factor during infection is difficult to ascertain due to its requirement in chronic not acute infection. However, the transgenic cystic fibrosis mouse model demonstrated that alginate is required for establishing *P. aeruginosa* oropharyngeal colonisation [114].

1.4.12. Biofilms

Another phenotype exhibited by *P. aeruginosa* that is believed to be important in disease is its ability to form biofilms. Biofilms are communities of bacteria organised in an extracellular polymeric substance matrix attached to a surface. Biofilm formation involves a number of steps. Initially free-swimming or planktonic bacteria attach to a surface, microcolonies form, which then develop into mature biofilms. The release of planktonic organisms from the mature biofilm allows seeding of a new biofilm.

It is thought that *P. aeruginosa* forms biofilms in the lungs of cystic fibrosis patients and on implanted medical devices such as intravenous and urinary catheters [115, 116]. Microcolonies of aggregates of *P. aeruginosa* in cystic fibrosis lungs have been observed. In addition to bacteria, these microcolonies contain an exopolymeric substance that may consist of alginate, or a mannose- or glucose-rich expolysaccharide encoded by the *psl* or *pel* loci respectively [117, 118]. A significant amount of nucleic acid also occurs in pseudomonal biofilms [119]. *P. aeruginosa* biofilm formation requires the infecting bacteria to be motile. Thus, mutants in pili and flagella were unable to establish biofilms [40]. During chronic infection in cystic fibrosis lungs, the *P. aeruginosa* switch to a mucoid (alginate producing) phenotype and lose their motility.

Biofilms probably contribute to the survival of *P. aeruginosa* because they render the bacteria more resistant to host defense systems. Biofilms are also more resistant to antibiotics, making these infections very difficult to treat [120].

1.4.13. Quorum Sensing

Quorum sensing controls the production of many of the virulence factors produced by *P. aeruginosa* and is believed to be involved in biofilm maturation. This mechanism

ensures that virulence genes are only induced when the bacterial population has reached a critical threshold or "quorum." Many virulence factors are not effective when produced at low levels, therefore quorum sensing acts to prevent the metabolically expensive manufacture of virulence factors until an unified attack can be mounted simultaneously by a large number of bacteria.

P. aeruginosa has two main quorum sensing systems, *las* and *rhl* (reviewed in [121]) (Fig. 1.3). The *las* system consists of LasI and LasR. LasI directs the synthesis of the N-acyl homoserine lactone (AHL), N-3-oxododecanoyl-homoserine lactone (3-oxo-C_{12}-HSL), which is transported out of the cell via the MexAB-OprM multidrug efflux pump. As the density of *P. aeruginosa* increases, the concentration of 3-oxo-C_{12}-HSL in the surrounding environment increases until a critical threshold is reached. The transcriptional activator LasR binds 3-oxo-C_{12}-HSL and induces the transcription a number of genes. The *rhl* system is similar to the *las* system but utilises RhlI, which directs the synthesis of N-butanoyl-homoserine lactone (C_4-HSL), and the transcriptional activator RhlR. *P. aeruginosa* cells are freely permeable to C_4-HSL. Thus, unlike 3-oxo-C_{12}-HSL, a specific method of secretion is not required. Both systems involve a positive feedback loop in which LasR bound to 3-oxo-C_{12}-HSL and LasI bound to C_4-HSL induce the expression of LasI/LasR and RhlI/RhlR respectively. LasR bound to 3-oxo-C_{12}-HSL also activates the transcription of *rhlR* and *rhlI*, therefore linking these systems in a hierarchical manner.

There are a number of other positive and negative regulators of *las* and *rhl* systems which fine tune the quorum sensing response of *P. aeruginosa* [121]. One of these additional levels of regulation involves a third quorum sensing molecule, the *P. aeruginosa* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone (Fig. 1.3). PQS induces RhlR and RhlI expression and is itself under the positive regulation of the *las* system and the negative regulation of the *rhl* system. The production of PQS is therefore dependent on the ratio of 3-oxo-C_{12}-HSL and C_4-HSL, suggesting a delicate balance between the *las* and *rhl* quorum sensing systems.

Many genes have been identified as being regulated by quorum sensing in *P. aeruginosa*. These include genes that encode membrane proteins, secreted enzymes, transcription factors, two-component regulators, and proteins involved in energy

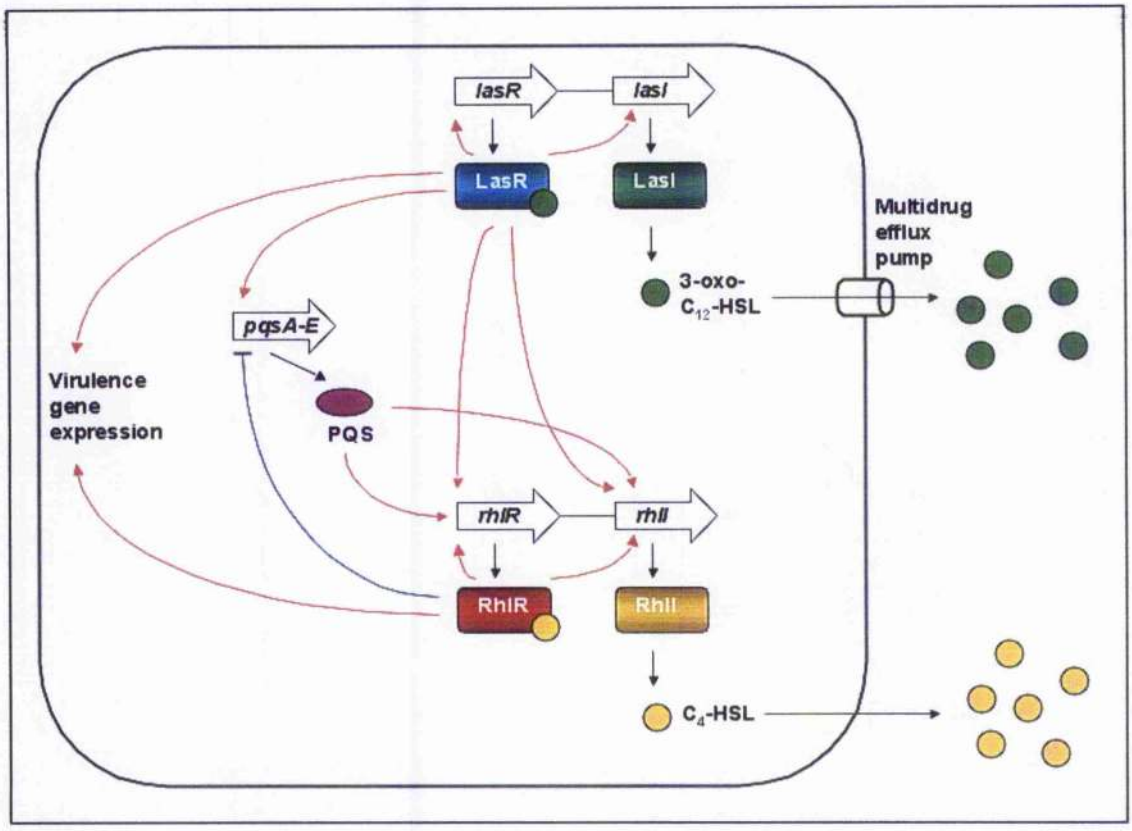


Fig. 1.3. Quorum sensing in *P. aeruginosa*

A simplified model of the hierarchical *las* and *rhl* quorum sensing systems in *P. aeruginosa* including the role of the *P. aeruginosa* quinolone signal (PQS). Red arrows indicate positive regulation and blue lines represent negative regulation. In the *las* system, LasI synthesises 3-oxo-C_{12}-HSL that is pumped out of the cell by the multidrug efflux pump. Once the amount of 3-oxo-C_{12}-HSL reaches a critical threshold, LasR bound to 3-oxo-C_{12}-HSL induces the expression of a number of genes including *lasR, lasI, rhlR, rhlI, pqsA-E* and various virulence genes. In the *rhl* system, RhlI synthesises C_4-HSL, which passively diffuses out of the cell. Once the amount of C_4-HSL reaches a critical threshold, RhlR bound to C_4-HSL induces the expression of a number of genes including *rhlR, rhlI* and various virulence genes. RhlR bound to C_4-HSL also represses expression of *pqsA-E*. PQS acts to increase expression of the *rhl* system.

metabolism and the transport of molecules in and out of the cell. Table 1.1. lists the virulence factors identified as being regulated by quorum sensing in *P. aeruginosa*.

Table 1.1. Virulence factors controlled by the quorum sensing in *P. aeruginosa*.

***las* controlled**	***rhl* controlled**	**PQS controlled**
PQS synthesis	PQS synthesis	*rhl* system
rhl system	Rhamnolipids	Rhamnolipids
Biofilm formation		Biofilm formation
Alkaline protease	Alkaline protease	
Elastase	Elastase	Elastase
	Pyocyanin	Pyocyanin
Lipase	Lipase	
	Lectins A and B	Lectins A and B
Hydrogen cyanide	Hydrogen cyanide	
Xcp secretion	Xcp secretion	
	Chitinase	
	RpoS	
Exotoxin A		
Neuraminidase		
Pvds-reg. endoprotease		
Catalase		
Superoxide dismutase		
Aminopeptidase		
Swimming		
	Exoenzyme S	
Swarming	Swarming	
Twitching	Twitching	

The importance of quorum sensing in pseudomonal virulence has been demonstrated in a number of mammalian and non-mammalian models. Strains containing mutations in quorum sensing genes induced less tissue destruction, pneumonia, dissemination and mortality compared with wild type *P. aeruginosa* in the burnt-mouse model and mouse models of acute and chronic lung infection [122-124]. Quorum sensing mutants also showed reduced virulence in the *C. elegans, D. discoideum* and *Arabidopsis thaliana* non-mammalian infection models. [92, 125, 126]

In addition to the reduced pathogenicity of quorum sensing mutants in a number of infection models, the direct involvement of quorum sensing during infection has also been investigated by studying sputum samples from cystic fibrosis patients colonised with *P. aeruginosa*. The sputum samples from these individuals contain AHLs and a correlation between the amount of messenger RNA (mRNA) transcripts for quorum

sensing genes and quorum sensing-regulated genes has been observed [127, 128]. In mouse models, the production of AHLs during infection has also been confirmed by co-infecting the mice with *P. aeruginosa* and an *E. coli* AHL reporter strain [129].

Quorum sensing is important in *P. aeruginosa* pathogenesis not only because it is involved in the regulation of virulence factors but also because the AHLs induce inflammation in the host. Injection of mice with 3-oxo-C_{12}-HSL stimulates the production of proinflammatory cytokines and arachidonic acid metabolites [130]. Also 3-oxo-C_{12}-HSL stimulates the production of interleukin 8, cyclooxygenase-2 and prostoglandin E2, and has immunomodulatory activity on cells *in vitro* [131].

1.5. Type III secretion systems

In addition to the virulence factors described above, *P. aeruginosa* produces four toxins that are secreted by the TTS system. For proteins to be successfully secreted by Gram-negative bacteria, they must be exported across the bacterial inner membrane through the peptidoglycan layer and across the outer membrane. Toxins that are subsequently targeted to the cytoplasm of host cells must also penetrate the plasma membrane of the eukaryotic cell, and in the case of plant cells, the plant cell wall. The TTS system enables Gram-negative bacteria to achieve this transport in a single step and deliver toxins from the bacteria directly into the cytoplasm of eukaryotic cells.

Bacteria that encode TTS systems include animal pathogens from the genera *Yersinia*, *Salmonella*, *Shigella*, *Bordetella*, *Chlamydia*, and *E. coli* and *P. aeruginosa* and plant pathogens belonging to the *Erwinia*, *Xanthomonas* and *Pseudomonas* genera. Some bacteria encode more than one TTS system as exemplified by *Salmonella enterica* serovar Typhimurium [132]. Although primarily required for virulence some endosymbiotic bacteria also utilise TTS systems, for example the insect endosymbiont *Sodalis glossinidus* [133].

In this section of my introduction I will describe the components of the TTS apparatus, paying particular attention to the TTS apparatus of *Yersinia*. The *Yersinia* TTS apparatus is the most extensively studied system and is homologous to the *P. aeruginosa* TTS apparatus. I will also consider research into the TTS systems of

Salmonella and *Shigella* when it contributes to a fuller understanding of the TTS systems of Gram-negative bacteria. I will discuss the signals that target toxins to the TTS apparatus and examine the role of chaperone proteins in TTS. I will then discuss regulation of expression and secretion via the TTS system. Finally, I will examine the effects of some of the TTS toxins on their eukaryotic targets. This broad overview of TTS will provide a framework on which the specific research on the TTS system of *P. aeruginosa* can be based.

1.5.1. Components of the type III secretion apparatus

The TTS toxins secreted by different Gram-negative pathogens vary widely in their structure and function. However, many of the components of the TTS apparatus are conserved. Of the 20 – 25 different proteins required for construction of the TTS apparatus, roughly half are conserved among the TTS systems of different bacteria. This conservation is evident both at the level of sequence homology and functional complementation. Evidence that some components of the TTS systems from different bacteria are functionally interchangeable is supplied by the *Yersinia* YopE protein that can be secreted into the extracellular medium and HeLa cells by *S. enterica* serovar Typhimurium [134]. Also the TTS inner membrane protein InvA of *S. enterica* serovar Typhimurium is complemented by the *Shigella flexneri* homolog MxiA [135]. Some components of the TTS apparatus, in addition to showing homology among different bacteria, also exhibit sequence similarity to the proteins that make up the basal body of the bacterial flagellum [136]. The relatedness between these two structures is further strengthened by the observation that flagella can secrete (but not translocate into a eukaryotic cell) some TTS toxins under specific circumstances [137, 138].

The basic TTS apparatus consists of a needle-like structure that projects from the bacterial surface and an inner and outer membrane ring (Fig. 1.4). There are also a number of additional proteins involved in translocation of toxins, many of which are predicted to be inner membrane proteins. Among the additional proteins required for TTS are translocator proteins that are predicted to facilitate the entry of toxins into the host cells.

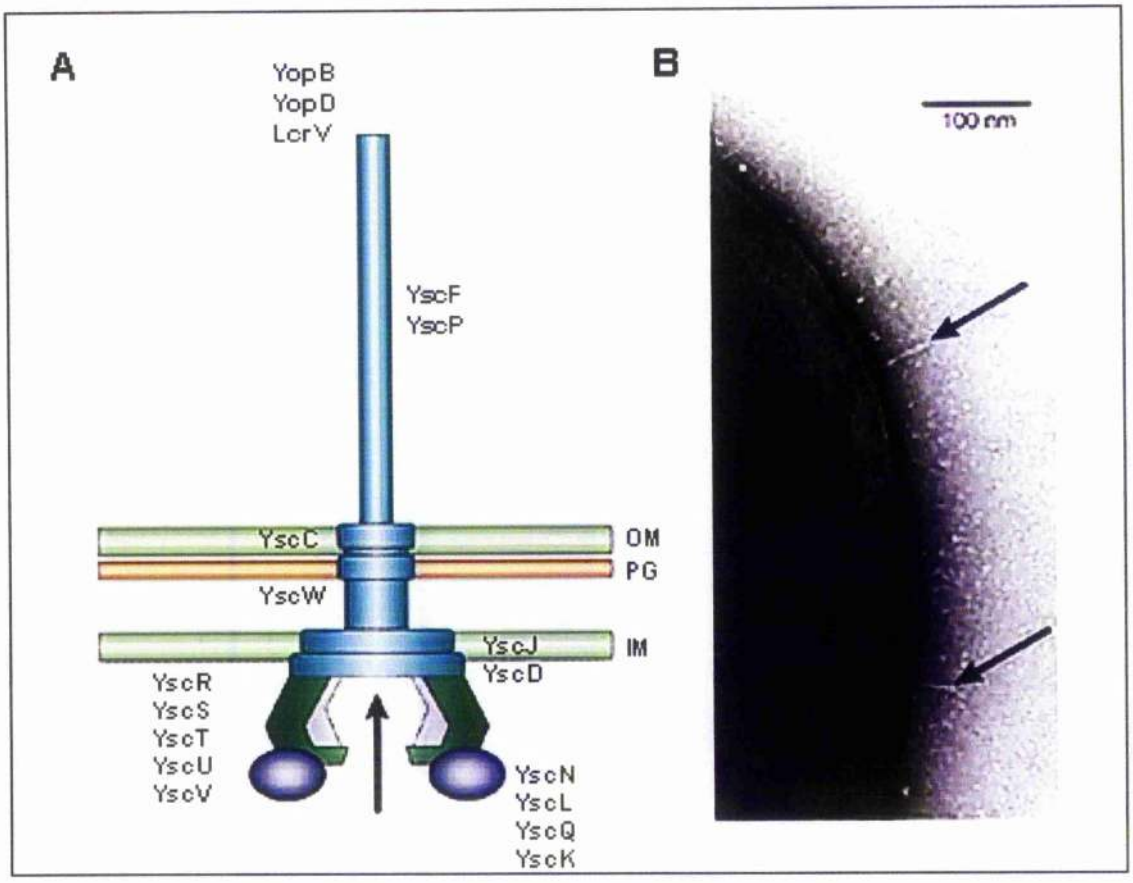


Fig. 1.4. The type III secretion apparatus of *Yersinia*.

(A) Schematic representation of the *Yersinia* TTS apparatus spanning the outer membrane (OM), the peptidoglycan layer (PG) and the inner membrane (IM) of the bacterium. YscF is the main constituent of the needle and YscP regulates its length. The inner membrane ring is predicted to comprise YscJ and YscD. The outer membrane ring is formed from the secretin, YscC, and YscW is involved in localisation of the ring. YscR, YscS, YscT, YscU and YscV are predicted inner membrane proteins that may make up the basal body of the TTS apparatus. YscN, YscL, YscQ and YscK are cytoplasmic proteins and YscN is an adenosine triphosphatase (ATPase) that supplies the energy for TTS. The translocators YopB, YopD and LcrV are secreted through the TTS apparatus and form proteinaceous pores in the host cell membrane. **(B)** An electron micrograph of TTS needles protruding from *Y. enterocolitica* E40. Taken from [139].

1.5.1.1. Needle-like structures

Proteins that are translocated from the bacterium to the host by the TTS system are believed to pass through a needle-like structure that extends from the bacterial surface (Fig. 1.4). Although there is no direct evidence that proteins are transported through the centre of these needles, secretion requires needle assembly [140] and egress of toxins from the distal end of the needle-like structures, Hrp pili, in bacterial phytopathogens has been observed [141, 142].

Extensive studies on the TTS needle of *Yersinia* have revealed a number of features that appear to be conserved among the other bacteria examined, for example *Salmonella* and *Shigella*. Electron microscopy of the *Yersinia* TTS needle suggests that these structures are straight, apparently rigid and hollow [143, 144]. The needles are homopolymers of the 9 kDa YscF protein [144] and are approximately 58.0 ± 10 nm in length [145]. YscP regulates needle length and it is believed to act by having one end tethered to the base of the TTS apparatus and the other end attached to the growing tip of the needle. Needle extension is then halted when YscP is fully extended [145]. In *Yersinia*, the number of needles per bacterium is dependent on the growth medium and varies between 50 and 100 needles that appear uniformly distributed over the surface of the bacterial cell [144]. Electron micrographic reconstruction of negatively stained *Y. enterocolitica* needles reveal an inner diameter of only 20 Å [143]. This narrow conduit, combined with the fact that the folded domains of TTS toxins often have diameters of 20 – 30 Å, suggests that the toxins may require partial or full unfolding in order to pass through the needle.

1.5.1.2. Inner and outer membrane rings

The needle-like structures of the TTS apparatus appear to be anchored to a protein ring embedded in the bacterial outer membrane that is itself attached to a larger protein ring in the inner membrane (Fig. 1.4). The inner and outer membrane rings probably form a single structure through which proteins can pass from the bacterial cytosol into the needle of the TTS apparatus.

In *Shigella* and the SPI-1 TTS system of *Salmonella* the components of the inner membrane ring have been partially purified and identified as PrgK/PrgH and MxiJ/MxiG respectively [140, 143, 146, 147]. PrgK, MixJ and their *Yersinia* homologue YscJ, are predicted to have a N-terminal cysteine that is lipid acylated, a periplasmic domain of about 200 residues followed by a C-terminal transmembrane region. PrgH, MxiG and their *Yersinia* homologue YscD, are predicted to contain a small N-terminal cytoplasmic domain, a single transmembrane region, and a large periplasmic domain.

The outer membrane ring is formed from an oligomer of a single protein related to members of the secretin protein family [148]. In *Yersinia*, the outer membrane ring structure is formed from YscC and exhibits 13-fold symmetry [149, 150]. In *Salmonella*, the ring is composed of the secretin InvG [151]. *Yersinia* and *Salmonella* encode YscW and InvH respectively that are involved in the localisation of the secretin rings to the outer membrane [151, 152]. YscW and InvH are not homologs of each other and are not widely conserved across TTS systems. It has also been demonstrated that InvH is not required for TTS.

1.5.1.3. Other components of the type III secretion apparatus

There are a number of other proteins that are predicted to form part of the TTS apparatus and are involved in the secretion of toxins. Although to date none of these proteins have been successfully copurified with the needle assembly, their homologues in the flagella system have been shown by biochemical means to associate with the flagellar apparatus [153-155]. A number of these proteins are predicted to be inner membrane proteins, for example the *Yersinia* proteins YscR, YscS, YscT, YscU and YscV. These predicted inner membrane proteins have the potential to interact with TTS proteins during transit and may act as receptors for the signal sequences on secreted proteins. In addition to the predicted inner membrane proteins there are a number of cytoplasmsic proteins involved in TTS including YscN, YscL, YscQ and YscK in *Yersinia*. One of these cytoplasmic proteins, YscN, is an ATPase that is involved in supplying the energy to drive protein transport through the TTS system [156].

1.5.1.4 Translocator proteins

Toxins that are successfully transported through the needle complex then need to gain entry into the eukaryotic cell through the cell membrane. It is believed that specific TTS proteins, termed translocators, facilitate this by forming proteinaceous pores in the host cell membrane. The translocators of *Yersinia*, YopB, YopD and LcrV, are required for translocation of toxins into host cells [157-160]. YopB is a 41.8 kDa protein that contains two central hydrophobic domains, YopD is a 33.3 kDa protein containing a single hydrophobic domain and LcrV is a 37 kDa protein that contains no clear hydrophobic domain. YopB and YopD together can form ion channels in lipid bilayers [161] and LcrV alone has been reported to form channels [162]. It has been postulated that LcrV may insert into the eukaryotic cell membrane in order to initiate the formation of a pore by the subsequent insertion of YopB and YopD [162]. In addition to its role in pore-formation, LcrV also regulates expression of certain components of the TTS system in *Yersinia* [163-166] and has an immuno-modulatory role in the host [167-171].

1.5.2. Type III secretion signals

For a protein to be translocated into the eukaryotic cell by the TTS system, it must first be targeted to this system. For most studied toxins, the TTS signal is located in the first ~ 15 mRNA codons or amino acids, although no consensus signal sequence at the nucleic acid or amino acid level has been identified. The ability of the first 15 codons to direct TTS has been demonstrated for a number of toxins by fusing this region to a reporter protein and observing secretion. For example, when the first 15 codons of YopE were fused to the N-terminus of Cya or neomycin phosphotransferase II, they were sufficient to drive secretion through the TTS system [172-174]. It is important to note that this sequence, although sufficient for secretion into the extracellular medium upon induction, was not sufficient for translocation of the fusion proteins into the eukaryotic cell [174, 175]. Although the location of the TTS signal is clear, its molecular composition remains controversial. Some evidence indicates that the signal is mRNA based, while other data suggests a protein signal.

1.5.2.1. Nucleic acid secretion signals

Support for an mRNA signal comes from two observations. Firstly, it is possible to introduce various frameshift mutations in the N-terminus of a number of TTS toxins, for example YopE, that do not affect the ability of these proteins to be targeted to the TTS system [172, 176, 177]. Frameshift mutations result in only a minor change in the mRNA sequence but drastically alter the protein sequence, therefore suggesting that the TTS signal is in the mRNA. Secondly, when a base in codon 3 of YopQ was mutated to another base that did not alter the protein sequence, a synonymous base change, this protein lost its ability to be secreted by the TTS system [178].

1.5.2.2. Protein secretion signals

Support for a protein-based signal sequence comes from experiments in which the signal sequence still targets the toxin to the TTS system despite dramatic changes in the mRNA sequence. For example, when 17 of 27 nucleotides in codons 2 to 10 of YopE are mutated to bases that leave the protein sequence intact, the secretion signal is still functional [179]. If the TTS signal is protein-based it appears to be highly degenerative as residues 2 to 8 of YopE (K-I-S-S-F-I-S) can be replaced by alternating serines and isoleucines (S-I-S-I-S-I-S) and still be functional [179]. Further evidence supporting a protein-based signal was obtained using this synthetic serine/isoleucine secretion signal. Altering the amphipathic nature of the synthetic secretion signal by substituting single amino acids abolished protein secretion despite there being very little change in the mRNA sequence [180]. Even if the TTS signal proves to be protein based, there is no clear consensus sequence at the amino acid level or at the structural level. However, the ability of some toxins to be secreted by the TTS apparatus of different bacteria suggests that, at least in some cases, there is some sort of conserved secretion signal.

1.5.3. Type III secretion system chaperones

Many TTS toxins require low molecular weight (<15 kDa), usually acidic (pI<5), cytosolic chaperones for their efficient secretion and the gene encoding the chaperone is normally located adjacent to the gene encoding its cognate toxin [181, 182]. Although chaperones are considered a hallmark of TTS, there are some TTS toxins that appear to

be translocated in a chaperone-independent manner, for example the YopM, YopO and YopP proteins of *Yersinia* [183]. The majority of chaperones are highly specific and bind to only a single toxin or two toxins with similar protein sequences. However, there are some promiscuous toxin chaperones that bind a number of different proteins that exhibit no obvious sequence relationship, for example the *S. flexneri* chaperone Spa15 that binds to IpaA, IpgB1 and OspC3 [184]. In addition to the chaperones responsible for translocation of toxins, there are also chaperones that promote the secretion of translocator proteins. For example SycD is the chaperone for the translocators YopB and YopD in *Yersinia* [185].

1.5.3.1. Chaperone structure

Although there is low sequence similarity between different TTS system toxin chaperones, X-ray crystallography of a number of chaperones has revealed a high degree of structural similarity [186-189]. Structural analysis has identified a unique, structurally conserved αβα sandwich fold and has revealed that these chaperones exist as homodimers. TTS system chaperones have four hydrophobic surface patches that act as toxin-binding sites and the residues that contribute to these patches are broadly conserved among chaperones. The translocator chaperones differ markedly in structure from the toxin chaperones as they lack the αβα sandwich fold and are predicted to have a tetratricopeptide-like repeat fold [190, 191].

1.5.3.2. Chaperone-toxin binding

The chaperones are physically associated with their cognate toxins in the bacterial cytoplasm and then dissociate and remain in the cytoplasm while the toxins are translocated through the TTS apparatus [192-194]. It has been demonstrated that the binding between the YopE toxin and its chaperone SycE [192] is very tight and this strong interaction is probably afforded by hydrophobic contacts between the hydrophobic surface patches of the chaperone and the toxin [189]. It has been postulated that the TTS system ATPase is involved in supplying the energy to break the strong interaction between chaperone and toxin to allow translocation of the toxin through the TTS apparatus [195]. Evidence to support this hypothesis has recently been gained from the study of InvC, an TTS system associated protein of *Salmonella enterica*

[196]. InvC belongs to the AAA ATPase class of enzymes and binds to the chaperone $SicP_2$ complexed with its cognate toxin, SptP. InvC hydrolyses adenosine triphosphate (ATP) and provides the energy for the dissociation of SptP from $SicP_2$. SptP is liberated in its unfolded state and is able to pass through the TTS needle.

Studies of the chaperone-binding region of YopE reveal that this region lies between the N-terminal secretion signal and the guanosine triphosphatase (GTPase) activating protein (GAP) catalytic domain and is distinct from both domains [189]. The chaperone-binding domain of YopE binds to SycE in a highly extended conformation [189]. The same highly extended conformation is observed when the *Salmonella* toxin SptP binds to its cognate chaperone SicP [188] although there is no sequence similarity between the YopE and SptP chaperone-binding regions and the SycE and SicP chaperones only possess about 10% sequence identity. There is no conformational change between free and complexed SycE suggesting that chaperones act as static binding platforms [186, 189]. The presence of a large number of hydrogen bonds between the chaperone and toxin may account for the specificity of binding between these two proteins. The chaperone residues that contribute to these hydrogen bonds are not conserved among chaperones so could be involved in determining which toxins are able to bind.

1.5.3.3. Models of chaperone action

A number of hypotheses have been put forward to explain the requirement of chaperones for translocation of toxins through the TTS system. It has been proposed that chaperones act as anti-aggregation and stabilising factors, signals for secretion or antifolding factors. There is also evidence that TTS chaperones act as regulators of expression of some components of the TTS system as described later. There is probably not one single reason explaining the requirement for all chaperones, rather it is likely that different chaperones have evolved to fulfil different roles.

1.5.3.3.1. Chaperones as anti-aggregation and stabilisation factors

Some proteins are stored in the bacterial cytoplasm prior to being secreted and this may lead to the requirement of chaperones to prevent premature aggregation or degradation.

An example of a chaperone preventing premature aggregation is provided by the *Shigella* translocators IpaB and IpaC and their chaperone IpgC [197]. When translocated through the TTS apparatus, IpaB and IpaC associate to form a pore in the eukaryotic membrane to allow TTS toxins access to the eukaryotic cell cytosol. In the bacterial cell the chaperone IpgC binds to both IpcB and IpcC to prevent premature aggregation. When IpgC is absent, IpcB binds IpcC and they are both degraded.

The stability of a number of TTS toxins has also been shown to be dependent on their cognate chaperones. For example, the half-lives of the toxins YopE, SptP and IpgD are all reduced in the absence of their respective chaperones SycE, SicP and IpgE [193, 198, 199]. It has been suggested that the chaperone-binding domain of YopE promotes aggregation and subsequent degradation and SycE is required to mask this region [175, 200]. However it is unclear what role the chaperone-binding domain of YopE has as it is not required for catalytic activity [201] and its only function appears to be for binding to the chaperone.

1.5.3.3.2. Chaperones as secretion signals

An alternative model for the role of chaperones is that they act as signals that target toxins for translocation via the TTS system. Although secretion is often dependent on the extreme N-terminal residues of toxins, it has been shown in some instances that chaperone binding is sufficient for secretion. For example, the chaperone-binding domain of YopE is sufficient for secretion of a number of fusion proteins when SycE is present [202]. However the conclusion that chaperone binding to YopE is sufficient for targeting the toxin for secretion is brought into question by the observation that the deletion of the N-terminal secretion signal of YopE drastically reduces or abolishes secretion even in the presence of SycE [179, 202].

Further evidence for the ability of chaperones to target their cognate toxins to the TTS system is supplied by the *Salmonella* toxins SptP and SopE. When the chaperone-binding domains of SptP or SopE are deleted, the toxins are still secreted but not translocated into eukaryotic cells. It is however apparent that the secretion of SptP and SopE lacking their chaperone-binding domains occurs through the flagellum. Thus, in

this case, chaperone binding appears to be required for targeting the toxins specifically to the TTS system instead of the flagellum [138].

It is not clear how chaperones could function to target their cognate toxins to the TTS system but it is possible that a three-dimensional motif could act as a TTS signal. Although lacking sequence homology the three-dimensional structures of the N-terminal region of YopE complexed with SycE and the N-terminal region of SptP complexed with SicP are very similar and thus may be involved in targeting.

A possible consequence of chaperones acting as targeting signals is the ability of bacteria to translocate toxins in a hierarchical manner. It has been suggested that chaperone bound toxins are preferentially targeted to the TTS system and thus secreted before toxins that lack chaperones and only rely on their N-terminal secretion signals for targeting [175]. Although only speculative, support for the hierarchy of secretion theory is supplied by comparing the translocation of hybrid proteins containing the N-terminal of YopE fused to adenylate cyclase (Cya) in a wild type and multi-TTS substrate mutant background [175]. When the N-terminal 15 amino acids of YopE were fused to Cya, translocation was low in a wild type background but high when injected by a *Yersinia* strain lacking the majority of the TTS substrates, suggesting competition between Yops for translocation. However, when the region of YopE containing both the N-terminal secretion signal and the chaperone-binding site was fused to Cya, translocation was high in both the wild type and multimutant strain. This suggests that chaperone binding confers a quantitative privilege in secretion in the presence of toxins that lack a chaperone. This hierarchy of secretion may be important in *Yersinia* to enable the toxins with chaperones (YopE, YopH and YopT) to be translocated immediately and the toxins that probably lack chaperones (YopM, YopP and YopO) to be translocated later. YopE, YopH and YopT are antiphagocytic Yops and therefore need to be translocated quickly upon activation to prevent phagocytosis. The other Yops may modulate longer-term responses such as inhibition of the host inflammatory response by YopP.

1.5.3.3.3. Chaperones as antifolding factors

Electron microscopy images of TTS needles indicate that they have an inner diameter of only about 20 Å [143]. Thus it has been predicted that larger proteins would need to partially or fully unfold in order to pass through this conduit. The extended conformation of the SptP or YopE chaperone-binding domains observed when these proteins were bound to their cognate chaperones suggests that chaperones may be involved in this unfolding [188, 189, 200].

Evidence that toxins need to be unfolded for TTS and that chaperones play a role in this event has been supplied by studying the secretion of a number of YopE-dihydrofolate reductase (DHFR) fusion constructs [200]. When the N-terminal secretion signal (residues 1 to 16) of YopE is fused to wild type DHFR, no secretion of the complex is evident. However, when the YopE N-terminal secretion signal is fused to a DHFR mutant with lowered stability, secretion occurs. In contrast, when the N-terminal region of YopE containing the secretion signal and the chaperone-binding site is fused to wild type DHFR, secretion occurs in the presence of the YopE chaperone SycE. These experiments suggest that DHFR needs to unfold in order to be secreted by the TTS system and that this is either achieved by decreasing the stability of DHFR by mutation or by SycE binding to the YopE chaperone-binding domain.

Various observations have brought into question the ability of chaperones to function in the unfolding of TTS toxins. Although chaperones may maintain the chaperone-binding domain of their cognate toxins in an extended conformation they do not appear to globally effect the folding of the toxin as illustrated by their inability to destroy the catalytic activity of the toxin. For example the *Salmonella* toxin SigD bound to its chaperone SigE has the same level of inositol phospholipase activity as unbound SigD [187]. Also binding of the *Yersinia* toxin YopE to its chaperone SycE does not alter the GAP activity of YopE [189].

1.5.4. Regulation of expression of type III secretion systems

TTS system genes are tightly regulated at the transcriptional and posttranscriptional level to ensure that they are only produced when and where required. This tight

regulation has probably evolved to prevent the inappropriate energy consumption required for expression of the 20 or more proteins required for TTS. Bacteria use a wide variety of different regulatory mechanisms to induce TTS genes, which reflects the wide variety of niches occupied by these pathogens. There are however some common regulators of TTS systems found in some or all the bacteria including the AraC family of transcriptional activators, phosphorelay two-component regulatory systems, quorum sensing systems, RNA binding proteins and alternative sigma factors.

It has been demonstrated that TTS genes are induced by a variety of different environmental stimuli that probably reflect the host environment encountered by the bacteria during infection. Environmental cues known to be involved in the regulation of expression of the TTS system include temperature, concentration of divalent cations, pH, cell density and host signals. In addition, TTS chaperones play a role in regulating expression of TTS genes.

1.5.4.1. Regulation of the type III secretion system by temperature

Expression of TTS systems in mammalian pathogens is usually optimal at 37°C as this correlates with body temperature [203, 204]. The TTS systems of plant pathogens are generally induced at much lower temperatures, for example 20°C, to accommodate the lower temperatures encountered by these pathogens in their hosts [205].

The ability of temperature to regulate TTS gene expression has been explored in a number of bacteria including *Shigella*. In *Shigella*, when the temperature is increased to 37°C, the AraC-like transcriptional activator, VirF, activates transcription of VirB that in turn induces expression of the TTS apparatus and toxins [204]. Expression of VirF is repressed in a temperature-dependent manner by the nucleoid protein, H-NS, which is encoded by the *virR* gene [206, 207]. At temperatures below 32°C, H-NS is able to bind to the *virF* promoter and repress VirF expression. When the temperature is increased, the DNA structure of the *virF* promoter is altered and H-NS is no longer capable of repressing VirF expression. A second nucleoid-associated protein, factor inversion stimulation, is involved in this regulation as it antagonises the repressive function of H-NS bound to the VirF regulator in a temperature dependent manner [208].

1.5.4.2. Regulation of the type III secretion system by divalent cation concentrations

In a number of bacteria, expression of the components of the TTS system is controlled by the concentration of divalent cations, for example Ca^{2+} or Mg^{2+}. This method of regulation is illustrated by *S. enterica* serovar Typhimurium that senses Mg^{2+} concentration and *Yersinia* that senses Ca^{2+} concentration.

S. enterica serovar Typhimurium is a facultative intracellular pathogen that uses the TTS system encoded by *Salmonella* pathogenicity island (SPI) –1 to invade host cells and the TTS system encoded by SPI-2 to survive and multiply in the vacuoles of these cells. *S. enterica* serovar Typhimurium uses the fact that Mg^{2+} concentrations outside the cell are high and Mg^{2+} concentrations inside the vacuole are low to reciprocally regulate these two TTS systems [209-211]. Low Mg^{2+} concentrations repress expression of SPI-1 genes and this downregulation is dependent on the PhoP/PhoQ two-component system. In contrast, the expression of several SPI-2 genes are induced by low Mg^{2+} concentrations [212], although the mediators of this regulation are unknown [213].

Low Ca^{2+} concentrations induce the expression of the *Yersinia* TTS toxins, the Yop proteins [213-215]. This is achieved indirectly by a negative-feedback control mechanism that results in *yop* gene expression only when the TTS is functional and active [216]. Low levels of Ca^{2+} trigger secretion of LcrQ via the TTS system, the repression of *yop* expression by LcrQ (either directly of indirectly) is released and these toxins can be synthesised and secreted [217].

1.5.4.3. Regulation of the type III secretion system by pH

Acidity levels can be an important regulator of TTS expression as exemplified by the intestinal pathogen enteropathogenic *E. coli* (EPEC). This pathogen needs to traverse the acidic environment of the stomach before it reaches the intestine where it secretes toxins via the TTS system encoded by the locus of enterocyte effacement (LEE) [218]. It has been shown that the AraC-like transcription factor, GadX, is important in the control of TTS expression in response to pH levels [219]. The expression of TTS genes

from LEE is induced by a cascade in which GadX induces expression of the *per* locus [219], the transcriptional activator encoded by the *per* locus [220] activates transcription of Ler (LEE-encoded regulator) [221], and Ler induces expression of the TTS genes. When pH is low, for example in the stomach, GadX is unable to induce expression of the *per* locus and as a result no TTS genes are induced. When the pH rises, for example in the intestine, GadX induces transcription of the *per* locus, therefore Ler is produced and the TTS genes in LEE are induced [219]. GadX also regulates the expression of the *gadAB* locus that encodes the glutamate decarboxylase system that enables survival of EPEC in the stomach [219]. As expected, the *gadAB* and *per* locus are reciprocally regulated, with expression from *gadAB* being induced by GadX under acidic conditions.

Another TTS system that utilises a change in pH to control expression and secretion of TTS toxins is the SPI-2 system in *S. enterica* serovar Typhimurium. Once *Salmonella* has induced its own uptake using the SPI-1 TTS system, it needs to establish a replicative niche within the vacuole it resides in. *Salmonella* achieves this by secreting toxins via the SPI-2 TTS system that act to alter the *Salmonella*-containing vacuole to prevent bacterial degradation and allow bacterial replication [222]. Within 20 minutes of formation of the *Salmonella*-containing vacuole inside infected host cells, the pH of this organelle drops below 5.5 [223] and *Salmonella* senses this change to activate SPI-2 gene expression and TTS [224-227]. Therefore the SPI-2 toxins are only produced and secreted when and where required to form a mature *Salmonella*-containing vacuole.

1.5.4.4. Regulation of the type III secretion system by quorum sensing

Quorum sensing describes the ability of bacteria to induce gene expression in response to cell density. There are a number of examples of TTS genes being controlled by quorum sensing including the regulation of the enterohemorrhagic E. coli (EHEC) TTS system [228]. In EHEC, LuxS makes autoinducer (AI)-2 and AI-3 that are secreted and provide a measure of cell density [229, 230]. Once the concentration of AI-3 (but not AI-2) has reached a certain threshold, TTS genes are induced [230].

1.5.4.5. Regulation of the type III secretion system by host cell factors

Expression of TTS toxins is induced in many bacteria by contact with eukaryotic cells and it is likely that the signals for cell contact are non-diffusible macromolecules located on the surface of eukaryotic cells. In most cases, for example in *Yersinia*, *Shigella* and *P. aeruginosa*, induction of TTS gene expression by cell contact requires an intact and functional TTS system. The TTS system may be involved in sensing and transmitting the signal directly or by allowing secretion of a negative regulator of TTS gene expression. In *Yersinia*, Yop production is induced by eukaryotic cell contact because this contact stimulates the secretion of the negative regulator of Yop genes, LcrQ [217].

In contrast to *Yersinia*, the TTS system of the plant pathogen *R. solanacearum* is induced and assembled upon cell contact independently of a functional TTS system [231]. This induction is achieved by a signal transduction pathway that consists of at least six genes: *prhA, prhR, prhI, prhJ, hrpG, hrpB*. It has been hypothesised that the outer membrane protein PrhA interacts with a non-diffusible plant cell wall component [232] and a signal is then transduced across the periplasm and inner membrane by PrhR and PrhI to the cytoplasmic regulator PrhJ [233]. PrhJ activates HrpG expression, which in turn activates HrpB expression [234, 235]. The AraC regulator, HrpB is then able to induce expression of the TTS system genes [235].

In addition to the ability of bacteria to induce TTS system genes in response to host cell contact, a number of other host signals have been identified that regulate this system. The SPI-2 TTS system in *S. enterica* serovar Typhimurium is induced by the natural resistance-associated macrophage protein-1 (Nramp1) [236]. During infection the host-defence protein, Nramp1 probably acts by transporting divalent cations including iron across the membrane of the *Salmonella* containing vacuole and thus starving the bacteria of essential nutrients [237-239]. Therefore *S. enterica* serovar Typhimurium appears to produce a virulence system, the SPI-2 TTS system, to counteract a host defence mechanism. Another example of the ability of bacteria to express their TTS system in response to a host signal is provided by EHEC. The hormone epinephrine activates the expression of the TTS system in EHEC probably by binding to the receptor for the quorum sensing autoinducer, AI-3 [230, 240].

1.5.4.6. Regulation of the type III secretion system by chaperones

In addition to their role in ensuring sufficient secretion of toxins or translocators, some chaperone proteins are also involved in feedback gene regulation of components of the TTS system. Induction of TTS, for example by cell-contact, results in an increase in concentration of unbound chaperones in the bacterial cytosol as the chaperones release their cognate toxins or translocators. The chaperones are then free to activate the expression of more TTS toxins for secretion. Chaperones that are capable of inducing expression of TTS systems are generally those that bind to translocators not toxins. The use of translocator chaperones in this regulatory context may be to establish a secretion and expression hierarchy so that translocators are secreted first to form pores in the eukaryotic membrane and then toxins are maximally expressed and secreted through these pores.

An example of this chaperone-mediated feedback gene regulation is supplied by the *Salmonella* SicA chaperone. SicA is a chaperone for the translocators SipB and SipC [240, 241]. Once secretion is initiated and SipB and SipC are secreted via the TTS system, the levels of free SicA in the bacteria rise. SicA is then free to act as a cofactor for the AraC-like transcriptional activator InvF that initiates transcription of a subset of TTS system-associated genes in *Salmonella* SPI-1 [242, 243].

As mentioned above, host cell contact or low Ca^{2+} levels result in secretion of LcrQ from *Yersinia* and subsequent upregulation of *yop* gene expression [217]. This regulation by LcrQ appears to be indirect and a number of experiments have implicated the LcrQ chaperone SycH and the translocator YopD and its chaperone SycD in the process. There is evidence that LcrQ and SycH inhibit the secretion of some Yops therefore imposing a hierarchy of secretion so that certain toxins can only be secreted when LcrQ has already been secreted [244]. One of these proteins secreted after LcrQ may be a negative regulator of Yop expression that acts at either the transcriptional or posttranscriptional level. It is postulated that this negative regulator is YopD bound to its cognate chaperone SycD as these proteins are required for LcrQ function [245, 246]. It has been demonstrated that YopD-SycD binds to the 5' untranslated region of *yopQ* and *yopE* mRNA and that LcrQ exerts its negative effect at the 5' UTR of *yop* genes [247, 248]. Thus, it is postulated that YopD-SycD either inhibits translation or leads to degradation of *yop* mRNAs and this inhibition is released when YopD is secreted. Free

SycD may also act directly by activating Yop gene expression in an analogous manner to its distant homologue, SicA.

1.5.5. Regulation of secretion via the type III secretion systems

As alluded to above, the TTS systems can be regulated at the level of secretion as well as at the level of expression. In fact, in *Yersinia*, it is the initial activation of secretion either by cell contact or low Ca^{2+} levels that lead to induction of *yop* genes. In contrast to the corregulation of secretion and expression in *Yersinia*, the TTS toxins of *S. flexneri* accumulate in the bacterial cytoplasm and are then secreted upon cell contact [249]. A second difference between TTS in *Yersinia* and *S. flexneri* is the polarity of secretion. Whereas upon induction by host cell contact, *Yersinia* only secrete TTS effectors in a polarised manner into eukaryotic cells [194, 250], *S. flexneri* secretes effectors in a non-polarised manner into the surrounding media [249].

The ability of *Yersinia* to secrete Yops in a polarised manner upon cell contact is mediated by the surface-exposed, TTS protein YopN. *Yersinia* mutants lacking a functional *yopN* gene secrete YopE and YopH into the surrounding media upon cell contact [194, 250]. These *yopN* mutants also secrete large amounts of Yops under high Ca^{2+} repressing conditions and thus YopN may function as a plug that blocks the TTS channel [251, 252]. It has been postulated that YopN is a surface exposed sensor that responds to a signal on the host cell surface and as a result allows localised opening of the secretion channels at the sites of cell contact.

Two models have been proposed to explain the secretion of accumulated Ipa proteins from the cytosol of *S. flexneri* upon cell contact. In the first model, the TTS channel is blocked by a complex between IpaB and IpaD. Upon cell contact, IpaB and IpaD dissociate and allow TTS of the accumulated Ipa proteins [249]. In the second model, IpaB, IpaC and IpaD are localised on the bacterial surface prior to cell contact and upon cell contact they are rapidly released. The other Ipa proteins are then slowly released via TTS from the intracellular stores [253]. There are data supporting both of these models and the differences may be the result of different secretion signals from different cell types being sensed.

1.5.6. Toxins secreted by type III secretion systems

Although many of the components of the TTS systems are widely conserved among Gram-negative bacteria, the toxins secreted by these systems exhibit a broad range of biochemical activities and interfere with a wide range of cellular processes. Most TTS toxins display sequence, structural or functional homology to eukaryotic proteins and are therefore able to subvert normal host cell processes to enhance survival of the pathogen. In many cases, the activity of the toxins is restricted to eukaryotic cells, either by acting on exclusively eukaryotic targets or due to the requirement of eukaryotic cofactors.

Despite the considerable variation between TTS toxins produced by different bacteria, there are a number of common pathways that they affect including disrupting the actin cytoskeleton and modulating inflammation.

1.5.6.1. Type III secreted toxins that disrupt the actin cytoskeleton

The function of many TTS toxins is to disrupt the actin cytoskeleton of eukaryotic cells. Some bacteria, for example *Shigella* and *Salmonella*, produce toxins that alter the cytoskeleton of nonphagocytic cells in order to promote their uptake by macropinocytosis. In contrast, other bacteria such as *Yersinia* produce toxins that block phagocytosis by disrupting the cytoskeleton of phagocytic cells. TTS toxins interfere with the eukaryotic cytoskeleton either directly by interacting with the components of this structure, or indirectly by modulating the activity of Rho GTPase proteins that control the cytoskeleton.

Rho GTPases are a family of small guanoisine triphosphate (GTP)-binding proteins that are master regulators of cytoskeleton dynamics and are exemplified by Cdc42, Rac1 and RhoA [254]. In common with other small GTP-binding proteins, Rho GTPases cycle between their inactive guanosine diphosphate (GDP)-bound state and their active GTP-bound state (Fig. 1.5). The exchange of GDP by GTP, and thus the activation of the small GTP-binding proteins, is catalysed by guanine nucleotide exchange factors (GEFs). The subsequent inactivation of the small GTP-binding proteins by hydrolysis of GTP to GDP occurs as a result of the intrinsic GTPase activity of these proteins and

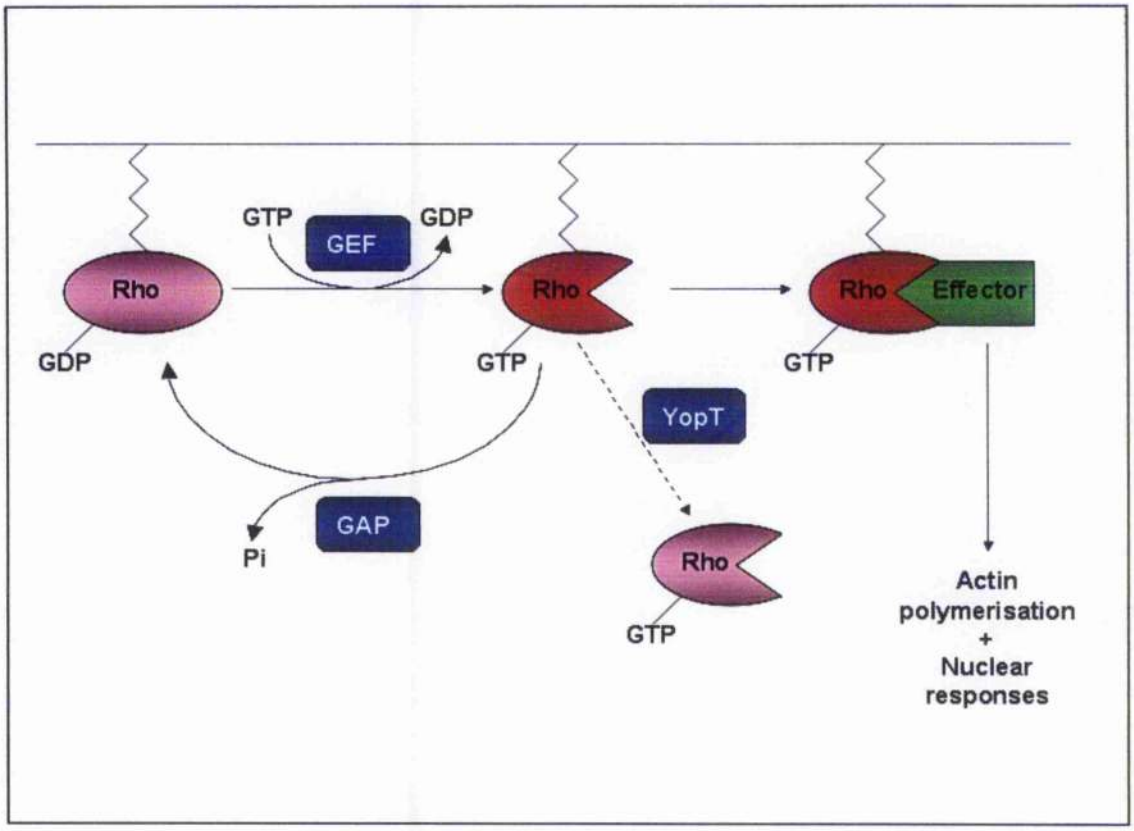


Fig. 1.5. Action of type III secreted toxins on Rho GTPases.

The Rho GTPases (Rho), Rho, Rac and Cdc42, are inactive in the GDP-bound state and active in the GTP- and membrane-bound state. The interaction of active Rho GTPases with their effectors results in various effects including actin polymerisation and nuclear responses. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by catalysing the exchange of GDP by GTP. GTPase activating proteins (GAPs) inactivate Rho GTPases by enhancing the hydrolysis of GTP. Some TTS toxins act as GEFs, for example the *Salmonella* toxins SopE and SopE2. Some TTS toxins function as GAPs, for example the *Salmonella* toxin SptP, the *Yersinia* toxin YopE and the *P. aeruginosa* toxins ExoS and ExoT. The *Yersinia* TTS toxin YopT inactivates RhoA by cleaving the isoprenylated RhoA near its carboxyl termini and abolishing its membrane binding. Adapted from [255].

is enhanced by GTPase activating proteins (GAPs). In addition to binding GTP, small GTP-binding proteins need to be membrane bound to be active and this binding is mediated by the post-translation modification of their carboxyl termini with a prenyl group.

Many TTS toxins act by modulating the activity of Rho GTPases either by functioning as GEFs, by exhibiting GAP activity or by abolishing membrane binding of the Rho GTPase. For example, the *Salmonella* SopE protein binds and functions as a GEF for Cdc42 and Rac1 [256]. The activation of Cdc42 and Rac1 by SopE results in cytoskeletal rearrangements and the subsequent uptake of *Salmonella* by non-phagocytic cells. *Salmonella* produces a second GEF, $SopE_2$, which is closely related to and exhibits similar properties to SopE [257]. In addition to SopE and $SopE_2$, *Salmonella* also secretes the TTS toxin SptP that exhibits GAP activity towards Cdc42 and Rac1 [258]. The ability of SptP to reverse the action of SopE promotes restoration of the host cell cytoskeleton after *Salmonella* invasion. Although both SopE and SptP are injected into the cytoplasm of the host cell at the same time, SptP is able to act after SopE as SopE is rapidly degraded by ubiquitin-mediated proteolysis [259].

Like *Salmonella*, *Shigella* also produces a TTS toxin, IpaC, which promotes entry into non-phagocytic cells by activating Rho GTPases. IpaC, in addition to its role as a translocator, is capable of activating Cdc42 that in turn activates Rac1 and consequently leads to actin polymerisation and bacterial uptake [260]. IpaC however is not a GEF and it is unclear how it activates Cdc42 and subsequently Rac1.

In contrast to *Salmonella* and *Shigella*, *Yersinia* produces a TTS toxin that blocks phagocytosis. The YopE protein of *Yersinia* exhibits GAP activity towards RhoA, Rac1 and Cdc42 [261]. The inactivation of these Rho GTPases leads to disruption of the actin cytoskeletion and subsequent inhibition of macrophage phagocytosis of the bacteria [262]. *Yersinia* also exploits the membrane-binding requirement of Rho GTPases to resist macrophage phagocytosis. The *Yersinia* TTS toxin YopT is a cysteine protease that cleaves isoprenylated RhoA near its carboxyl termini [263]. The non-membrane bound RhoA is inactive and the resulting depolymerisation of actin in the macrophage prevents phagocytosis of the pathogen [264, 265].

In contrast to interfering with the Rho GTPases, a number of TTS toxins are able to disrupt the eukaryotic cytoskeleton by binding directly to actin. For example, the actin-binding proteins (ABPs) of *Salmonella*, SipA and SipC, promote entry of *Salmonella* into non-phagocytic cells by disrupting the actin cytoskeleton [266]. It has been demonstrated that purified SipC nucleates actin polymerisation and bundles F-actin into cables *in vitro* [267]. SipA is also able to bind F-actin and acts to stimulate actin polymerisation and prevent actin disassembly by a number of direct and indirect mechanisms [268-271].

The ability of EPEC and EHEC to induce pedestal formation in epithelial cells is another example of the modulation of the actin cytoskeleton by TTS toxins (Fig. 1.6). EPEC and EHEC both secrete Tir (translocated intimin receptor) via their TTS systems. Tir is inserted into the plasma membrane of host cells and facilitates attachment of the bacteria by binding to the bacterial outer membrane protein intimin [272, 273]. The interaction between intimin and Tir triggers a signalling cascade that alters the normal regulation of the host cytoskeleton and leads to the formation of the pedestal-like structures. In both EHEC and EPEC, actin assembly is induced by the actin-related protein 2/3 (Arp 2/3) complex, which is activated by the neuronal Wiskott-Aldrich syndrome protein (N-WASP) [274]. In EPEC, N-WASP recruitment is dependent on tyrosine phosphorylation of the injected Tir and recruitment of the mammalian adaptor proteins Nck1 and Nck2 [274, 275]. However in EHEC, the TTS toxin EspFu/TccP recruits N-WASP to Tir in tyrosine phosphorylation and Nck-independent manner [273, 276, 277].

A number of the TTS toxins target the actin cytoskeleton of eukaryotic cells by phosphorylating or dephosphorylating components of the signal transduction pathways involved in the control of the cytoskeleton. For example, the TTS toxin YopH is a protein tyrosine phosphatase (PTPase) that contributes to the ability of *Yersinia* to resist macrophage phagocytosis [278]. It has been demonstrated that YopH dephosphorylates a number of proteins involved in the formation of focal adhesions in macrophages and it is likely that these actions contribute to the antiphagocytic action of this TTS toxin [279-282]. The autophosphorylating serine-threonine kinase, YpkA, is also able to contribute to the virulence of *Yersinia* [283]. YpkA induces morphological changes and disrupts the cytoskeleton in cultured cells [284], although the targets of YpkA and its

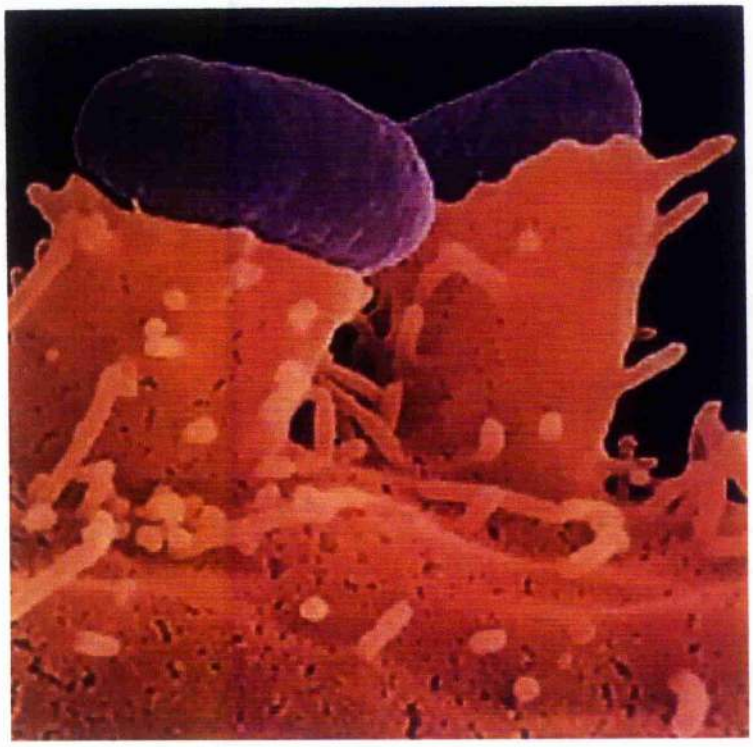


Fig. 1.6. Scanning electron micrograph of the pedestals induced by EPEC.
The rod-shaped EPEC bacteria are coloured in purple and the epithelial cell surface extruding two pedestals is coloured in orange. Taken from [285].

mode of action remain unclear. *Salmonella* produces an inositol phosphatase SopB that causes rearrangement of the actin cytoskeleton and stimulates bacterial cell entry [286]. SopB mediates the conversion of inositol 1,3,4,5,6-pentakisphosphate to inositol 1,4,5,6-tetrakisphosphate which appears to activate Cdc42 and result in actin reorganisation [286]. SopB is also required for the maturation of the *Salmonella*-containing vacuole [287] and this may be due to its ability to promote membrane fission by eliminating phosphatidylinositol-4,5-bisphosphate [288]. The *Shigella* toxin IpgD is another inositol phosphatase responsible for dramatic morphological changes in the host cell that are believed to promote bacterial entry [289]. IpgD transforms phosphatidylinositol-4,5-bisphosphate into phosphatidylinositol-5-phosphate and this conversion is somehow responsible for the induction of the morphological changes.

Two other *Shigella* toxins that modify the host cell cytoskeleton and enable efficient bacterial entry into epithelial cells are VirA and IpaA. VirA binds to tubulin and promotes microtubule destabilisation [290] and IpaA binds vinculin and induces F-actin depolymerisation [291].

1.5.6.2. Type III secreted toxins that modulate inflammation

A second common mechanism of action for TTS toxins is to modulate inflammation by interfering with mitogen-activated protein kinase (MAPK) and NF-κB signalling pathways. MAPK and NF-κB signalling pathways are phosphorylation cascades that result in the transcription of a number of genes including pro-inflammatory cytokines and anti-apoptotic genes. Some TTS toxins act to promote the production of pro-inflammatory cytokines, while others act to downregulate cytokine production.

The inositol phopshatase SopB and the GEF SopE, in addition to their roles in promoting *Salmonella* uptake by modulating the eukaryotic cytoskeleton, are also able to induce inflammation. Both SopB and SopE promote the production of pro-inflammatory cytokines by inducing activation of the MAPK, c-Jun NH_2-terminal kinase (JNK) [256, 286].

As described above, the GAP domain of SptP reverses the cytoskeletal changes induced by *Salmonella*. Similarly, SptP also appears to downregulate the *Salmonella*-induced

activation of the MAPKs, JNK and ERK [258, 292]. SptP is a bifunctional protein that possesses a carboxyl-terminal PTPase domain in addition to its GAP domain and it appears to be the tyrosine phosphatase activity of SptP that is responsible for inhibiting ERK activation [292]. The only potential target identified so far for the PTPase domain of SptP is the intermediate filament protein vimentin [292]. It is unclear how phosphorylation of vimentin could reverse the *Salmonella*-induced cellular responses and therefore other target(s) of SptP may need to be identified.

A number of the TTS toxins produced by *Yersinia* are involved in the downregulation of the inflammatory response induced by infection, for example the PTPase YopH and the GAP YopE [293-295]. The *Yersinia* toxin YopJ (YopP in *Y. enterocolitica*) is also capable of counteracting the pro-inflammatory response in various cell culture systems [296, 297]. YopJ/P achieves this anti-inflammatory response by inhibiting MAPK and NF-κB signalling pathways [298]. YopJ/P binds to MAPK kinases (MKKs) and inhibits their activation by blocking their phosphorylation. YopJ/P also binds to the inhibitor-kappa B kinase β (IKKβ) and this interaction probably prevents the phosphorylation and subsequent activation of IKKβ. YopJ/P belongs to the CE clan of cysteine proteases and there is some evidence that it may act as an ubiquitin or SUMO (small ubiquitin-related modifier) protease [299, 300]. However, it is unclear how this de-ubiquitinylating or de-SUMOylating activity could contribute to the ability of YopJ/P to disrupt MAPK and NF-κB signalling. YopJ/P also induces apoptosis in macrophages [301] and this may be the result of the inactivation of NF-κB signalling that leads to the inhibition of host cell survival factors [302]. Alternatively the protease activity of YopJ/P may act directly to promote apoptosis [303].

1.5.6.3. Other functions of type III secreted toxins

In addition to the modulation of the cytoskeleton and inflammation signalling, TTS toxins have many other roles in altering the eukaryotic cell to facilitate their own survival. Other classes of TTS toxins include the caspase-1-binding proteins and a family of toxins containing leucine-rich repeats. Although a vast amount of work has been done to elucidate the biological activity and targets of the TTS toxins, the action of many is still unknown.

The TTS toxins IpaB and SipB, from *Shigella* and *Salmonella* respectively, induce apoptosis of macrophages by a caspase-1 dependent mechanism [304, 305]. In addition to their role as translocators, IpaB and SipB are delivered into the cytosol of infected cells where they interact with and presumably activate caspase-1 [306, 307].

Another class of TTS toxins are those that possess a leucine-rich repeat. Members of this family include YopM from *Yersinia*, SspHs and SlrP from *Salmonella* and IpaHs from *Shigella*. The most extensively studied of this group of toxins is YopM, which has been shown to interact with and stimulate protein kinase C-like 2 and ribosomal S6 protein kinase 1 [308]. There is also evidence that YopM causes a depletion of natural killer cells *in vivo*, probably by affecting the expression of interleukin (IL)-15 receptor α and IL-15 [309].

1.6. The type III secretion system of *Pseudomonas aeruginosa*

Having reviewed the TTS systems of Gram-negative bacteria, I will now focus on what is known about the TTS system in *P. aeruginosa*. I will describe the components of the TTS apparatus in *P. aeruginosa* and discuss how this system is regulated. I will then discuss each of the toxins secreted by the TTS system of *P. aeruginosa* in turn and consider what is known about their enzymatic activity and role in infection.

1.6.1. The type III secretion machinery in *Pseudomonas aeruginosa*

The genes encoding the pseudomonal TTS system are located in the 55 min region of the *P. aeruginosa* PAO1 bacterial chromosome [310]. The genes are clustered in 5 operons: *pscNOPQRSTU*, *popNpcr1234DR*, *pcrGVHpopBD*, *exsCEBA*, and *exsDpscBCDEFGHIJKL*. Most of the components have homologues in the *Yersinia* TTS system and are predicted to fulfil the same functions (Fig. 1.7). The *psc* and *pcr* genes encode components of the TTS apparatus and regulatory proteins, the *exs* genes encode regulators of TTS and the *pop* gene products are secreted proteins.

The pseudomonal TTS needle is mainly composed of PscF and this protein is able to form robust needle-like structures when expressed on its own [311]. PscF forms a 1:1:1 stable, soluble complex with PscE and PscG in the cytoplasm of *P. aeruginosa* and this

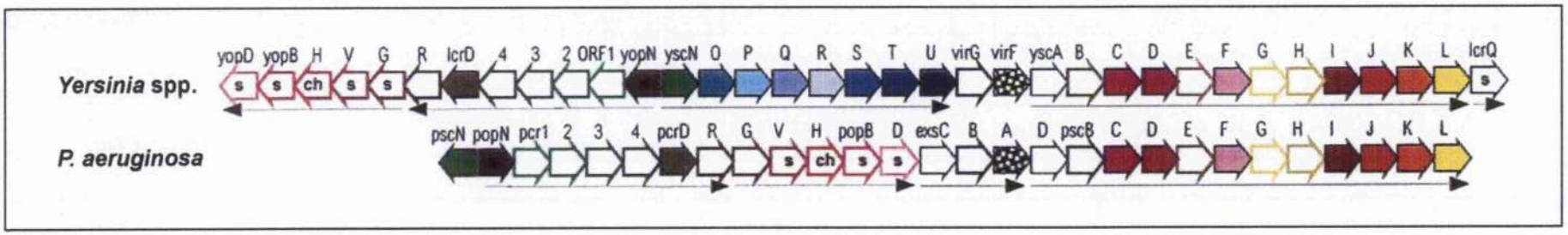


Fig. 1.7. Genetic organization of type III secretion systems of *Yersinia* spp. and *P. aeruginosa*

Diagram showing the similarity in sequence and organization of the genes encoding the TTS systems of *Yersinia* spp. and *P. aeruginosa*. Homologies between encoded proteins are indicated by color code. The filled in arrows indicate genes that are widely conserved among TTS systems of Gram-negative bacteria, while the unfilled arrows indicate genes that are not widely conserved. The black and white checked filling patterns indicate genes that encode transcription factors. A small **s** inside a gene symbol indicates secretion of the encoded protein, while the genes which encode chaperonic proteins are labeled **ch**. Transcriptional units are indicated by arrows underneath the genes. Taken from [310].

complex is required to prevent premature polymerisation of PscF in the bacterial cytoplasm and to maintain PscF in a secretion-prone conformation [311]. The length of pseudomonal PscF needles appears to be controlled, because although TTS needles from *P. aeruginosa* are about 80 nm in length, PscF can generate needles of over 1 μm when expressed in *E. coli* [311]. It has been proposed that the needle length is controlled by the YscP homologue in *P. aeruginosa*, PscP [313].

In addition to the four toxins secreted by the TTS system of *P. aeruginosa,* PcrV, PopB, PopD and PopN are also secreted via this system [314]. PopB and PopD are translocators that form pores in the host cell membrane [315, 316] and the chaperone PcrH is required for their presecretory stabilisation and efficient secretion [317]. PcrV is also required for the functional assembly of the membrane-inserted PopB/PopD translocon complex, although it does not form part of the pore [318]. It has been demonstrated that PcrV in concert with PopN and PcrG is involved in establishing efficient polarised translocation of toxins and negatively regulating toxin expression [319].

1.6.2. Regulation of expression of the type III secretion system in *Pseudomonas aeruginosa*

In *P. aeruginosa*, expression of the TTS system is induced by low calcium concentrations, the presence of serum or host cell contact [310, 320]. Expression of the TTS system is highly regulated by a number of pathways that converge on the AraC-like transcriptional activator, ExsA [321]. ExsA binds to the consensus sequence, T-X-A-A-A-A-X-A, about 50 bp upstream of the transcription start site of the TTS system genes to induce their expression [322].

Induction of the TTS system occurs only in the presence of a functional TTS system, thus suggesting a direct link between secretion and transcription [323, 324]. It is apparent that when the secretion channel is closed in the presence of high calcium, transcription of the TTS system is repressed, but when the secretion channel is opened by low calcium levels, the TTS system is expressed [323]. This suggests the presence of a negative regulator of the TTS system that is secreted when the secretion channel is open in a manner reminisant of LcrQ/YopD secretion by *Yersinia.* It has been recently

demonstrated that the secreted negative regulator of TTS gene expression in *P. aeruginosa* is ExsE [325, 326] (Fig. 1.8). ExsE is a small, highly charged protein that binds to ExsC in the bacterial cytoplasm. ExsC is a chaperone-like protein required for the efficient secretion and stability of ExsE [325, 326] and also acts as an anti-anti-activator of TTS expression. ExsC induces TTS gene expression by binding to an anti-activator of TTS expression, ExsD [327]. ExsD represses TTS gene expression by directly binding to ExsA and inhibiting the activity of this transcriptional activator [323]. Thus it has been proposed that under high calcium conditions (Fig. 1.8, A), the secretion channel is closed and ExsE is not secreted and therefore binds to its chaperone, ExsC. ExsD is free to bind to ExsA and repress TTS gene expression. When the channel is open, for example due to low calcium levels (Fig. 1.8, B), ExsE is secreted via the TTS apparatus and ExsC is able to bind ExsD. The binding of ExsC to ExsD releases ExsA, which is then free to activate transcription of the TTS system [325, 326].

In addition to the ExsECDA signalling cascade that controls TTS gene expression in *P. aeruginosa*, an increasing number of positive and negative regulatory components have been identified. For example, positive regulators of the TTS system include the membrane-associated adenylate cyclase CyaB and the cAMP dependent transcription factor Vfr that are required for expression of the TTS system in response to calcium depletion and act upstream of or in parallel to ExsA [328]. The recently identified FimL protein also appears to regulate TTS gene expression by intersecting with the Vfr-modulated pathway [329]. Another protein required for TTS gene expression is the predicted inner membrane protein RtsM that may act as a two-component signalling protein that links environmental sensing with activation of TTS [330]. The pseudouridinase enzyme TruA is also required for expression of TTS genes, possibly because pseudouridination of tRNAs is critical for the translation of TTS genes or their regulators [331]. Finally, the periplasmic thiol/disulphide oxidoreductase DsbA affects multiple virulence factors and is required for TTS gene expression probably because the absence of this enzyme results in abnormal protein folding due to the lack of disulphide bonds [332].

Negative regulators of the TTS system include PtrA, which specifically suppresses TTS gene expression by directly binding to ExsA [333]. The expression of *ptrA* is highly

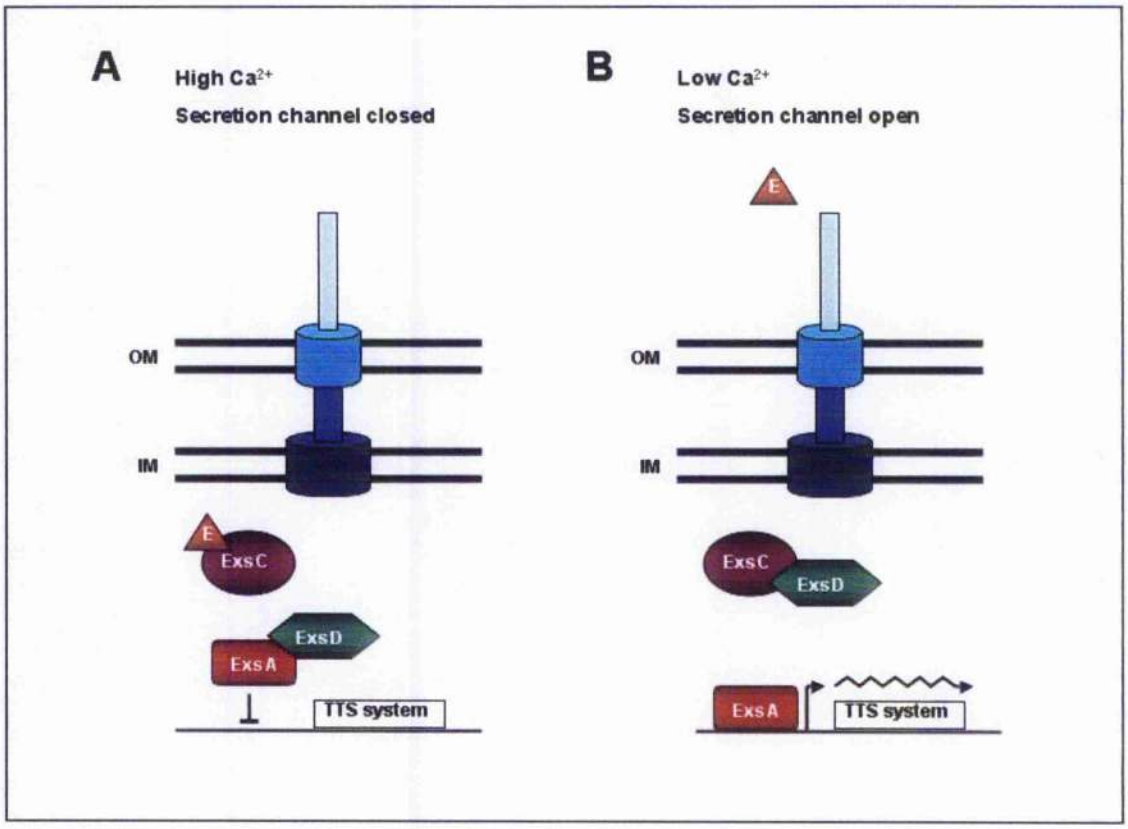


Fig. 1.8. A model for the regulation of the type III secretion regulon in *P. aeruginosa*.

The TTS apparatus is indicated in blue spanning the inner membrane (IM) and outer membrane (OM) of the bacterial cell. At high Ca^{2+} concentrations, the secretion channel is closed and ExsE (E) is bound to ExsC. ExsD is therefore free to bind ExsA and repress transcription of the TTS system regulon. Under low Ca^{2+} conditions, the secretion channel is open and ExsE is secreted. ExsC binds ExsD, and ExsA is free to induce expression of the TTS system regulon. Adapted from [326].

and specifically induced by high copper levels through the CopR-CopS two-component regulatory system, suggesting that the TTS system is repressed under conditions of copper stress [333]. TTS gene expression is also negatively regulated by the Rhl quorum sensing system and the stationary phase sigma factor RpoS, which likely contributes to the observed repression of ExoS in pseudomonal biofilms [334, 335]. The TTS system is reciprocally regulated compared to alginate synthesis, with a mutation in the *mucA* gene suppressing expression of the TTS system and this suppression being dependent on AlgU and AlgR [336].

There is also evidence that the metabolic status of the bacterial cell effects expression of TTS genes in *P. aeruginosa*. For example, the *aceAB* genes, which encode the subunits of pyruvate dehydrogenase, are required for TTS gene expression in response to low calcium [337]. Also a mutation that results in overexpression of histidine utilization genes abolishes cytotoxicity mediated by the TTS system [338]. This cytotoxicity defect can be partially suppressed by an insertion mutation in *cbrA*, which encodes the sensor kinase in a two-component system implicated in sensing and responding to carbon-nitrogen imbalance [338].

1.6.3. The type III secreted toxins of *Pseudomonas aeruginosa*

There are four known toxins secreted by the TTS system of *P. aeruginosa*: ExoS, ExoT, ExoU and ExoY. ExoS and ExoT are bifunctional proteins containing an N-terminal GAP domain and a C-terminal ADPRT domain. ExoU is a phospholipase and ExoY is an adenylate cyclase. Not all strains of *P. aeruginosa* encode all the TTS toxins. A study of over 100 clinical and environmental strains revealed that they all contained *exoT*, 89% contained *exoY*, 72% contained *exoS* and 28% contained *exoU* [339]. An inverse correlation between the presence of the *exoS* and *exoU* genes was observed where all but two isolates contained either *exoS* or *exoU* but not both [339].

1.6.3.1. Exoenzyme S

ExoS was first identified in 1978 as an ADPRT secreted by *P. aeruginosa* strain 388 [340]. It was known at that time that *P. aeruginosa* produced and secreted another ADPRT named Exotoxin A, which inactivated protein synthesis by ADP-ribosylating

EF-2. ExoS however did not target EF-2 but instead ADP-ribosylated a number of proteins in crude extracts of wheat germ or rabbit reticulocytes [340].

The ADPRT activity of ExoS co-purified with two proteins with a molecular weight of 49 kDa and 53 kDa [341]. These two proteins were immunologically cross-reactive [342] and shared similar N-terminal amino acid and proteolytic peptide sequences [343]. The 53 kDa protein appeared inactive in comparison to the 49 kDa protein that exhibited potent ADPRT activity [342, 343]. It was therefore proposed that the 53 kDa protein was a precursor of the 49 kDa form and that ExoS required proteolytic cleavage to become active. The *exoS* gene was subsequently cloned [344] and a *P. aeruginosa* mutant was generated that lacked *exoS* [345]. This *exoS* deletion mutant did not secrete the 49 kDa protein and exhibited no ADPRT activity but still expressed and secreted the 53 kDa form [345]. This suggested that different genes encoded the 49 kDa and 53 kDa proteins, a fact that was later proved by the identification of the gene encoding the 53 kDa protein, *exoT* [346].

ExoS does not contain a typical N-terminal signal peptide and it is secreted without N-terminal processing [344, 346]. It was therefore apparent that ExoS was not secreted by the general secretory pathway and it was suggested that it might be secreted by a TTS system, typified at that time by the TTS system that exports Yops in *Yersinia* [347]. Identification of *P. aeruginosa* mutants that were unable to secrete ExoS and contained a transposon insertion in an operon that contained homologues of the Yop TTS apparatus, confirmed that ExoS was indeed secreted by the TTS system [324].

1.6.3.1.1. The consequences of exoenzyme S expression

Initial studies using purified ExoS showed that this protein was not toxic when added directly to cell culture or animal model systems [343, 348]. This apparent lack of toxicity is the result of the requirement for a functional TTS system to transport ExoS from the cytoplasm of *P. aeruginosa* into the cytoplasm of eukaryotic cells [324]. Therefore, the consequences of ExoS expression on eukaryotic cells were examined either by comparing the effects of co-culturing cells with an ExoS-producing *P. aeruginosa* strain and an isogenic non-ExoS-producing mutant or by introducing ExoS into eukaryotic cells through the TTS system of *Y. pseudotuberculosis*. ExoS

production correlated with a decrease in DNA synthesis and cell viability, disruption of the actin cytoskeleton that led to cell rounding, loss of cell adhesion and microvillus effacement [349-352]. ExoS also had an anti-phagocytic effect on macrophages and inhibited pseudomonal invasion of corneal epithelial cells [352, 353]. A number of studies have suggested a role for ExoS in triggering apoptosis [354, 355]. ExoS appears to activate a pro-apoptotic pathway through JNK-mediated cytochrome *c* release and inhibit anti-apoptotic pathway(s) controlled by ERK1/2 and maybe p38 [356].

Early studies examining the effects of ExoS in mammalian models of *P. aeruginosa* infection suggested that ExoS contributed to bacterial dissemination in a burned mouse model and to pathology in a rat model of chronic lung infection [342, 357-359]. However, the mutant strain used in these experiments contained a transposon in an operon encoding a portion of the TTS apparatus [324]. Thus, the observed effects were the result of inhibition of secretion of all the TTS toxins in the strain not just ExoS. A more recent study that examined the effect of a targeted disruption of the *exoS* gene failed to detect any contribution of ExoS to virulence in a burned mouse model [360]. However, ExoS has been shown to contribute to virulence in a mouse model of acute pneumonia [361, 362]. ExoS facilitated both bacterial persistence and dissemination to extrapulmonary sites such as the liver [361, 362].

1.6.3.1.2. The domain structure of exoenzyme S

ExoS is a 453 amino acid protein, which exhibits ADPRT activity. In addition to its ADPRT activity, ExoS is also able to act as a GAP. These two enzymatic activities are located in separate functional domains of ExoS, with the N-terminus containing the GAP domain and the C-terminus containing the ADPRT domain (Fig. 1.9). In addition to these two catalytic domains, ExoS also possesses sequences required for secretion, chaperone binding and membrane localisation, N-terminal to the GAP domain (reviewed in [363]).

1.6.3.1.3. The secretion domain of exoenzyme S

As previously discussed, toxins that are transported via the TTS system require targeting to this system by a TTS signal located in the first ~ 15 mRNA codons or

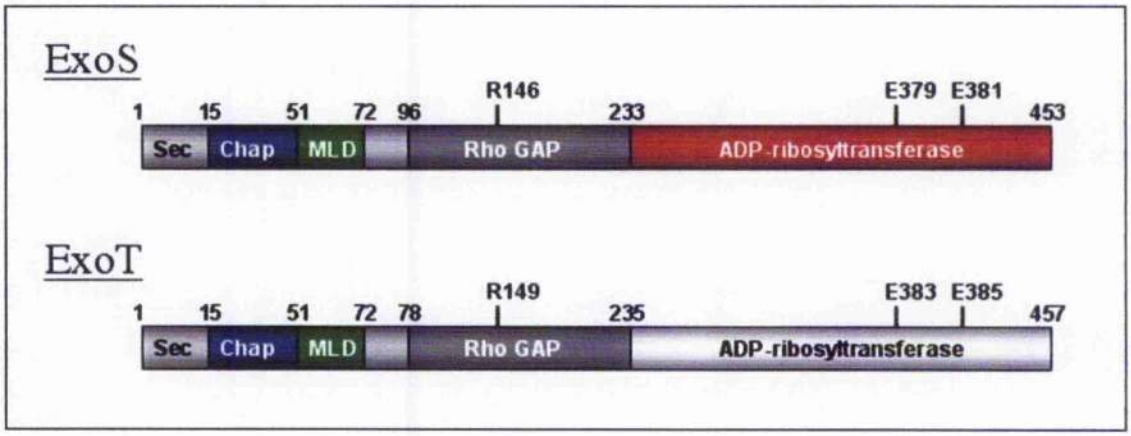


Fig. 1.9. The domain structure of ExoS and ExoT.

ExoS and ExoT are bifunctional toxins containing an N-terminal Rho GTPase activating protein (Rho GAP) domain and a C-terminal ADP-ribosyltransferase domain. N-terminal to the GAP domain is a secretion domain (Sec), a chaperone binding domain (Chap) and a membrane localisation domain (MLD). The GAP active site arginines (R146 and R149) and the ADP-ribosytransferase active site glutamic acids (E379/E381 and E383/E385) are indicated. Taken from [363].

amino acids of the toxin. It has been demonstrated that the N-terminal 9 amino acids of ExoS are required for export [324], thus suggesting that the signal for secretion by the TTS system of *P. aeruginosa* is also located at the extreme N-terminus of ExoS.

1.6.3.1.4. The chaperone-binding domain of exoenzyme S

Analysis of the region upstream of *exoS* revealed a locus that was co-regulated with *exoS* [364]. This locus included three open reading frames (ORFs), one of which, ORF 1, showed significant similarity to the gene encoding the YopE chaperone, *sycE*. SycE is required for the efficient secretion of YopE and, as previously discussed, acts by preventing the aggregation and subsequent degradation of YopE [175, 193, 200, 365]. The hypothesis that OFR 1 is a chaperone for ExoS is supported by the observation that when ExoS was expressed in *Y. pseudotuberculosis*, ORF 1 was required for its efficient secretion [352]. The lack of accumulation of ExoS in the *Yersinia* strain expressing ExoS but not ORF 1 also suggests that ExoS is less stable in the absence of ORF 1 [352]. When purified, ExoS occurs as a high-molecular–weight aggregate and a region within the N-terminal 99 amino acids is responsible for this phenotype [366]. Therefore, ORF 1 may act in the same way as SycE, to mask the aggregation prone chaperone-binding site of its cognate toxin and prevent its degredation.

1.6.3.1.5. The membrane localisation domain of exoenzyme S

A number of studies using non-toxic ExoS mutants, that either lack the entire ADPRT domain or that have an inactive ADPRT domain, have revealed that ExoS localises to the perinuclear region of eukaryotic cells in a punctate pattern [26]. ExoS fractionates with the particulate fraction of eukaryotic cells suggesting that it is targeted to a membranous region around the nucleus [367]. Studying the localisation of various ExoS deletion mutants has defined the region responsible for this intracellular targeting as lying between amino acids 51 and 72 [368]. This region has been called the membrane localisation domain (MLD) and is necessary and sufficient for membrane localisation as illustrated by its ability to direct a green fluorescent protein (GFP) fusion protein to the perinuclear membrane domain [368]. Deletion of the MLD did not alter the TTS of ExoS from *P. aeruginosa* but did result in a protein that is localised to the cytosol of eukaryotic cells [368]. Therefore, this region is distinct from the secretion

and chaperone binding domains described above. The MLD does not appear to be essential for ExoS action as its deletion does not alter the ability of ExoS to elicit cytotoxicity or morphological changes [367, 368]. Abolishing the MLD may however alter the targets of ExoS, as ExoSΔMLD is unable to ADP-ribosylate Ras (one of the targets of the ADPRT domain) [368].

1.6.3.1.6. The ADP-ribosyltransferase domain of exoenzyme S

The ExoS ADPRT domain acts by catalysing the cleavage of NAD^+ at the glycosidic bond and covalently attaching the ADP-ribose moiety to a number of eukaryotic target proteins. ExoS is a poly-substrate specific ADPRT that has been shown to modify a wide range of proteins. A number of small GTPases are ADP-ribosylated by ExoS including the Ras and Ras-like proteins Ras, Rap1, Rap2, and RalA, the Rab proteins Rab3, Rab4, Rab5, Rab8 and Rab11 and the Rho proteins Rho, Rac and Cdc42 [369-373]. Other targets of the ExoS ADPRT domain include the intermediate filament protein vimentin [374], the extracellular proteins IgG and apolipoprotein A1 [375] and the actin-binding proteins ezrin, radixin and moesin [376]. Not all the proteins identified as *in vitro* targets of ExoS are ADP-ribosylated *in vivo*. For example Rab4 and Rho are modified *in vitro* but not in eukaryotic cells [372, 373]. There also appear to be cell-specific differences in the range of proteins ADP-ribosylated by ExoS. For example, a larger number of small GTPases were ADP-ribosylated in human epithelial cells compared to human macrophages or rodent cell lines [377, 378]. In addition to the ADP-ribosylation of eukaryotic proteins, ExoS is also auto-ADP-ribosylated both *in vitro* and in Chinese hamster ovary (CHO) cells [379]. The ADPRT domain ADP-ribosylates the catalytic arginine residue 146 of the GAP domain and reduces the activity of the GAP domain *in vitro* [379]. Thus there may be intramolecular regulation of the two functional domains of ExoS in intact cells.

The ADPRT domain of ExoS resides within the C-terminal 222 amino acids of the toxin (Fig. 1.9) [366]. ExoS is a biglutamic acid ADPRT and the glutamic acid residues 379 and 381 are important for its enzymatic action [380, 381]. Mutation of the glutamic acid at position 381 inhibits both NAD glycohydrase and ADPRT activity whereas mutation of the glutamic acid residue 379 inhibits just ADPRT activity [381]. Therefore the glutamic acid at position 381 is the catalytic residue and the glutamic acid

at position 379 facilitates the transfer of the ADP-ribose to the target protein. ExoS ADP-ribosylates specific arginine residues in the target proteins and may modify one or more residues [369, 382]. For example, K-Ras and N-Ras are modified once whereas H-Ras can be modified up to three times [382]. The preferred site for H-Ras ADP-ribosylation is arginine 41, with the second site being arginine 128 and an alternative site being arginine 135 [383, 384].

ExoS absolutely requires a eukaryotic cofactor for its ADPRT activity [385], thus preventing the action of the ExoS ADPRT domain in *P. aeruginosa*. This protein was initially named FAS (factor activating ExoS) and has since been identified as a 14-3-3 protein [386]. Of the seven mammalian 14-3-3 isoforms, all of those studied (β ζ η σ and τ) activate ExoS with similar efficiency [387]. The 14-3-3 proteins are a group of highly conserved helical, intracellular proteins ubiquitously expressed in all eukaryotes from fungi to humans to plants. 14-3-3 proteins can interact with over 200 target proteins and are involved in controlling many cellular processes including cell cycle, cell growth, differentiation, survival, apoptosis, migration and spreading [388]. 14-3-3 proteins exist as dimers with each monomer containing an amphipathic groove that mediates binding. Although many binding partners of 14-3-3 proteins contain a phosphoserine motif [389, 390], 14-3-3 is also able to interact with nonphosphorylated substrates [391]. ExoS is one such nonphosphorylated substrate that requires the basic residues lining the amphipathic groove for binding [387]. It has been demonstrated that amino acid 420–429 of ExoS are important for its interaction with 14-3-3 and that the D-A-L-D-L motif (amino acids 424–428) is essential for binding [392]. In addition to abrogating 14-3-3 binding *in vitro*, mutation of the D-A-L-D-L motif prevents Ras ADP-ribosylation *in vivo* [392]. The 14-3-3 binding domain is also required for ExoS to elicit cytotoxicity and morphology changes in eukaryotic cells [392, 393].

In order to dissect which of the consequences of ExoS expression can be attributed to the ADPRT domain, a number of groups have studied the effect of ExoS with a deleted or mutated GAP domain [378, 394, 395]. Phenotypes linked to the ADPRT domain of ExoS include cytotoxicity, decreased DNA synthesis, cell morphology changes, loss of adherence and apoptosis [355, 378, 394, 395]. The ADPRT domain has also been linked to the virulence caused by ExoS in the mouse model of acute pneumonia [361]. It is not clear how the ADPRT domain of ExoS acts to cause this array of phenotypes

but a number of hypothesis have been proposed. The Ras family of small GTPases are involved in controlling critical cellular processes including proliferation, differentiation and apoptosis. Therefore inhibition of these proteins by ADP-ribosylation may account for the decreased DNA synthesis and cell death caused by ExoS. The Rho GTPases are involved in the control and reorganisation of the cytoskeleton, and the actin binding proteins ezrin, radixin and moesin contribute to cytoskeleton dynamics [396]. Thus the ADP-ribosylation of any of these may be responsible for the morphological consequences of the ExoS ADPRT domain.

An example of the ability of the ADPRT domain of ExoS to alter the function of one of its targets is provided by Ras. Bacterially translocated ExoS ADP-ribosylates Ras preferentially at arginine residue 41 and this modification blocks the interaction of Ras with its GEF, Cdc25 [383, 397]. As Cdc25 is unable to catalyse the exchange of GDP for GTP on ADP-ribosylated Ras, the amount of endogenous GDP-bound Ras accumulates [398]. GDP-bound Ras is inactive and unable to interact with its downstream partners such as Raf. Thus ADP-ribsoylation of Ras inhibits its ability to interact with Raf and prevents downstream signalling [398]. ADP-ribosylation of arginine residue 41 of Rap1b by ExoS also inhibits the ability of its GEF, C3G to stimulate guanine nucleotide exchange [399].

Although ADP-ribosylation of Ras does inhibit Ras-mediated signal transduction pathways, it has been demonstrated that this is not the cause of the cytotoxic phenotype of ExoS. As described above, deletion of the MLD of ExoS abrogates the ability of ExoS to ADP-ribosylate Ras [368]. However deletion of the MLD does not decrease ExoS induced cytotoxicity in infected cells [368]. Therefore ADP-ribosylation of Ras is uncoupled from the cytotoxic phenotype elicited by ExoS.

1.6.3.1.7. The GTPase activating protein domain of exoenzyme S

The presence of a second catalytic domain in ExoS was suggested by the ability of an ADPRT mutant to cause morphological changes [352]. ExoS with a mutation of the catalytic glutamic acid residue 381 possessed only 0.02% of the ADPRT activity of wild type ExoS but was still able to elicit actin cytoskeleton disruption and inhibit phagocytosis in epithelial cells and macrophages respectively [346, 352]. It was

subsequently shown that the N-terminal 234 amino acids of ExoS were able to disrupt the actin cytoskeleton and cause cell rounding without causing cytotoxicity when transfected into CHO cells [400].

An indication of the function of the N-terminal domain was provided by the observation that cytotoxic necrotizing factor 1, which activates Rho GTPases, reversed the cytoskeletal rearrangements caused by the N-terminal 234 amino acids of ExoS [400]. This suggested that ExoS might disrupt signal elements upstream of Rho GTPases or directly disrupt Rho GTPase function. It was then demonstrated that ExoS acted directly on Rho GTPases as a GAP, stimulating the GTP hydrolysis by Rho, Rac and Cdc42 *in vitro* [401]. The arginine residue 146 of ExoS was shown to be essential for this GAP activity and three-dimensional structural analysis revealed that this residue functions as an arginine finger that stabilises the transition state of the Rac-GTPase reaction [401, 402].

The RhoGTPases, Rho, Rac and Cdc42 are molecular switches involved in the regulation of actin cytoskeletal rearrangements and a large number of signalling processes. Rho regulates focal adhesion formation, the assembly of actin filaments into stress cables and contributes to cell contractility [403, 404]. Rac regulates the formation of lamellipodia (membrane ruffles) and contributes to cell motility [403]. Cdc42 regulates filopodium formation and contributes to cell polarity [403]. The ability of ExoS to act as a GAP for these proteins *in vivo* was investigated by examining the effect of co-expressing dominant active RhoGTPases and the ExoS GAP domain [405]. Co-expression of dominant active Rac1 and Cdc42 inhibited the reorganisation of the actin cytoskeleton by the ExoS GAP domain and expression of dominant active Rho stimulated the formation of stress cables in the presence of ExoS [405]. Therefore, the GAP domain of ExoS is active *in vivo* and acts to stimulate the reorganisation of the actin cytoskeleton by inhibition of Rac and Cdc42 and inhibit actin stress cable formation by inhibition of Rho.

As discussed above, the ADPRT domain of ExoS is also able to elicit morphological changes in eukaryotic cells in the absence of a functional GAP domain. Thus the relative contribution of each domain to the phenotype of ExoS remains unclear. In some cell lines, for example the HT-29 epithelial cell line, the GAP domain has

minimal effects on cell morphology [395]. However in other cell lines, such as J774A.1 macrophages, the GAP domain is responsible for the antiphagocytic phenotype [378]. The relative contributions of each domain may be influenced by the cellular environment, for example the localisation and accessibility of target proteins [406]. A further complication is the apparent intramolecular regulation of the GAP and ADPRT domains. As stated above, the ADPRT domain of ExoS ADP-ribosylates the catalytic arginine residue 146 of the GAP domain and reduces the activity of the GAP domain *in vitro* [379]. Also there is evidence that the GAP domain down-regulates the action of the ADPRT domain *in vivo* [395].

1.6.3.2. Exoenzyme T

As previously described, ExoT was identified by virtue of its similarity to ExoS. ExoT and ExoS are 75% identical at the amino acid level and exhibit the same domain structure (Fig. 1.9) with an N-terminal GAP domain and a C-terminal ADPRT domain [346, 363]. Although not explicitly studied for ExoT, the high degree of identity between the C-terminal 72 amino acids of ExoS and ExoT suggests that the same regions within these two proteins are required for secretion, chaperone binding and membrane localisation. With regards to chaperone binding, the absence of a chaperone-like gene in the vicinity of *exoT* in addition to the homology between the chaperone binding domain of ExoS and the same region in ExoT, indicates that ExoT may utilise the same chaperone as ExoS, ORF 1 [346]. For intracellular localisation, it has been proposed that both ExoS and ExoT experience a common intracellular trafficking pathway and localise to the same region within eukaryotic cells. ExoT has a similar intracellular fractionation pattern as ExoS and it has the same targets for its GAP domain [405, 407]. Also if the ADPRT domains of ExoS and ExoT are switched, the *in vivo* targets of ADP-ribosylation remain dependent on the specific ADPRT domain and not on the identity of the rest of the protein, indicating that their targeting is not altered [408].

1.6.3.2.1. The consequences of exoenzyme T expression

In common with ExoS, ExoT is able to elicit actin reorganisation, cell rounding and detachment of a number of cell lines [349, 407, 409]. ExoT is also able to inhibit the

internalisation of *P. aeruginosa* by macrophages and epithelial cells and inhibit wound healing [353, 407, 410, 411]. However unlike ExoS, ExoT is not cytotoxic to mammalian cells [412].

Unlike the other TTS toxins of *P. aeruginosa*, ExoT is not a variable trait with all strains containing the *exoT* gene [339]. The retention of this gene in all strains suggests that it plays an important role in some aspect of bacterial survival. However, ExoT appears to contribute very little to *P. aeruginosa* virulence in a mouse model of acute pneumonia or burns [360-362]. The only effect that has been attributed to ExoT in an mammalian model is to facilitate bacterial dissemination to the liver [361, 410].

1.6.3.2.2. The GTPase activating protein domain of exoenzyme T

The GAP domain of ExoT is very similar in structure and function to that of ExoS. Residues 78-237 of ExoT exhibit GAP activity towards RhoA, Rac1 and Cdc42 both *in vitro* and *in vivo* [407, 409]. Also the arginine at position 149 (analogous to the arginine at residue 146 in ExoS) is required for this activity [407, 412]. It is evident that the GAP domain of ExoT contributes to the cytoskeleton disruption and anti-internalisation properties of this toxin. When *P. aeruginosa* secretes ExoT with the arginine at position 149 mutated, more bacteria are internalised compared to *P. aeruginosa* that secretes wild type ExoT [410]. However, ExoT lacking a functional GAP domain is still able to cause morphological changes in eukaryotic cells and cause a reduced but significant inhibition of internalisation [410, 412, 413]. This suggests that the ADPRT domain is also involved in the cytoskeletal disruption and anti-phagocytic phenotypes of ExoT.

1.6.3.2.3. The ADP-ribosyltransferase domain of exoenzyme T

When ExoT was first identified it was thought to have little ADPRT activity. Using the soyabean trypsin inhibitor as an artificial substrate, recombinant ExoT only exhibited 0.2% of the ADPRT activity of ExoS [346]. In addition, the ADPRT activity of ExoT on Ras was only 1-3% compared to that of ExoS *in vitro* and no Ras ADP-ribosylation by ExoT was detected *in vivo* [412, 414]. This lack of Ras ADP-ribosylation correlated with the inability of ExoT to inhibit receptor-mediated Ras activation and signalling to

Raf [412]. In common with ExoS, the small amount of ExoT ADPRT activity detected was absolutely dependent on the presence of a 14-3-3 protein and required the glutamic acid residues 383 and 385 (analogous to the glutamic acid residues 379 and 381 in ExoS) [346, 414, 415].

Although the ADPRT domain of ExoT did not appear very active, the observation that the ExoT GAP mutant caused morphological changes and inhibited internalisation and wound healing suggested that the ADPRT domain was indeed functional but targeted host proteins distinct from Ras [410, 412, 413]. It was subsequently shown that ExoT was able to effectively ADP-ribosylate two eukaryotic proteins, Crk (CT10 regulator of kinase)–I and Crk-II both *in vitro* and *in vivo* [415]. ExoT was capable of ADP-ribosylating Crk-I at a rate similar to the rate at which ExoS ADP-ribosylates the soyabean trypsin inhibitor and ExoS was unable to ADP-ribosylate Crk-I [415]. Like ExoS, ExoT is also capable of auto-ADP-ribosylation, although what effects this has on the activity of the toxin is unknown [415].

The ability of ExoT to ADP-ribosylate Crk-I and Crk-II provided a link between the enzymatic activity of the ADPRT domain and its ability to inhibit phagocytosis (Fig. 1.10). Crk-I and Crk-II are alternatively spliced products of the human *CRK* gene that contain Src homology (SH) 2 and SH3 domains [416]. They are adaptor proteins that play a central role in integrin-mediated phagocytosis, focal adhesion and cell migration [417-419]. Upon ligand binding, the α- and β-subunits of the integrin transmembrane receptors dimerise and recruit focal adhesion complex proteins. These proteins include focal adhesion kinase (FAK) and Src family kinases and the scaffolding proteins Paxillin and p130Cas. Paxillin and p130Cas are tyrosine phosphorylated by FAK or Src and are then able to bind to the SH2 domain of the Crk proteins, which recognises phosphorylated tyrosines in the motif pY-X-X-P [420-424]. The SH3 domain of Crk binds the downstream factor DOCK180 that, in conjunction with ELMO, activates Rac1 by virtue of its GEF activity [425, 426]. Activated Rac1 can then stimulate actin reorganisation that eventually leads to cell migration and phagocytosis.

It was recently demonstrated that ExoT inhibits integrin-mediated phagocytosis by ADP-ribosylating arginine 20 of Crk [427] (Fig. 1.10). Arginine 20 is found in the SH2 domain of Crk and when it is ADP-ribosylated by ExoT, Crk is no longer able to

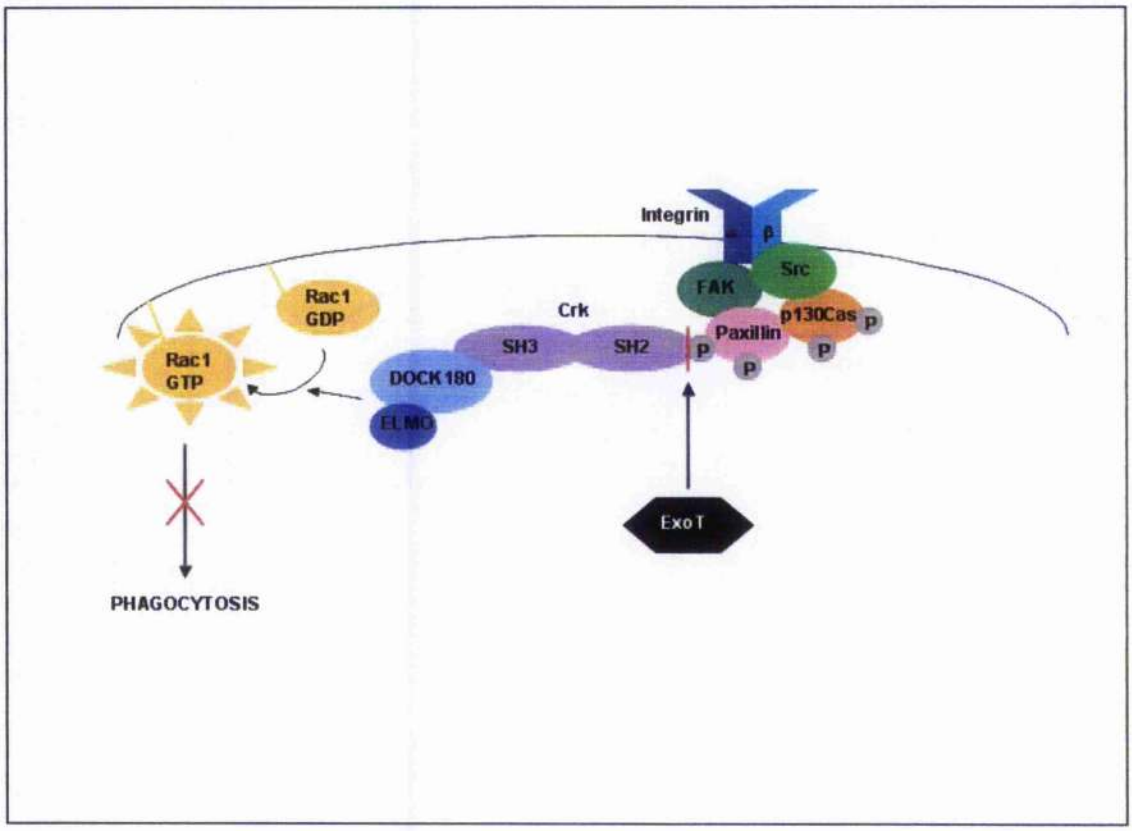


Fig. 1.10. The action of the ADP-ribosyltransferase domain of ExoT.

Ligand binding to integrin leads to the recruitment of focal adhesion complex proteins including FAK, Src, Paxillin and p130Cas. Paxillinn and p130Cas are phopshorylated (P) by FAK or Src and are able to bind to the SH2 domain or the Crk proteins. The SH3 domain of Crk then binds to DOCK180, which in conjunction with ELMO, activates Rac1 by exchanging GDP for GTP. Activated Rac1 stimulates phagocytosis. ExoT inhibits this process by ADP-ribosylating arginine 20 of Crk, thus inhibiting the interaction between Paxillin or p130Cas and the SH2 domain of Crk (red line). Adapted from [363].

associate with Paxillin or p130Cas [427]. Therefore the integrin receptor activated p130Cas-Crk-DOCK180 pathway that would lead to Rac1-mediated phagocytosis is severed. The observation that overexpression of Crk-I or expression of dominant-active Rac1 reduced cell rounding by ExoT supports this model of ExoT action [427].

1.6.3.3. Exoenzyme U

ExoU was the third TTS toxin to be discovered in *P. aeruginosa* as the factor responsible for the cytotoxic phenotype of the clinical lung isolate PA103. It was observed that a PA103 mutant strain with an inactivated *exsA* allele was attenuated in virulence in a rabbit model of lung infection [428]. ExsA was known to activate the expression of ExoS and ExoT [321], therefore it was predicted that one of these TTS toxins was responsible for the virulence phenotype of PA103. However, PA103 lacks *exoS* [429] and a PA103 strain with a transposon mutation in the *exoT* gene was still cytotoxic *in vitro* and caused lung epithelial injury in an acute lung infection mouse model [430]. The observation that neither ExoS nor ExoT accounted for the cytotoxicity of PA103 and the requirement for ExsA suggested that another TTS toxin was responsible for this phenotype. Two independent groups demonstrated that this additional TTS toxin was a secreted protein of about 74 kDa, ExoU [430] (or PepA as it was originally called by one group [431]). Mutation of the *exoU* gene abrogated the *in vitro* cytoxicity and *in vivo* virulence properties of PA103 and complementation of this *exoU* mutant strain with a plasmid encoding ExoU restored these phenotypes [430, 431].

A number of observations suggested that ExoU was secreted by the TTS system and was co-ordinately regulated with the other TTS toxins of *P. aeruginosa*. In common with ExoS and ExoT, ExoU secretion occurred without cleavage of a signal peptide and was induced by low calcium conditions [431]. ExoU also failed to be secreted by isogenic mutants defective in the TTS pathway. For example a PA103 strain with a mutation in the gene encoding the TTS apparatus component PscJ was not capable of secreting ExoU [431]. The requirement of ExsA for ExoU secretion and the presence of the ExsA consensus element (T-X-A-A-A-A-X-A) 84 bp 5' of the translation start codon of *exoU* suggested that, like ExoS and ExoT, ExoU expression was activated by ExsA [430, 431]. It was also observed that the first 5 amino acids of ExoU were

identical to those of ExoS and ExoT, indicating that there might be a common N-terminal motif required for pseudomonal toxins to be recognised by the TTS apparatus [430, 431].

1.6.3.3.1. The consequences of exoenzyme U expression

ExoU has been associated with the death of many different cell types including fibroblasts, epithelial cells, neutrophils, macrophages and the yeast, *Saccharomyces cerevisiae*. By comparing the cytotoxicity of isogenic *P. aeruginosa* mutants that either secrete ExoU or do not secrete ExoU, a number of studies have indicated the role of this toxin in eliciting cell death [349, 430-434]. It has also been demonstrated that transformation of a non-toxic, *exoU* minus strain with a plasmid encoding ExoU rendered this strain toxic to cultured epithelial cells [435]. In addition to cell death resulting from infection with an ExoU-secreting strain, transfection of various cell lines with an ExoU-expressing plasmid [436-439] or syringe loading of CHO cells with recombinant ExoU [440] also results in cell death.

Before the catalytic activity of ExoU was known it was noted that ExoU cytotoxicity occurred rapidly and was characteristic of necrosis as opposed to apoptosis [434, 441, 442]. Fully toxic ExoU has never been detected in infected or transfected cells by Western blot analysis, fluorescence microscopy or flow cytometry analysis indicating that only a small amount of ExoU is required for activity [436, 437, 439]. In fact it has been demonstrated that half-maximal cytotoxicity appears to require only ~300-600 ExoU molecules per cell [440].

In addition to being cytotoxic to cells, ExoU is required for the *in vivo* virulence of a number of *P. aeruginosa* strains. Murine models of acute lung infection have been used to demonstrate the role that ExoU plays in bacterial persistence, dissemination and mortality [361, 430, 431, 435]. Deletion of *exoU* reduces the virulence of pseudomonal strains and adding *exoU* to non-toxic strains confers a virulent phenotype on these bacteria. A role for ExoU in inducing systemic inflammation and septic shock has also been suggested using a rabbit model of lung infection [443]. In addition to the role that ExoU plays in lung infections, it has also been shown to contribute to bacterial survival and disease severity in a murine scarification model of corneal infection [444].

The presence of ExoU appears to correlate with more severe disease in humans. For example, patients with hospital-acquired pneumonia who were infected with ExoU secreting *P. aeruginosa* had a poorer prognosis than patients who were infected with isolates that did not secrete TTS toxins [445].

1.6.3.3.2. Exoenzyme U possesses phospholipase activity

When ExoU was first identified and analysed using BLAST, no genes or proteins with significant similarity were found [430, 431]. The lack of similar proteins and the rapid and potent cytotoxicity of ExoU made it difficult to determine the enzymatic action of this toxin. However, the development of the *S. cerevisiae* transfection model provided a suitable system in which tostudy ExoU as the presence of a thick cell wall prevented immediate cell lysis and allowed observation of internal changes within the yeast [439]. ExoU caused a number of internal changes in *S. cerevisiae* including the accumulation of numerous vesicles that appeared as dimples or pockmarks by Nomarski differential interference contrast microscopy and alterations in immunofluorecent staining with several organelle markers [439]. Staining with markers for the yeast vacuole and vacuolar membrane revealed a vacuolar fragmentation phenotype upon ExoU induction and ExoU also caused an increase in the immunofluorescent signal intensities for several organelle markers including the vacuole, mitochondria, late Golgi and endosome. These results indicated that yeast vacuoles were a major target of ExoU action and that this toxin might also cause exposure of a number of epitopes present on yeast organelles.

Two mechanisms were suggested to account for the effects that ExoU had on vacuoles, either ExoU interfered with vacuolar biogenesis or ExoU disrupted membranes [439]. In order to distinguish between these two possible activities, a number of inhibitors were examined to determine whether they abolished ExoU toxicity including chemicals that affected the function of vacuolar ATPases, chloride channels, serine and cysteine proteases and phospholipases. It was noted that inhibitors of human cytosolic phospholipase A_2 ($cPLA_2$) and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) but not secreted phospholipase A_2 ($sPLA_2$) eliminated or greatly reduced ExoU toxicity [439, 440]. Therefore, ExoU either possesses lipase activity or activates cellular lipases. The

inability of specific inhibitors of endogenous $cPLA_2$ or $iPLA_2$ to protect against ExoU-mediated cytotoxicity and the observation that pre-treatment of ExoU with methyl arachidonyl fluorophosphonate, an irreversible inhibitor of $cPLA_2$, was partially protective, suggests that ExoU was itself acting as a phospholipase [440].

Further evidence supporting the action of ExoU as a phospholipase was supplied by the homology displayed between amino acids 107-357 of ExoU and plant patatins (which posses phospholipase activity), mammalian $cPLA_2$ and $iPLA_2$ (Fig. 1.11) [439, 440]. Three highly conserved regions were observed between these phospholipases: 1) a glycine-rich nucleotide-binding motif, G-X-G-X-X-G, at position 111-116 of ExoU, 2) a serine hydrolase motif, G-X-S-X-G at position 140-144 of ExoU and 3) an active site aspartate residue in the conserved motif D-X-G/A at position 344-346 of ExoU. In $cPLA_2$, the serine-aspartate catalytic dyad is required to hydrolyse the sn-2 ester bond of phospholipids and release free fatty acids and lysophospholipids. The glycine-rich nucleotide-binding motif is responsible for polarising the sn-2 ester and stabilising the negative charge that develops upon nucleophilic attack by the catalytic serine during substrate cleavage [446]. As expected if ExoU acts as a phospholipase, mutation of either the predicted catalytic serine residue 142 or the catalytic aspartate residue 344 abolished the cytotoxic activity of ExoU in both infection and transfection studies [439, 440].

ExoU appears to be a broad substrate specific lipase that is able to hydrolyse neutral lipids and phospholipids [439]. Phospholipids with saturated fatty acids and/or acid head groups however, seem less suitable substrates for this lipase [447]. ExoU may also exhibit lysophospholipase A activity therefore removing the second fatty acid from a phospholipids that has already had its first fatty acid removed [448].

In addition to the homology shown to patatin and mammalian $cPLA_2$ and $iPLA_2$, ExoU also shows significant homology to a number of other bacterial proteins that contain the conserved catalytic dyad of $cPLA_2$ [449]. For example, the ORFs RP534 of *Rickettsia prowazekii* and BA0745, BA1992 and BA4137 of *Bacillus anthracis* all contain the glycine rich motif and the active site serine and aspartate domains. In addition the *Yersinia entercolitica* YplA protein secreted by the flagellar TTS system and the B-

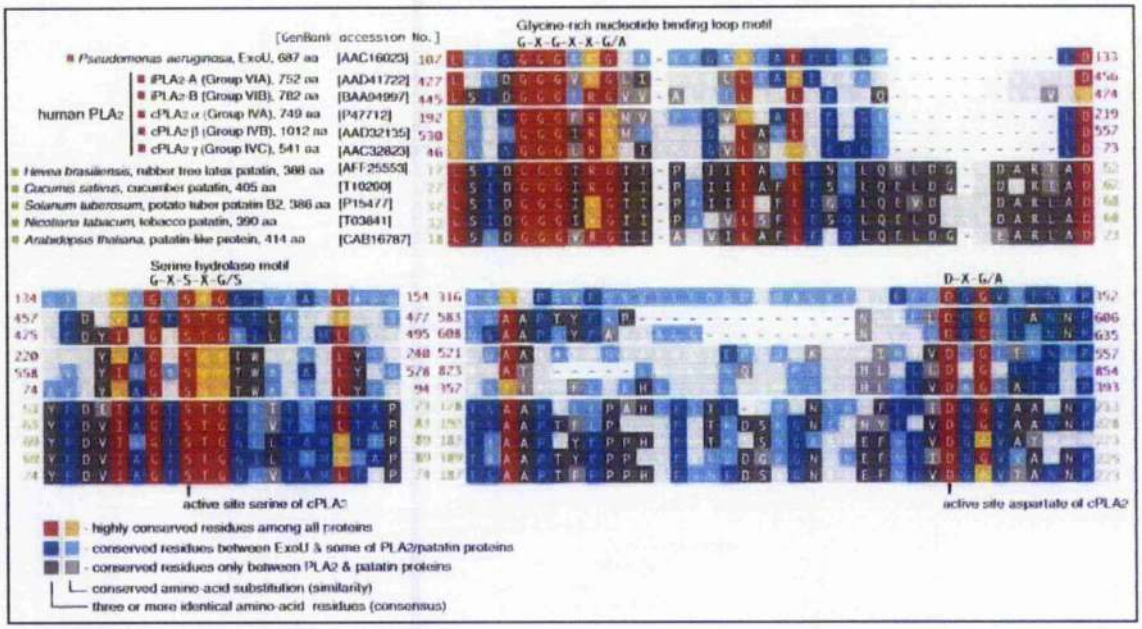


Fig. 1.11. Alignment of ExoU with patatins and patatin-like phospholipase A_2 domains

Alignment of the primary sequences of *P.aeruginosa* ExoU (amino acids 107-154 and 316-352), and patatin-like phospholipase A_2 domains of human $iPLA_2$ (A and B), human $cPLA_2$ (α, β and γ) and plant patatins using the NCBI Conserved Domain Database. A color index for conserved amino acid residues among listed proteins was based on the PAM250 substitution score matrix. The conserved glycine-rich nucleotide-binding motif (G-X-G-X-X-G), the serine hydrolase motif (G-X-S-X-G) and the active site aspartate residue motif (D-X-G/A) are indicated. Taken from [439].

chain of ricin produced by *Ricinus communis* also contain the serine-aspartate catalytic dyad and are known to posses phospholipase activity [450-452].

1.6.3.3.3. Exoenzyme U requires a eukaryotic cofactor for activation

When recombinant ExoU (rExoU) was added to ^{14}C-labelled liposomes, no fatty acid release was observed by thin-layer chromatography [439, 440]. The purified rExoU was only active if pre-incubated with yeast or mammalian cell extract, which suggests that ExoU requires a eukaryotic cofactor or modification to be catalytically active [439, 447, 448]. ExoU is able to act on the phospholipids found in bacterial membranes but bacterial soluble extract is unable to activate rExoU [447]. Thus, it is apparent the requirement for a eukaryotic activating factor prevents ExoU from being toxic to the *P. aeruginosa* that manufactures it.

Although attempts to identify the eukaryotic activating factor of ExoU have so far failed [439], a number of experiments have been carried out to identify its characteristics. Activation of human $cPLA_2$ requires calcium ions, but cations such as Ca^{2+}, Mg^{2+} or Zn^{2+} were unable to activate rExoU suggesting a different mechanism of activation [447]. When rExoU is pre-incubated with cell extract and then purified it is unable to act as a lipase because the activator must be present during substrate hydrolysis [447]. This suggests that the activator is a cofactor not a modifier of ExoU. The identity of the eukaryotic cofactor as a protein is indicated by the reduced ability of cell extract heated to high temperatures or treated with chymotrypsin to activate rExoU [447]. Finally, size exclusion filtration with a spin column suggests that the activating factor is over 100 kDa in size [447] implying that the cofactor is either a large protein or protein complex.

1.6.3.3.4. The requirement of the C-terminus of exoenzyme U for activity

When ExoU was first identified, in addition to a non-toxic transposon mutant that failed to secrete ExoU due to a transposon insertion near the beginning of the *exoU* gene, a non-toxic mutant that secreted a truncated form of ExoU was also identified [431]. In this mutant, deletion of the last 88 bp of *exoU* was sufficient to render the strain non-toxic. Therefore the C-terminus of ExoU appears to be required for its cytotoxic activity. Further experiments on the domains required for ExoU action in mammalian

and yeast cells revealed that the C-terminus was indeed required as were domains in the N-terminus and domains in the middle of the protein [436-439]. When the catalytic activity of ExoU was determined, the requirement for the N-terminal and middle domains became apparent as these regions occurred within the PLA_2 homology domain [439]. The C-terminal domain required for ExoU activity however resides outside the PLA_2 homology domain and is therefore not believed to contribute directly to catalytic phospholipase activity (Fig. 1.12). It has been postulated that the C-terminus of ExoU may be required for cofactor binding or modification or to enable the toxin to interact with its phospholipid substrates, but there is currently no evidence to support these hypotheses [449].

1.6.3.3.5. Exoenzyme U requires a chaperone for efficient secretion

It was observed that a sequence downstream of *exoU* was required for the efficient secretion but not for the synthesis of ExoU [453]. Analysis of this genomic region identified three ORFs, one of which was predicted to encode a protein with the typical characteristics of a TTS chaperone. This ORF was named *spcU* (specific Pseudomonas chaperone for ExoU) and encoded a 137 amino acid protein with a predicted molecular weight of 14.9 kDa and a predicted acidic pI of 4.4 [453]. The C-terminal region of SpcU aligns with a common leucine-rich motif found within the chaperone family providing further proof of its role as a chaperone [453]. It is evident that the ExoU locus is organised as an operon encoding both *exoU* and *spcU*. The ExoU mRNA is about 400 bp larger than expected and the start codon of *spcU* overlaps the stop codon of *exoU* [453]. There is also a putative ribosome-binding site 5 bp upstream of the *spcU* start codon within the *exoU* coding sequence [453].

When ExoU and SpcU were co-expressed in *E. coli*, they appeared to associate in a non-covalent complex with amino acids 3 to 123 of ExoU being required for this interaction [453] (Fig. 1.12). As previously discussed, chaperones may act in a number of different ways: as anti-aggregation and stabilisation factors, as secretion signals, as antifolding factors and as regulators of expression of the TTS system. In the case of SpcU, there is evidence that it acts to prevent ExoU aggregation because histidine-tagged ExoU appears to form fibular structures in the absence of SpcU [453].

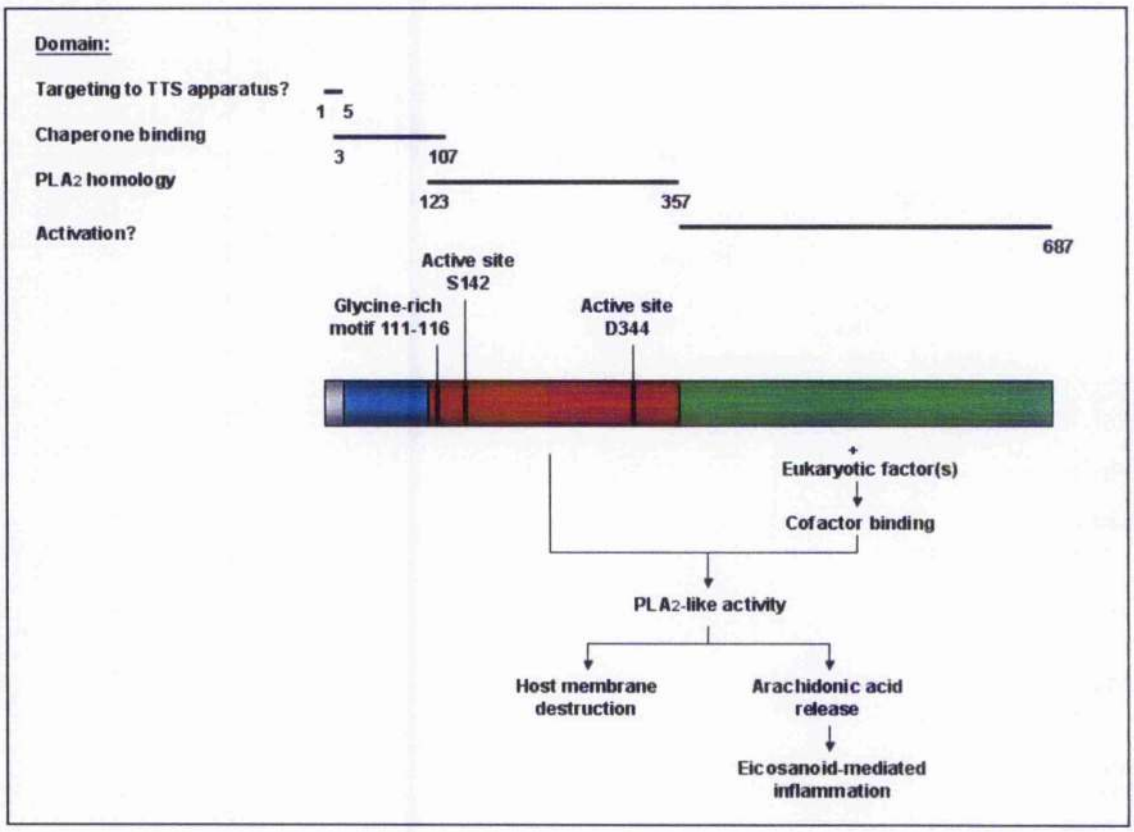


Fig. 1.12. Domain structure and action of ExoU

ExoU exhibits phospholipase A_2 (PLA_2) activity and is homologous to Patatin and Patatin-like PLA_2 enzymes between amino acids 123 and 357. The glycine-rich motif, active site serine (S142) and aspartate (D344) residues are indicated. The first five amino acids of ExoU might be involved in targeting the toxin to the TTS apparatus. Amino acids 3 to 107 include the region responsible for binding of the ExoU chaperone, SpcU. The C-terminus of ExoU is required for activity and may be involved in binding the essential eukaryotic cofactor. ExoU causes host membrane destruction and eicosanoid-mediated inflammation due to its PLA_2 activity. Adapted from [449].

1.6.3.3.6. Exoenzyme U is encoded on a pathogenicity island

As previously noted, ExoU is a variable trait in clinical isolates [339] and therefore may be acquired by these strains by horizontal transfer. *exoU* appears to occur within a pathogenicity island as there is a putative insertion element homologous to IS407 94 bp 5' of the *exoU* transcriptional start site [430, 431]. The GC content of the putative insertion element, *exoU* and *spcU* (60%, 59% and 56% respectively) is also markedly lower than that of the *P. aeruginosa* genome (67.2%) suggesting its acquisition from another source [430, 431, 453]. A specialised cloning vector, designed to capture chromosomal regions that may serve as sites for amino acid deletions or insertions by virtue of their high degree of polymorphisms, identified an 80 kb island of *P. aeruginosa* genomic DNA that includes *exoU* [454].

The presence of *exoU* and *exoS* in *P. aeruginosa* isolates is almost completely mutually exclusive [339, 445, 454-458]. Clinical and environmental isolates generally contain either *exoU* or *exoS* and only a very few strains have been identified that contain genes for both exoenzymes [339, 361]. It is not clear how *P. aeuriginosa* ensures that it encodes only ExoU or ExoS. It has however been observed that strains containing *exoU* also contain the *exoS* chaperone whereas strains encoding *exoS* do not encode the *exoU* chaperone, *spcU*. This suggests that horizontal transfer of the pathogenicity island containing *exoU* and *spcU* into a strain containing *exoS* is followed by excision of the *exoS* gene. The mutually exclusive expression of either ExoU or ExoS by *P. aeruginosa* implies that in specific environments it is advantageous to produce either one or other of the toxins but it is disadvantageous to produce both.

1.6.3.3.7. Possible secondary roles of exoenzyme U during infection

The ability of ExoU to act as a lipase enables it to trigger membrane disruption that results in cell death. In addition to its direct cytotoxic effect, ExoU may play additional roles in pseudomonal pathogenesis. For example, the ability of ExoU to induce systemic inflammation and rapid septic shock in a rabbit lung infection models may be caused by the release of cytokines from the damaged alveolar epithelial cells [443]. Also the lipolytic activity of ExoU may enable it to act on the major lipid lung surfactant, dipalmitoyl phosphatidylcholine. The lysophospholipids released from the

hydrolysis of lipids in lung surfactant can cause damage to cell membranes [459]. Thus ExoU may contribute to the systemic spread of *P. aeruginosa* by indirectly damaging cell membranes through its action on lipid lung surfactant. Another way that ExoU may contribute to pathogenesis is through the release of arachidonic acid. Some mammalian phospholipids contain arachidonic acid as one of their fatty acids and once released by the action of phospholipases, this molecule can be metabolised to eicosanoids. Eicosanoids, including prostaglandins and leukotrienes, are capable of mediating signal transduction pathways that activate inflammatory responses [460, 461]. Therefore, ExoU may contribute to inflammation in mammals by catalysing the release of arachadonic acid from phospholipids (Fig. 1.12). Evidence to support this hypothesis was provided by microarray analysis of genes regulated by ExoU, which showed that a number of genes involved in cellular transcription and signal regulation are induced by ExoU [462]. Although the generation of secondary messengers by ExoU may contribute to the pathogenesis of *P. aeruginosa* in mammals, the absence of arachadonic acid phospholipids and eicosanoid-mediated signal transduction pathways in yeast and plant cells [463, 464] suggests that the toxicity in these systems is dependent on direct disruption of lipid membranes.

1.6.3.4. Exoenzyme Y

A comparison of the extracellular protein profiles of wild type and a TTS mutant *P. aeruginosa* strain identified a fourth toxin secreted by the TTS system, ExoY [314, 324, 465]. ExoY is a 378 bp, 42 kDa protein that is only secreted from bacteria expressing a functional TTS apparatus [465]. Analysis of the chromosomal region encoding ExoY revealed that there is an ExsA binding site located upstream of *exoY*, indicating that expression of this toxin is co-ordinately regulated with the other TTS toxins of *P. aeruginosa* [465].

1.6.3.4.1. Exoenzyme Y is an adenylate cyclase

ExoY shows significant similarity to two adenylate cyclase toxins produced by pathogenic bacteria, CyaA from *Bordetella pertussis* and EF (edema factor) from *Bacillus anthracis* [465]. The homology is mainly confined to two regions known to be required for the catalytic activity of CyaA and EF (conserved regions I and II).

Conserved region I extends from residues 41-107 of ExoY and contains an ATP/GTP-binding site A motif that is thought to participate in contacting the α-phosphate of the bound nucleotide in CyaA [466]. The conserved region II occurs between amino acids 209-221 of ExoY and is predicted to be involved in interacting with the β- and γ-phosphates of bound nucleotides [466]. CyaA and EF also have a third short stretch of homology (conserved region III) and a calmodulin-binding domain that are not found in ExoY.

In agreement with the function predicted from sequence analysis, it was demonstrated that recombinant ExoY possessed adenylate cyclase activity and catalysed the formation of 3'5'-cAMP from ATP [465]. A number of amino acids are known to be required for the adenylate cyclase activity of CyaA including lysine residues 81 and 88 and aspartate residues 212 and 214 (residues numbered relative to position in ExoY) [467-470]. The lysines are found within conserved domain I and the aspartate residues occur within conserved domain II. All four residues are conserved between CyaA, EF and ExoY and are believed to be involved in contacting the bound nucleotide. The individual mutation of any one of these four residues within ExoY rendered the protein inactive because it was no longer able to act as an adenylate cyclase [465]. Thus, lysine 81 and 88 and aspartate 212 and 214 are essential for the adenylate cyclase activity of ExoY as well as that of CyaA.

1.6.3.4.2. ExoY requires a eukaryotic cofactor for activation

It was noted that addition of CHO cell extract stimulated the adenylate cyclase activity of ExoY by at least 500 fold and that heating the extract to 100°C destroyed its effect [465]. This suggests that, in common with the other TTS exoenzymes of *P. aeruginosa*, ExoY requires a proteinaceous eukaryotic cofactor for activity. Both CyaA and EF utilise the eukaryotic protein calmodulin for their activity. Calmodulin is absolutely required for the adenylate cyclase activity of EF [471] and stimulates CyaA by 500 – 1000 fold [472]. Addition of calmodulin however does not affect the activity of ExoY, which correlates with the lack of a calmodulin binding site in this protein [465]. The eukaryotic cofactor of ExoY remains unknown.

1.6.3.4.3. The consequences of ExoY expression

Injection of ExoY into CHO cells results in elevated intracellular cAMP levels and a rounded morphology but no cytotoxicity [349, 362, 465]. When the critical lysine residue 81 is mutated, ExoY is no longer capable of eliciting cell rounding [349], which correlates the morphological changes observed in the eukaryotic cells to the adenylate cyclase activity of ExoY. A recent study [473] has linked actin disruption by ExoY with inhibition of bacterial invasion but this only seems to occur at early time points, for example 2 h post-infection. At a later time point, 4 h post-infection, actin is still disrupted but the phagocytic properties of the mammalian cell are unaffected. This study also shows that the adenylate cyclase inactive lysine 81 mutant of ExoY causes actin disruption that is associated with inhibition of bacterial invasion, albeit at a lower level than wild type ExoY [473]. This suggests that ExoY, like ExoS and ExoT, might have more than one catalytic domain. Alternatively, the ExoY lysine 81 mutant may not be completely inactive with regards to its adenylate cyclase activity.

ExoY is widespread among both clinical and environmental isolates of *P. aeruginosa* with about 90% of tested strains containing the *exoY* gene [339]. Despite its prevalence, the contribution of ExoY to pathogenesis is unclear. A couple of studies that compared the pathogenesis of *P. aeruginosa* strains expressing all possible combinations of the TTS toxins, ExoS, ExoT and ExoY, suggest that ExoY plays only a very minor part in colonisation and dissemination in an acute pneumonia infection model [362, 474]. However a role in dissemination is suggested by an *in vitro* study that illustrated that ExoY induces pulmonary microvascular endoethelial gap formation and increases permeability in the isolated perfused lung [475]. Both CyaA of *B. pertussis* and EF of *B. anthracis* contribute to pathogenesis by disrupting the bactericidal functions of the immune effector cells thus disabling the host defence mechanisms (reviewed in [476]). It is however unclear if ExoY also acts in this way although its ability to inhibit phagocytosis suggests it might.

1.7. Aims

The central aim of this study was to determine the detailed mechanisms of action of the pseudomonal TTS toxins ExoS and ExoU using the yeast *Saccharomyces cerevisiae* and

a human epithelial cell line. Chapter 2 details our development of the *S. cerevisiae* model to investigate the consequences of ExoS expression. Our use of the *S. cerevisiae* model to discover host genes required for the action of the pseudomonal TTS toxins ExoS, ExoU and ExoY is described in Chapter 3. Chapter 4 explains how we used a human epithelial cell line to examine ExoU intracellular localisation, modification and toxicity and to identify the regions of the toxin required for these activities.

CHAPTER 2: STUDYING THE MECHANISM OF ACTION OF EXOENZYME S IN *SACCHAROMYCES CEREVISIAE*.

2.1. INTRODUCTION

As previously discussed in Chapter 1, ExoS is a bifunctional toxin with an N-terminal GAP domain and a C-terminal ADPRT domain. The GAP domain enhances the GTP hydrolysis of the Rho GTPases: Rho, Rac and Cdc42 [401, 405] and the ADPRT domain ADP-ribosylates many targets including small GTPases of the Ras, Rab and Rho family [370-373]. A variety of phenotypes have been attributed to ExoS expression in mammalian cells, including a decrease in DNA synthesis and cell viability, apoptotic death, disruption of the actin cytoskeleton that leads to cell rounding, inhibition of phagocytosis, loss of cell adhesion and microvillus effacement [349-352, 354, 355].

What remains unclear about ExoS action, however, is how modification of the known targets results in the observed consequences of ExoS expression and what contribution each domain makes to ExoS action. Although, ExoS ADP-ribosylates Ras *in vivo* and disrupts Ras-mediated signalling through Raf [397, 398], it has been demonstrated that this is not the cause of the cytotoxic phenotype of ExoS [368]. Therefore, the major target(s) of ExoS and its precise mechanism of action remain unknown. Also, the contribution of the GAP and ADPRT domain of ExoS to the observed phenotypes is uncertain. Although the ADPRT domain is definitely responsible for cytotoxicity [394], the relative roles of the GAP and ADPRT domains in causing cytoskeletal disruption is ambiguous [378, 395]. The apparent intramolecular modulation of the activity of each domain by the opposing domain confuses the picture further [379, 395].

Many models have been used to elucidate the function of the TTS toxins in *P. aeruginosa*. TTS toxin function can be studied in two ways, either by examining the effect of knocking out the toxin gene on bacterial pathogenicity in a eukaryotic infection model or by studying the consequences of expressing the toxin in eukaryotic cells. A number of mammalian models have been used to examine the relevance of various *P. aeruginosa* TTS toxins during infection. These include the burned mouse model, the mouse model of acute pneumonia and the corneal scratch-injury eye model [360, 361,

444, 474]. The effect of the TTS toxins on cells has also been assessed by introducing individual toxins into mammalian cell lines. The toxins have been introduced into mammalian cells by a variety of procedures including transient transfection, microinjection, and infection with *P. aeruginosa* or *Y. pseudotuberculosis* secreting just one TTS toxin [350, 352, 436, 440]. Although these models are useful for observing the effects of the toxin and determining which regions of the toxin are required for the observed phenotypes, they have limited use in studying the effects of mutations in host genes on the progression of disease. Generating a transgenic knockout mouse or a stable mutant mammalian cell line is time consuming and not amenable to high throughput screening of host proteins required for toxin action. Thus, it is difficult to study the mammalian side of bacterial invasion in these traditional models.

To circumvent the problems of using genetically unwieldy models for *P. aeruginosa* infection, for example mice or mammalian culture cells, genetically tractable model systems have been developed. These include the worm *Caenorhabditis elegans*, the social amoeba *Dictostelium discoideum*, the fruit fly *Drosophila melanogaster* and the plant *Arabidopsis thaliana* [92, 477-479]. Certain strains of *P. aeruginosa* are able to infect and cause disease in all these organisms and many of the pseudomonal virulence factors that are important in mouse models of infection are also required for infection of these model organisms. For example, *P. aeruginosa* virulence factors shown to be important in infection of *D. discoideum* include the *las* and *rhl* quorum sensing systems, rhamnolipids, the TTS system and the TTS toxin ExoU [92, 480]. The susceptibility of these " simple" organisms to pseudomonal infection and the requirement for many of the same virulence factors to establish infection, suggests that the toxins are targeting conserved eukaryotic processes. These models are fast growing and cheap and can therefore be used for rapid screening for novel virulence factors. Their susceptibility to genetic manipulation also enables these organisms to be used to study mammalian genes that are involved in infection [481].

Probably the simplest eukaryotic organism that had been used to study the effects of bacterial toxins is the yeast *Saccharomyces cerevisiae* [482]. In contrast to the other model systems, *P. aeruginosa* is unable to infect *S. cerevisiae* although it has been shown to infect the yeast *Candida albicans* [483]. However, the effects of individual virulence factors can be studied by expressing these toxins in *S. cerevisiae*. The validity

of using yeast as a model to study toxin action is provided by the conservation from yeast to mammals of many of the molecular mechanisms regulating cellular processes that are affected during bacterial infection. For example, the molecular components of DNA metabolism, programmed cell death, cell cycle control, cytoskeletal dynamics and membrane trafficking show a high degree of conservation between yeast and mammals [482]. Table 2.1. highlights this conservation of the molecular components that regulate the cytoskeleton and membrane traffic in mammals and yeast and lists the bacterial pathogens that exploit these cellular processes.

There are many advantages of using *S. cerevisiae* as a model in which to study bacterial virulence factors. This simple eukaryote is easy to transform with DNA, can grow as a haploid or a diploid, is fully sequenced and is straightforward to genetically manipulate. A great deal is known about the cellular processes in yeast, for example how the cell cycle is regulated and how the cytoskeleton is controlled. These processes can also be readily manipulated either by using specific mutant strains or chemicals. For example, there are chemicals that arrest *S. cerevisiae* at specific points in the cell cycle that can be used to synchronise a population of yeast cells. Also there are overexpression and deletion libraries available that can be used for rapid genetic screening for mutants with altered susceptibility to bacterial toxins (see Chapter 3).

A number of studies over the past five years have utilised *S. cerevisiae* to study the action of TTS toxins. The *Yersinia* toxins YopE, YopM, YopJ and YpkA, the *Salmonella* toxins SptP, SopE2 and SipA, the *P. aeruginosa* toxins ExoT and ExoU, and the *P. syringae* toxins AvrPtoB and HopPtoE-G have all been examined in yeast (reviewed in [482]). The results from these studies have confirmed that these bacterial toxins are toxic to yeast cells and that they inhibit similar pathways in both yeast and mammalian cells. For example YopE, which functions as a Rho GAP protein that disrupts the actin cytoskeleton in mammalian cells, is highly toxic to *S. cerevisiae* and interferes with its actin cytoskeleton [484]. It appears that in yeast, YopE cytotoxicity results from inhibition of the Rho1p-regulated pathway [261]. Overexpression of an activator of Rho1p (its GEF, Rom2p) or overexpression of a downstream effector of Rho1p-dependent signalling (Bck1p) suppressed the cytopathic effect of YopE expression. Also overexpression of the mammalian homologue of Rho1p, RhoA, or

activated forms of mammalian Rac1 or Cdc42 suppressed the cytotoxic effects of YopE in yeast.

Table 2.1. Conservation among eukaryotic processes relevant in bacterial pathogenesis.

Cellular process	Molecular components in:		Associated
	Mammals	Yeast	pathogen(s)
Cytoskeleton			
Actin dynamics	Rho proteins	Rho proteins	*Salmonella*,
	RhoGAP and GEF	Cdc24p, Rom1-3p, Bem	*Yersinia*,
	Arp2/3 complex	Arp2/3 complex	*Pseudomonas*,
	Type 1 myosins	Myo3p, Myo5p	EPEC, and
	Fimbrin	Sac6p	*Listeria* spp.
	AAK, BIK	Ark1p, Prk1	
	WASP	Las17p	
Microtubule dynamics	CDC42	Rho/Cdc42p	*Campylobacter*,
	Dynein	Dyn1-3p	*Chlamydia*, and
	Dynactin	Nip100p	*Shigella* spp.
	MDia	Bni1p	
	APC (?)	Kar9p	
	EB1	Bim1p	
Membrane traffic			
ER transport	Coatamer	COPI	*Legionella* and
	COPII	COPII	*Brucella spp*
	hSar1	Sar1p	
	ARF1	Arf1p	
	ARF GAP and GEF	ARF GAP and GEF	
	Rabs	Rab1p	
Endosomal transport	Hrs, TSG101	ESCRT I to III	*Salmonella*,
	PIKFYVE	Fab1p	*Mycobacteria*,
	HVps34	Vps34p	*Legionella*, and
	Clathrin	Clathrin	*Chlamydia* spp.
	AP-1 and AP-3	AP-1 and AP-3	
	Rabs	Ypt6p, Ypt31-32p	
	Syntaxins	Pep12p, Tlg1p, Tlg2p	
Endocytosis	Clathrin	Clathrin	*Salmonella*,
	AP-2, AP180	AP-2, AP180A	*Shigella*, and
	Hip1	Sal2p	*Listeria* spp.
	Epsin	Ent1-2p	
	Synaptojanin	Inp51-53p	
	Amphiphysisn	Rsv	
Autophagy	Tor pathway	Tor kinases	*Legionella* and
	Beclin 1	Apg6p	*Salmonella* spp.
	p150	Apg14	
	Ulk1	Apg1p	
	MAP1LC3	Apg8p	

In addition to studying toxins that interfere with cytoskeletal function, *S. cerevisiae* has been used to study TTS toxins that inhibit MAPK signalling [485], toxins that inhibit membrane structure and function [439], and to study the intracellular trafficking of toxins [486]. Indeed the utility of this system is highlighted by its contribution to determining the mechanism of action of the *P. aeruginosa* TTS toxin ExoU, as discussed in Chapter 1 [439].

As mentioned above, one of the advantages of using *S. cerevisiae* as a model in which to study bacterial virulence factors is the wealth of knowledge on the control of the actin cytoskeleton in this organism. If expression of a bacterial toxin perturbed the normal actin structures within yeast, it may be possible to determine what component of the cytoskeletal regulatory mechanism was being targeted. Comparing a toxin-induced actin phenotype with known *S. cerevisiae* mutant phenotypes may provide a clue as to the mechanism of action of the toxin. Also, as illustrated with YopE [261], overexpression (or deletion) of suspected toxin targets or components in a toxin-targeted pathway might abrogate toxin cytotoxicity, therefore providing proof of a biologically relevant target.

The actin cytoskeleton of *S. cerevisiae* comprises primarily of two structural components, cortical patches and actin cables. Cortical patches are punctate F-actin-rich bodies, while cables are bundles of F-actin filaments. Cortical patches and actin cables lie at the cell cortex and their localisation patterns change in a cell cycle-dependent manner. The cell cycle of *S. cerevisiae*, like all eukaryotes, is divided into four stages (Fig. 2.1). The G_1 (Gap1) phase allows time for growth, DNA is replicated during S phase, the G_2 (Gap2) phase provides a second period of growth, and nuclear division (mitosis) and cell fission (cytokinesis) occur during M phase. The actin cytoskeleton is responsible for guiding secretory vesicles to the cell surface, where they accumulate and fuse, thus polarising growth in the direction of the polarised cortical patches and actin cables [487, 488]. As a yeast cell commits to a new cell cycle in G_1 at START, a bud site is selected and cortical patches cluster and actin cables polarise at this site (Fig. 2.1, a). During G_2, the cortical patches cluster at the bud tip and the actin cables pass from the mother cell into the bud causing apical bud growth (Fig. 2.1, b). The cortical patches cables then randomly redistribute throughout the bud during M phase allowing isotropic growth, while cables in the mother cell still extend to the cell

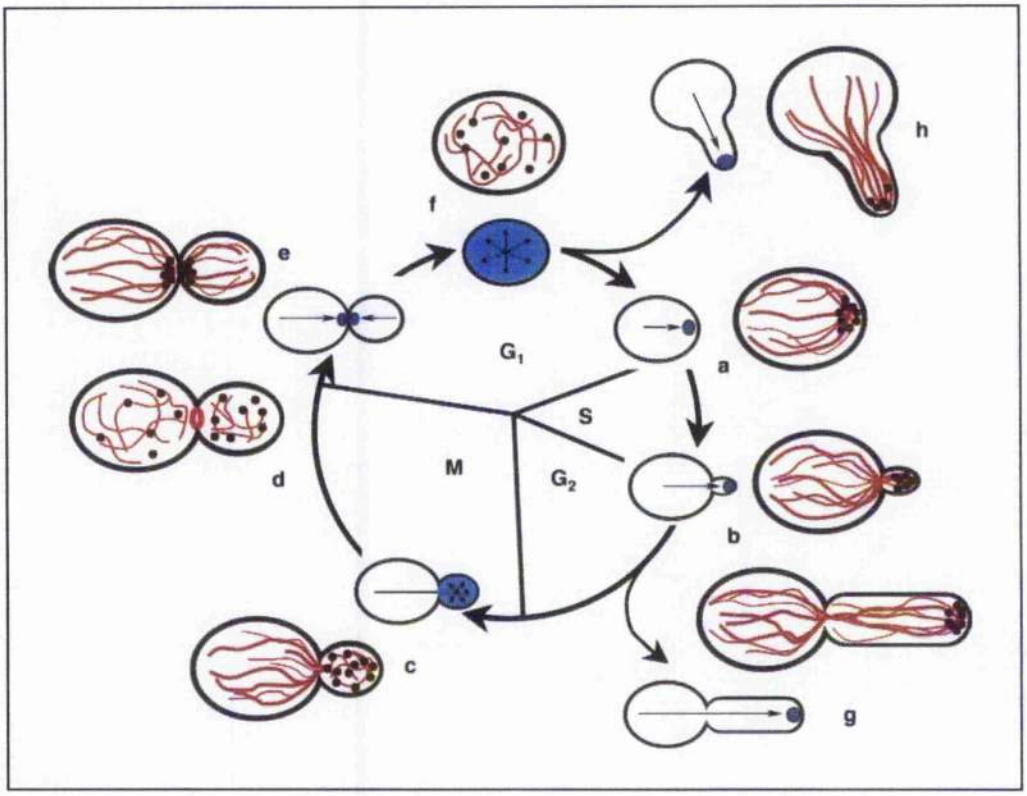


Fig. 2.1. Actin distribution throughout the cell cycle of *S. cerevisiae*.

Cell polarity in budding yeast is established by the localized plasma membrane recruitment of the Rho GTPase Cdc42p (blue) and proteins related to its function. These proteins orient the actin cytoskeleton, which consists of actin cables (red) and cortical patches (brown). In turn, the actin cytoskeleton guides secretory vesicles to the cell surface, where they accumulate (also blue) and fuse, thus polarizing growth (arrows). **(a)** The cell cycle begins in G_1 with establishment of a nascent bud site. **(b)** Clustering of Cdc42p directs early bud growth toward the tip. **(c)** Redistribution of Cdc42p over the bud surface during G_2-M redirects bud growth isotropically, and results in an ellipsoidal shaped bud. **(d)** With the completion of bud growth, cables and patches disorganize, and a cytokinetic ring forms, then contracts and disassembles after mitosis. **(e)** Cdc42p reorients actin and growth between the two new cells to generate new cell walls. The mother cell resumes budding immediately. **(f)** The new daughter undergoes a period of undirected growth. **(g)** Under certain growth conditions, some strains of *S. cerevisiae* differentiate into a filamentous state that forgoes the transition in G_2-M from tip-directed to isotropic growth. The resulting cells are highly elongated. **(h)** Mating pheromones arrest haploid yeast in G_1 and polarize Cdc42p toward potential mating partners to generate a mating projection. Taken from [488].

neck (Fig. 2.1, c). At the end of bud growth the cortical patches and actin cables in the mother cell also randomly redistribute and a cytokinetic F-actin ring assembles at the bud neck (Fig. 2.1, d). The cytokinetic ring contracts and disassembles after mitosis separating the contents of the mother and daughter cell. The cortical patches and actin cables in the mother and daughter cell repolarise to the former bud neck after cytokinesis to facilitate synthesis of the cell walls between the two new cells (Fig. 2.1, e). The cortical patches and actin cables in the mother and daughter cell randomly redistribute again in G_1 and undirected growth of the daughter cell occurs (Fig. 2.1, f). The Rho GTPase Cdc42 is central to polarising the actin cytoskeleton in yeast at all stages of the cell cycle. Cdc42 is recruited to growth sites on the plasma membrane and activates effectors that signal to the actin cytoskeleton [489]. Fig 2.1. illustrates the distribution of Cdc42 throughout the cell cycle and clearly demonstrates that the areas of Cdc42 localisation are the areas of growth. The distribution of Cdc42 is regulated by a number of proteins including the cyclin-dependent protein kinase, Ras, and heterotrimeric G proteins [488].

In addition to this cycle of vegetative growth, there are two other types of yeast growth. The first, filamentous growth is induced in some *S. cerevisiae* strains by a variety of conditions including nitrogen starvation [490]. During filamentous growth, apical extension of the bud is prolonged to form highly elongated cells (Fig. 2.1, g). The second type of alternative growth is induced during mating [491]. Haploid yeast can exist as either mating type a or α (genotypes *MATa* and *MATα*, respectively), and yeast of opposite mating types can fuse to form a *MATa/MATα* diploid. Haploid yeast can sense a potential mating partner and prepare for fusion by responding to a pheromone produced by the yeast of the opposite mating type. *MATa* cells secrete an a-Factor pheromone that *MATα* yeast respond to, and *MATα* cells secrete an α-Factor pheromone that *MATa* yeast respond to. When a yeast cell is stimulated by the pheromone of its opposite mating type, it undergoes a series of physiological changes in preparation for mating including expression of about 200 genes, arrest in G_1, orientated growth towards the mating partner and finally fusion with the mating partner. The growth stimulated by the pheromone of the opposite mating type results in formation of a mating projection polarised in the direction of the pheromone gradient (Fig. 2.1, h). In common with vegetative growth, Cdc42 localisation polarises the cortical patches and

actin cables to the area of growth in both filamentous growth and mating projection formation (Fig. 2.1) [488].

The ability of the yeast mating pheromones to arrest cells of the opposite mating type in G_1 provides another exploitable phenotype of *S. cerevisiae* useful for studying the actions of bacterial toxins. The yeast cell cycle can be arrested by treatment with purified mating pheromone and then released from this arrest as a synchronised population of cells [492]. Therefore, if toxins act to inhibit growth at a particular point in the cell cycle, this can be easily monitored in the synchronised yeast. The ability of toxins to prevent DNA synthesis can also be assessed by using mating factor arrest. Cells arrested in G_1 will all have a single copy of their DNA and if the toxin effects DNA synthesis, the cells will be unable to replicate their DNA. Analysing the DNA content of a synchronised population of cells released from mating pheromone arrest over a period of time will therefore indicate whether DNA replication has been targeted. When experiments are carried out using mating factor arrest, the α-Factor pheromone is generally used to arrest growth of *MATa* yeast. This is because a-Factor is a farnesylated 12-residue peptide (sequence Y-I-I-K-G-V-F-W-D-P-A-C) that is difficult to purify or synthesise, whereas α-Factor is a 13-residue unmodified peptide (sequence W-H-W-L-Q-L-K-P-G-Q-P-M-Y) that is straightforward to manufacture.

The work described in this chapter explains how we developed the *S. cerevisiae* model to study the effects of the *P. aeruginosa* TTS toxin, ExoS. It details how we used this model to assess the effects of ExoS on cell growth, the actin cytoskeleton and DNA synthesis. The relative contribution of the GAP and ADPRT domains to the observed actions of ExoS was also determined. Our results reveal that ExoS was extremely cytotoxic to *S. cerevisiae*. Both domains were toxic to yeast but the ADPRT domain was much more cytotoxic. ExoS was shown to disrupt the actin cytoskeleton and inhibit DNA synthesis and in both cases the ADPRT domain was responsible. ExoS also increased mating projection formation after treatment with α-Factor and inhibited normal bud formation after release from α-Factor arrest.

2.2. METHODS

2.2.1. Materials

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Griener Bio-One (Kremsmuenster, Austria) supplied the plasticware unless otherwise stated. The primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA) and DNA sequencing was performed by the Dundee University Sequencing Service (Dundee, UK).

2.2.2. *S. cerevisiae* strains

The three strains of *S. cerevisiae* used in this study were the diploid strain INVSc1 (Invitrogen, Carlsbad, CA, USA) and the haploid strains BY4741 (Euroscarf, Frankfurt, Germany) and BMA64-1A (Euroscarf). The genotypes of these strains are:

- INVSc1: *his3Δ1/his3Δ1; leu2/leu2; trp1-289/trp1-289; ura3-52/ura3-52*
- BY4741: *MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*
- BMA64-1A: *MATa; ura3-52; trp1Δ2; leu2-3_112; his3-11; ade2-1; can1-100*

2.2.3. Maintenance and growth of *S. cerevisiae*

Yeast strains were grown in Yeast Peptone Dextrose (YPD) broth containing 1% Yeast Extract (Oxoid, Basingstoke, UK), 2% Peptone (Oxoid) and 2% Dextrose. Transformed yeast were grown in synthetic dropout (SD) media to maintain the selective pressure on the plasmid. Glucose containing Minimal SD Base (BD Biosciences, Palo Alto, CA, USA) and galactose/raffinose containing Minimal SD Base GAL/RAF (BD Biosciences) were supplemented with the appropriate Dropout Supplement (BD Biosciences). For plates, 4% agar (Oxoid) was added to the YPD and SD media. All yeast incubations were carried out at 30°C, with shaking at 250 revolutions per minute (rpm) for liquid cultures. Working stock plates of yeast were kept for up to two months at 4°C and for long-term storage, yeast strains were stored in 25% glycerol at -80°C.

2.2.4. Construction of plasmids

This study utilised three yeast expression vectors to examine the effect of ExoS and the GAP and ADPRT domains of ExoS on *S. cerevisiae*. These three vectors were pYES2/NT (Invitrogen), pYC2/NT (Invitrogen) and pCM252 (Euroscarf). pYES2/NT and pYC2/NT contain the *URA3* gene and pCM252 contains the *TRP1* gene, therefore *S. cerevisiae* transformed with these plasmids were selected for on SD media lacking uracil and tryptophan respectively. Wild type ExoS, ExoS with the GAP domain mutated, ExoS with the ADPRT domain mutated and ExoS with both the GAP and ADPRT domains mutated were expressed from all three vectors. Table 2.2 list the primers used in the construction of the plasmids and Table 2.3. summarises how each construct was made.

Table 2.2. Primers

PRIMER	SEQUENCE (5'-3')
ExoS-Fw	GCGGTACCTCAAGCATATGCATATTCAATCGCTTCAGCAG
ExoS-Rev	CTCTCGAGGGATCCGCTGCCGAGCCAAGAATC
R146A-Fw	CGGAGATGGGGCGCTAGCTTCGCTGAGCACCG
R146A-Rev	CGGTGCTCAGCGAAGCTAGCGCCCCATCTCCG
E379A-E381A-Fw	CGAACTACAAGAATGCAAAAGCGATTCTCTATAACAAAG
E379A-E381A-Rev	CTTTGTTATAGAGAATCGCTTTTGCATTCTTGTAGTTCG
T7prom	GTAATACGACTCACTATAGGGC
CYC1R	GCGTGAATGTAAGCGTGAC
ExoS-Int	CGTGTTCAAGCAGATGGTG

Table 2.3. Plasmid construction

PLASMID	CONSTRUCTION
pYES2/NT-ExoS	The *exoS* gene was amplified[a] from PA01 genomic DNA using the ExoS-Fw and ExoS-Rev primers and cloned into the *Kpn*I and *Xho*I sites of pYES2/NTA.
pYES2/NT-ExoS_GAPM	The R146A mutation was introduced into the GAP domain of ExoS by site-directed mutagenesis[b] using the R146A-Fw and R146A-Rev primers.
pYES2/NT-ExoS_ADPRTM	The E379A and E381A mutations were introduced into the ADPRT domain of ExoS by site-directed mutagenesis[b] using the E379A-E381A-Fw and E379A-E381A-Rev primers.
pYES2/NT-ExoS_GAPM_ADPRTM	The *Sac*II/*Xho*I fragment from pYES2/NT-ExoS_ADPRTM was sub-cloned into the *Sac*II/*Xho*I sites of pYES2/NT-ExoS_GAPM.
pYC2/NT-ExoS	*exoS* was sub-cloned from pYES2/NT-ExoS into the *Kpn*I and *Xho*I sites of pYC2/NTA.
pYC2/NT-ExoS_GAPM	*exoS*_GAPM was sub-cloned from pYES2/NT-ExoS_GAPM into the *Kpn*I and *Xho*I sites of pYC2/NTA.
pYC2/NT-ExoS_ADPRTM	*exoS*_ADPRTM was sub-cloned from pYES2/NT-ExoS_ADPRTM into the *Kpn*I and *Xho*I sites of pYC2/NTA.
pYC2/NT-ExoS_GAPM_ADPRTM	*exoS*_GAPM_ADPRTM was sub-cloned from pYES2/NT-ExoS_GAPM_ADPRTM into the *Kpn*I and *Xho*I sites of pYC2/NTA.
pUC19-ExoS	*exoS* was sub-cloned from pYES2/NT-ExoS into the *Kpn*I and *Xba*I sites of pUC19.
pUC19-ExoS_GAPM	*exoS*_GAPM was sub-cloned from pYES2/NT-ExoS_GAPM into the *Kpn*I and *Xba*I sites of pUC19.
pUC19-ExoS_ADPRTM	*exoS*_ADPRTM was sub-cloned from pYES2/NT-ExoS_ADPRTM into the *Kpn*I and *Xba*I sites of pUC19.
pUC19-ExoS_GAPM_ADPRTM	*exoS*_GAPM_ADPRTM was sub-cloned from pYES2/NT-ExoS_GAPM_ADPRTM into the *Kpn*I and *Xba*I sites of pUC19.
pCM252-ExoS	*exoS* was sub-cloned from pUCP19-ExoS (blunt *Kpn*I and *Pst*I) into the blunt *Stu*I and *Pst*I sites of pCM252.
pCM252-ExoS_GAPM	*exoS*_GAPM was sub-cloned from pUCP19-ExoS_GAPM (blunt *Kpn*I and *Pst*I) into the blunt *Stu*I and *Pst*I sites of pCM252.
pCM252-ExoS_ADPRTM	*exoS*_ADPRTM was sub-cloned from pUCP19-ExoS_ADPRTM (blunt *Kpn*I and *Pst*I) into the blunt *Stu*I and *Pst*I sites of pCM252.
pCM252-ExoS_GAPM_ADPRTM	*exoS*_GAPM_ADPRTM was sub-cloned from pUCP19-ExoS_GAPM_ADPRTM (blunt *Kpn*I and *Pst*I) into the blunt *Stu*I and *Pst*I sites of pCM252.

[a] Explained in 2.2.5

[b] Explained in 2.2.6

2.2.5. Amplification of *exoS*

2.5 U of *PfuTurbo*® Hotstart DNA polymerase (Stratagene, La Jolla, CA. USA) was used to polymerase chain reaction (PCR) amplify *exoS* from PA01 genomic DNA in a reaction mixture containing 1 x cloned *Pfu* DNA polymerase reaction buffer (Stratagene), 25 mM of each deoynucleotide triphosphate (dNTP), 1 pmole/µl ExoS-Fw and 1 pmole/µl ExoS-Rev primers (Table 2.2) and 5% dimethyl sulfoxide (DMSO) in a total volume of 100 µl. The mixture was subjected to one denaturing cycle of 5 min at 94°C and then 30 cycles using the following conditions: a 45 s denaturing step at 94°C, followed by a 45 s annealing step at 52°C and a 1 min extension step at 72°C, and completed by a final extension cycle of 10 min at 72°C. After cloning into the *Kpn*I and *Xho*I sites of pYES2/NTA, the sequence of *exoS* was confirmed by sequencing with the T7prom, CYC1R and ExoS-Int primers (Table 2.2).

2.2.6. Site-directed mutagenesis

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was used to incorporate the R146A and E379A-E381A mutations into ExoS in pYES2/NT-ExoS. For each site-directed mutagenesis reaction, two complementary primers were designed (Table 2.2) with nucleotide mutations that would result in the desired amino acid substitutions and would also introduce a new restriction site into pYES2/NT-ExoS. 125 ng of each high performance liquid chromatography (HPLC) purified primer was added to 5 ng of pYES2/NT-ExoS, 1 µl dNTP mix, 1 x reaction buffer and 2.5 U of *PfuTurbo* DNA polymerase in a final volume of 50 µl. This reaction mixture was subjected to one 30 s denaturing cycle at 95°C followed by 18 cycles of a 30 s denaturing step at 95°C, a 1 min annealing step at 55°C and a 15 min extension step at 68°C. *PfuTurbo* DNA polymerase replicated both plasmid strands with high fidelity and without displacing the mutant primers and thus synthesised mutated plasmids containing staggered nicks. After amplification, the non-mutated parental DNA template was digested with 10 U of *Dpn* I for 1 h at 37°C. The *Dpn* I endonuclease (target sequence 5'-Gm^{6}ATC-3') is specific for methylated and hemimethylated DNA and as the pYES2/NT-ExoS was isolated from a dam+ *E. coli* strain, it was sensitive to digestion by *Dpn* I. A 1µl aliquot of the *Dpn* I-treated DNA was transformed into XL1-Blue supercompetent cells and the nicks in the mutated plasmids were repaired in these cells. Plasmid DNA was prepared

from the transformed *E. coli* using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany) and the success of the mutagenesis was assessed. Site-directed mutagenesis of ExoS with the R146A primers introduced an *Nhe*I site and site-directed mutagenesis with the E379A-E381A primers introduced a *Bsm*I site. Therefore it was possible to ascertain whether the site-directed mutagenesis had worked by using these restriction enzymes in diagnostic digests. Mutations were subsequently confirmed by sequencing the constructs with the T7 prom, CYC1R and ExoS-Int primers (Table 2.2).

2.2.7. Yeast transformation

S. cerevisiae cells were transformed with plasmids using the Frozen-EZ Yeast Transformation II™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturers instructions. Briefly, competent cells were prepared by centrifuging 10 ml of mid-log phase yeast at 500 x g for 4 min then washing the pellet in 10 ml EZ 1 solution and resuspending in 1 ml EZ 2 solution. For transformation, 0.2-1 μg of plasmid DNA was mixed with 50 μl of competent cells and 500 μl EZ3 solution. The transformation reaction was incubated at 30°C for 45 min and subjected to vigorous mixing 3 times during incubation. 100 μl of the transformation mixture was plated out onto appropriate SD agar and allowed to grow at 30°C for 2-4 days.

2.2.8. Integration of pCM242 into the *leu2* locus of the *S. cerevisiae* genome

The pCM242 plasmid was integrated into the genome of INVSc1 and BMA64-1A by homologous recombination between the functional *LEU2* gene in pCM242 and the mutated *leu2* locus in the yeast chromosomes. The pCM242 plasmid was digested with *Eco*RV and purified using the QIAquick PCR Purification Kit (Qiagen) according to manufacturers instructions. The *Eco*RV linearised pCM242 was transformed into INVSc1 or BMA64-1A (see section 2.2.7) and the yeast that had successfully integrated the plasmid were selected on SD-leucine+glucose agar plates.

2.2.9. Recovery of plasmid DNA from yeast

The Zymoprep™ Yeast Plasmid Miniprep Kit (Zymo Research) was used to prepare plasmid DNA from *S. cerevisiae*. A 1 ml aliquot of an overnight culture was

centrifuged at 600 x g for 2 min and 150 µl of Solution 1 and 2 µl of Zymolase were added to the pellet. The pellet was resuspended and incubated at 37°C for 30 min before being mixed with 150 µl of Solution 2 and then 150 µl of Solution 3. The mixture was centrifuged at maximum speed for 2 min and the resulting supernatant was mixed with 400 µl of isopropanol. After centrifugation at maximum speed for 8 min all the supernatant was removed and the plasmid pellet was resuspended in 35 µl Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer (10 mM Tris.Cl pH 8.0, 1 mM EDTA).

Plasmid DNA prepared from *S. cerevisiae* is not very pure and therefore not suitable for sequencing. To prepare DNA for sequencing, 5 µl of the yeast plasmid preparation was used to transform TransforMax™ EC100™ Electrocompetent *E. coli* (Epicentre Biotechnologies, Madison, WI, USA). Electroporation was carried out in a 0.2 cm electroporation cuvette (Invitrogen) using the GenePulser Xcell™ (Bio-Rad Laboratories, Hercules, CA) set to the following parameters: 25 µFD, 200 Ω and 2.5 kV. Plasmid DNA was isolated from 3 ml of an overnight culture of the transformed *E. coli* using the QIAprep® Spin Miniprep kit (Qiagen) according to the manufacturers instructions.

2.2.10. Growth assays

For the plate growth assays, overnight cultures of yeast grown in the appropriate SD media supplemented with glucose were diluted in phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4) to OD_{600} = 1. These cultures were serially 10-fold diluted 4 times in PBS and 5 µl of each dilution was spotted onto appropriate agar plates. The plates were incubated at 30°C for 4 days before being photographed using a Kodak DX4530 digital camera. For the galactose inducible system, INVSc1 or BY4741 containing either the pYES2/NT- or the pYC2/NT-based vectors were grown overnight in SD-uracil+glucose media and spotted onto SD-uracil+glucose and SD-uracil+galactose/raffinose agar plates. For the tetracycline inducible system, INVSc1 or BMA64-1A containing the integrated pCM242 plasmid and a pCM252-based plasmid were grown overnight in SD-leucine-tryptophan+glucose media and spotted onto agar plates of the same media supplemented with 0, 0.1, 0.5, 1 and 2 µg/ml doxycycline.

For the liquid growth assay, cultures of INVSc1+pCM242 transformed with either pCM252 or pCM252-ExoS were grown overnight in SD-leucine-tryptophan+glucose media. These cultures were diluted in fresh media to OD_{600} = 0.3. After 4 hours of growth, doxycyline was added at a final concentration of 2 µg/ml. OD_{600} readings were taken every hour to assess the growth of the cultures.

2.2.11. Immunofluorescence

Cultures of INVSc1+pCM242 containing a pCM252-based plasmid were grown and induced as described for the liquid growth assay. Four hours after addition of doxycycline the *S. cerevisiae* were fixed by adding 5% formaldehyde to 5 ml of culture and incubating for 30 min at room temperature with occasional inversion. The cells were centrifuged at 1500 x g for 5 min and the pellets were washed three times in PBS. The pellets were resuspended in 200 – 800 µl of PBS (to roughly normalise cell density) and 150 µl aliquots were washed in 1 ml of Solution B (100 mM K_2HPO_4, 100mM KH_2PO_4 and 1.2 M sorbitol). Each pellet was incubated in 1 ml Solution B containing 0.2% 2-mercaptoethanol and 2 µg/ml lyticase at 37°C for 30 min to permeabilise the yeast. After permeabilisation, the cells were centrifuged at 500 x g and washed once in 1 ml Solution B. To stain for actin, the fixed and permeabilised *S. cerevisiae* were incubated in 50 µl Solution B containing 0.4 U of AlexaFluor 488 Phalloidin (Invitrogen) at 37°C overnight in the dark. The cells were washed three times in 1 x PBS before being spread onto a Poly-Prep™ Slides (Sigma-Aldrich) and allowed to dry for 10 min. A drop of vector shield (Vector Laboratories, Burlingame, CA, USA) was used to mount the yeast under a coverslip. The slides were viewed using a Nikon Eclipse E600 microscope with a Nikon p/an Fluor 100x lens and captured using an Optronics digital camera and MagnaFire software (Meyor Instruments, Houston, TX, USA).

2.2.12. Synchronisation of *S. cerevisiae*

The haploid *MAT*a strain BMA64-1A containing the integrated pCM242 plasmid and a pCM252-based plasmid were grown to mid-log phase in SD-leucine-tryptophan+glucose media. The cultures were diluted to OD_{600}=0.2 and arrested in G_1 with 20

μg/ml α-factor (Zymo Research). Two hours after addition of the α-factor, ExoS or mutant ExoS was induced by adding 2 μg/ml doxycycline. The yeast were released from α-factor arrest an hour after the doxycycline was added by centrifuging the cells at 1500 x g for 5 min then washing twice in pre-warmed SD-leucine-tryptophan+glucose media containing 2 μg/ml doxycycline. The cells were resuspended in pre-warmed SD-leucine-tryptophan+glucose containing 2 μg/ml doxycycline and cells were fixed for immunofluorescence (see section 2.2.11.) or flow cytometry analysis (see section 2.2.13.) every 30 min.

2.2.13. Flow cytometry analysis

Synchronised BMA64-1A+pCM242 containing a pCM252-based plasmid were prepared for flow cytometry analysis. The cells from 1 ml of culture were harvested by centrifugation at 500 x g for 5 min and the pellet was resuspended in 1.5 ml double distilled water (DDW). The yeast were fixed by adding 3.5 ml of 95% ethanol and incubating overnight at 4°C. The cells were centrifuged at 500 x g for 5 min and washed in 1 ml DDW. The RNA was degraded by incubating the yeast with 0.5 ml 2 mg/ml RNase A in 50 mM Tris.Cl, pH 8.0 for 1- 2 hours at 37°C. The cells were centrifuged at 500 x g for 5 min and the pellet was resuspended in 200 μl of 5 mg/ml pepsin and 0.45% concentrated HCl and incubated for 30 - 60 min at 37°C to degrade the proteins. The yeast were harvested by centrifugation at 500 x g for 5 min and the pellet was resuspended in 0.5 ml 1 x propidium iodide solution (180 mM NaCl, 70 mM $MgCl_2$, 75 μM propidium iodide, 100 mM Tris.Cl, pH 7.5). The yeast were incubated in the 1 x propidium iodide solution overnight at 4°C to stain the DNA. A 50 μl aliquot of cells was diluted in 0.1 x propidium iodide solution diluted in 50 mM Tris.Cl, pH 7.5 for flow cytometry analysis. Samples were analysed on a FACscan flow cytometer using CELL QUEST software to obtain and analyze the data (BDIS, San Jose, CA, USA).

2.3. RESULTS

2.3.1. Use of galactose inducible expression systems to assess the toxicity of ExoS in *S. cerevisiae*

In order to utilise *S. cerevisiae* as model system in which to elucidate the molecular mechanism of action of ExoS, we first needed to establish whether *S. cerevisiae* was sensitive to the cytotoxic effects of ExoS. To control the expression of ExoS in *S. cerevisiae* we began our studies using the yeast expression vector pYES2/NT. pYES2/NT is a high copy number vector that allows expression of a gene of interest to be controlled by altering the carbon source on which the yeast is grown. Cloning the gene downstream of the *GAL1* promoter enables the gene to be induced to high levels in the presence of galactose, repressed in the presence of glucose and neither induced nor repressed when grown on raffinose. Therefore, we planned to ligate *exoS* into pYES2/NT, transform the construct into *S. cerevisiae* and induce expression of ExoS by switching the carbon source from glucose to galactose.

The *exoS* gene was amplified from *P. aeruginosa* strain PA01 and ligated in-frame into pYES2/NT. Sequence analysis of the construct revealed that *exoS* had five nucleotide polymorphisms compared to the database sequence of *exoS* from PA01 (X99471). Four of these nucleotide changes (169 T-C, 174, T-C, 222 G-A, 1161 G-A) were silent and did not alter the amino acid sequence of ExoS. The fifth nucleotide polymorphism was a substitution of an adenine at position 184 with a guanine, which led to an amino acid change at residue 62 from a methionine to a valine. All of these allelic changes have been previously identified in the *exoS* sequence of *P. aeruginosa* strain 388 (L27629), which also has a number of additional changes [493].

To determine the contribution of the GAP and ADPRT domains to the toxicity of ExoS in *S. cerevisiae*, the active site residues of each domain in pYES2/NT-ExoS were mutated. Arginine 146 was mutated to an alanine to abolish activity of the GAP domain (pYES2/NT-ExoS_GAPM) and the glutamic acids at residues 379 and 381 were mutated to alanines to render the ADPRT domain inactive (pYES2/NT-ExoS_ADPRTM). A double mutant was also generated that lacked both an active GAP and ADPRT domain (pYES2/NT-ExoS_GAPM+ADPRTM)

When we attempted to transform the *S. cerevisiae* strains INVSc1 and BY4741 with the constructs we had made, we experienced some difficulties. Although, we were able to transform INVSc1 and BY4741 with the empty pYES2/NT vector, pYES2/NT-ExoS_ADPRTM and pYES2/NT-ExoS_GAPM+ADPRTM (efficiency of transformation > 10^5), we were unable to transform the yeast with pYES2/NT-ExoS or pYES2/NT-ExoS_GAPM. These results suggested that the ADPRT domain of ExoS was so toxic to *S. cerevisiae* that even when grown on glucose, which should repress expression, enough toxin was manufactured as a result of leaky transcription from the *GAL1* promoter to kill the transformed yeast.

Although leaky expression from the *GAL1* promoter in pYES2/NT made it an unsuitable vector for assessing the toxicity of the ADPRT domain of ExoS, it was still useful for examining the toxicity of the GAP domain. As expected both the diploid yeast INVSc1 and the haploid yeast BY4741 transformed with the empty pYES2/NT vector were able to grow equally well on glucose or galactose and raffinose (Fig. 2.2, pYES2/NT). Expression of ExoS with both the GAP and ADPRT domains mutated did not affect yeast cell viability. This was illustrated by the yeast strains INVSc1 and BY4741 transformed with pYES2/NT-ExoS_GAPM+ADPRTM growing as well as the strains containing the empty vector control under all conditions (Fig. 2.2, GAPM + ADPRTM). When INVSc1 or BY4741 containing pYES2/NT-ExoS_ADPRTM were grown on glucose, they grew as well as the strains containing the empty vector. However, when these strains were grown on galactose and raffinose their growth was much inhibited compared to the empty vector control (Fig. 2.2, ADPRTM). Therefore, the GAP domain of ExoS was toxic to *S. cerevisiae* when induced by growth on galactose.

In an attempt to overcome the problem of leaky expression from the *GAL1* promoter in pYES2/NT, we sub-cloned *exoS* and the mutant *exoS* genes into the low copy number yeast expression vector pYC2/NT. pYC2/NT and pYES2/NT are identical apart from the sequences used for maintenance and replication in yeast. pYES2/NT contains the 2μ origin that allows the plasmid to be episomally maintained and replicated at high copy numbers (generally 10-40 copies per cell). In contrast, pYC2/NT contains the

CEN6/ARSH4 sequence that allows non-integrative centromeric maintenance and low copy number replication of the plasmid (generally 1-2 copies per cell).

Transformation of INVSc1 and BY4741 with pYC2/NT-ExoS or pYC2/NT-ExoS_GAPM failed. Therefore, even when ExoS expression was repressed by glucose and there were only about 1 or 2 copies of the gene per cell, enough of the ADPRT domain was produced to kill the yeast. After repeated attempts at transforming INVSc1 and BY4741 with either pYC2/NT-ExoS or pYC2/NT-ExoS_GAPM, a small number of transformants were obtained. However when the plasmids were recovered from these transformants and the *exoS* gene sequenced, they were shown to contain mutations. For example, a pYC2/NT-ExoS plasmid recovered from INVSc1 contained a mutation of nucleotide 793 from a guanine to a thymidine; this resulted in a substitution of the glutamine residue at 265 to a stop codon. Truncating ExoS at residue 265 would remove the ADPRT domain, therefore enabling the construct to be transformed into yeast. Also, a pYC2/NT-ExoS plasmid recovered from BY4741 contained a thymidine to cytosine substitution of nucleotide 1148; this caused the leucine residue at position 383 to be substituted with a proline. Proline has a cyclic structure and can dramatically influence protein structure, therefore the introduction of this residue two amino acids away from the active site glutamic acid residue 381 probably rendered the ADPRT domain inactive.

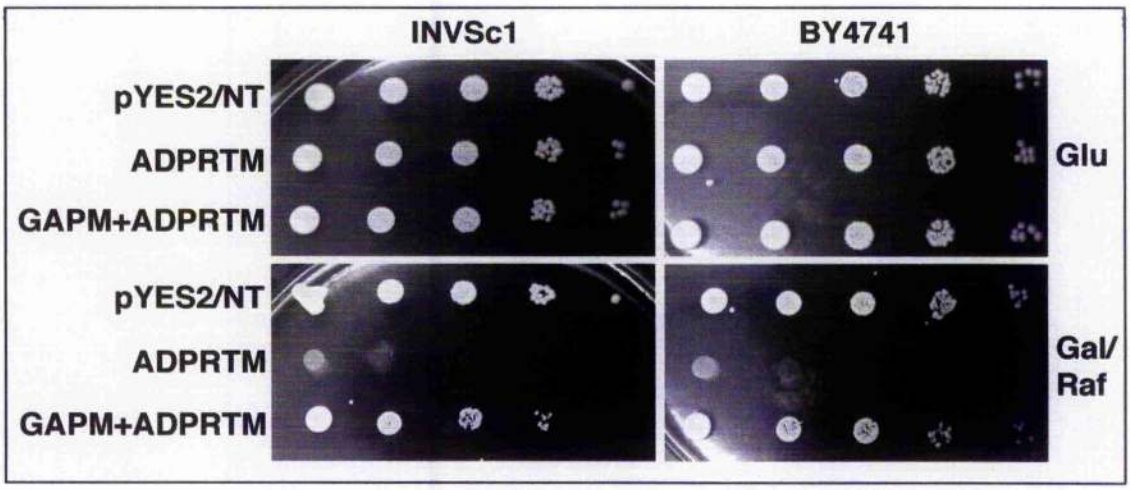


Fig. 2.2. Toxicity of the GAP domain of ExoS in *S. cerevisiae*.

A diploid and haploid strain of *S. cerevisiae* (INVSc1 and BY47471 respectively) were transformed with the empty pYES2/NT vector (pYES2/NT), this vector encoding ExoS with the ADPRT domain mutated (ADPRTM) or pYES2/NT encoding ExoS with both the GAP and ADPRT domains mutated (GAPM+ADPRTM). The growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

2.3.2. Use of tetracycline inducible expression systems to assess the toxicity of ExoS in *S. cerevisiae*

The ADPRT domain of ExoS was so cytotoxic that the tiny amount of protein expression that resulted from leaky expression of the *GAL1* promoter under glucose repressing conditions was sufficient to cause yeast cell death. Even the use of a low copy number vector did not reduce the expression of ExoS to levels compatible with yeast transformation. A system that allowed tighter control of ExoS expression was therefore required. One such yeast expression system is the tetracycline-regulated activator-repressor dual system [494]. There are two variations of this system, one of which enables the gene of interest to be induced by the removal of tetracycline and one of which allows the gene to be induced by the addition of tetracycline. We decided to utilise the latter system because *exoS* can be induced simply by the addition of tetracycline without the requirement for a change of medium.

The tetracycline inducible system requires two plasmids (Fig. 2.3, A). One plasmid (pCM252) encodes a *tetR*'-VP16 transactivator and contains a hybrid of seven repeats of the bacterial Tn10 transposon-derived tetracycline-responsive *tetO* promoter fused to the *S. cerevisiae CYC1* TATA region upstream of a multiple cloning site. The *tetR*'-VP16 transactivator is a fusion protein consisting of the mutant *tetR*' DNA binding domain from Tn10, that recognises *tetO* only in the presence of tetracycline, fused to the VP16 activator moiety from herpes simplex virus. The second plasmid (pCM242) encodes a fusion protein of the wild type *tetR* moiety, which recognises *tetO* only in the absence of tetracycline, fused to Ssn6, a component of a general repressor complex in yeast. The pCM252 plasmid is maintained episomally while the pCM242 plasmid requires integration into the mutant *leu2* locus of *S. cerevisiae* by homologous recombination.

When yeast containing pCM252 and pCM242 are grown without tetracycline (Fig. 2.3, B), the *tetR*-Ssn6 repressor binds to $tetO_7$ and represses transcription of the gene of interest. When tetracycline is added to the system (Fig. 2.3, C), tetracycline binds to *tetR* altering its conformation and making it unable to bind to $tetO_7$. Tetracycline also binds to the mutant *tetR*' moiety and the resulting conformation change enables the

tetR'-VP16 transactivator to bind to $tetO_7$ and induce expression of the gene downstream of the hybrid $tetO_7$-*CYC1* promoter.

To assess the usefulness of this tetracycline-inducible activator-repressor system, we integrated pCM242 into the *leu2* locus of INVSc1 and transformed the resulting strain with pCM252 containing *exoS* or mutated *exoS*. The transformations of INVSc1+pCM242 with pCM252, pCM252-ExoS, pCM252-ExoS_GAPM, pCM252-ExoS_ADPRTM or pCM252-ExoS_GAPM+ADPRTM were all successful. This suggested that the tetracycline-inducible activator-repressor system was tightly regulated to ensure that there was no leaky expression of ExoS when the yeast were grown in the absence of tetracycline. It is interesting to note that when we attempted to transform INVSc1 that did not contain pCM242 with pCM252-ExoS or pCM252-ExoS_GAPM, no transformants were obtained. Therefore, the use of this system in studying ExoS is dependent on the strong *tetR*-Ssn6 repressor.

When serial dilutions of INVSc1+pCM242 transformed with pCM252 were spotted onto agar containing varying concentrations of doxycycline (a tetracycline derivative), as expected there was no difference in yeast growth (Fig. 2.4, pCM252). When ExoS expression in INVSc1+pCM242+pCM252-ExoS was repressed by growth on media lacking doxycycline, the growth of the yeast equalled that observed in yeast not expressing ExoS. However, when ExoS was induced by increasing concentrations of doxycycline, the *S. cerevisiae* were no longer viable (Fig. 2.4, ExoS). Induction of ExoS by as little as 0.1 μg/ml doxycycline resulted in maximal cell death. When ExoS with a mutated GAP domain was expressed in INVSc1+pCM242, the pattern of cell death matched that observed when the wild type ExoS was expressed (Fig. 2.4, GAPM). When the ADPRT domain of ExoS was mutated and only an active GAP domain was expressed in INVSc1+pCM242, yeast cell death was induced but only at higher concentrations of doxycycline (Fig. 2.4, ADPRTM). The cytotoxicity of the GAP domain was only evident when induced by at least 0.5 μg/ml doxycycline and even when induced by 2 μg/ml doxycycline, yeast cell death did not match that caused by ExoS induced by 0.1 μg/ml doxycycline. Mutation of both the GAP and ADPRT domains rendered ExoS as non-toxic as the empty pCM252 vector (Fig. 2.4, GAPM+ADPRTM). Thus, ExoS is cytotoxic to *S. cerevisiae* and its expression can be adequately controlled by the tetracycline-inducible activator-repressor system. Both the

GAP and ADPRT domains exhibit cytotoxicity, but the ADPRT domain has a more potent toxic effect on yeast than the GAP domain.

The toxicity of ExoS in *S. cerevisiae* was also demonstrated in liquid culture. Overnight cultures of INVSc1+pCM242 containing either pCM252 or pCM252-ExoS were diluted to an OD_{600} of approximately 0.3. After four hours of growth, 2 μg/ml doxycycline was added to each culture and the growth assessed by taking OD_{600} reading every hour (Fig. 2.5). Even before the addition of doxycycline, the growth rate of INVSc1+pCM242+pCM252-ExoS appeared slower than that of INVSc1+pCM242+ pCM252. After the addition of doxycycline, the growth of INVSc1 expressing ExoS was much reduced compared to the yeast not expressing ExoS. The apparent growth inhibition observed before the addition of doxycycline may be explained by a small amount of leaky expression of ExoS even from the tightly controlled tetracycline-inducible activator-repressor system.

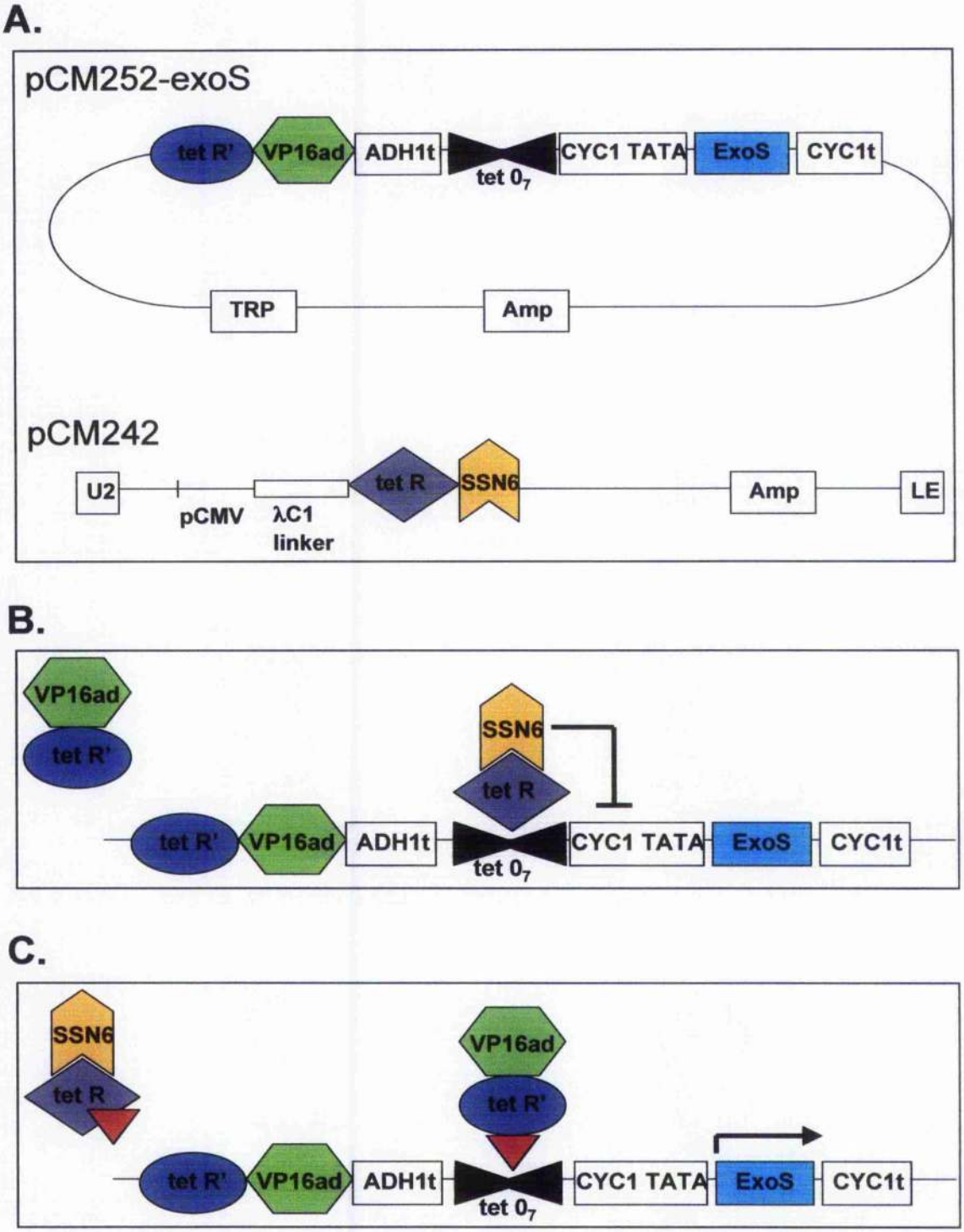


Fig. 2.3. The tetracycline-inducible activator-repressor expression system.

(A) A diagram of the pCM252 and pCM242 plasmids utilised in the tetracycline-inducible activator-repressor system to control of expression of ExoS in *S. cerevisiae*. **(B)** ExoS is repressed in the absence of tetracycline by the *tetR*-Ssn6 repressor and **(C)** induced in the presence of tetracycline (red triangles) by the *tetR*'-VP16 transactivator. Details of the system are described in the text.

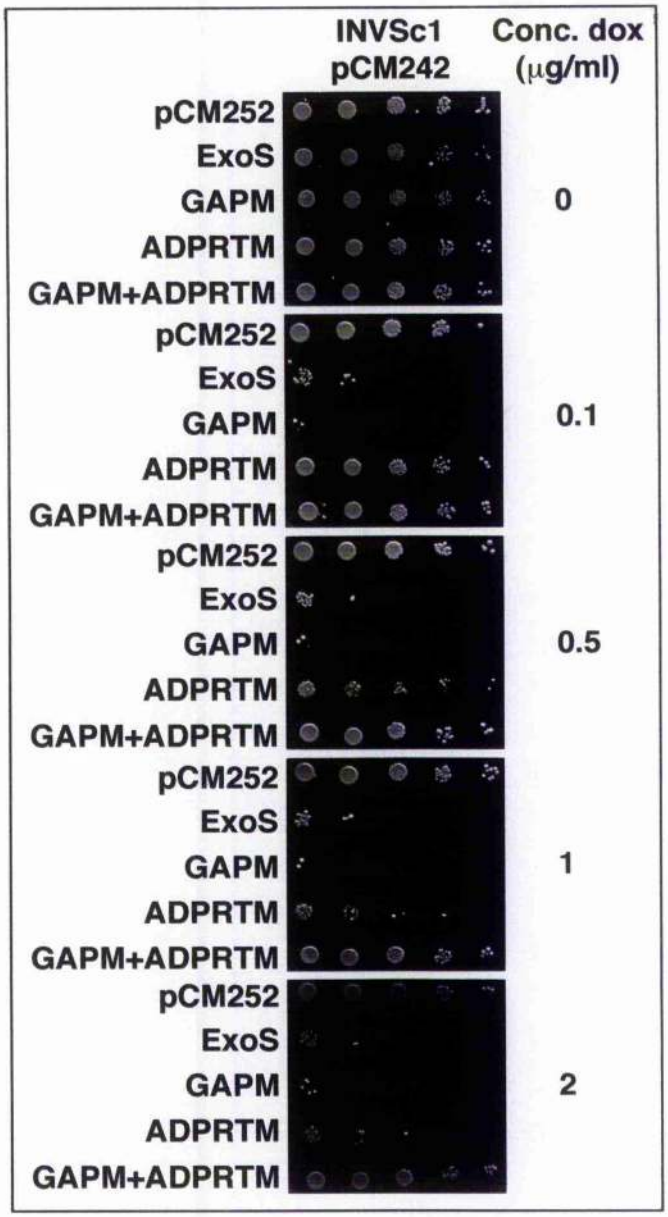


Fig. 2.4. Toxicity of ExoS and the GAP and ADPRT domains of ExoS *in S. cerevisiae*.

INVSc1 with pCM242 integrated into its *LEU2* locus was transformed with the empty vector pCM252 or this vector encoding ExoS, the ExoS GAP mutant (GAPM), the ExoS ADPRT mutant (ADPRTM) or the ExoS GAP and ADPRT mutant (GAPM+ADPRTM). Toxicity of each construct to *S. cerevisiae* was assessed by spotting ten-fold serial dilutions of culture onto agar containing varying concentrations of doxycycline and assessing growth after three days.

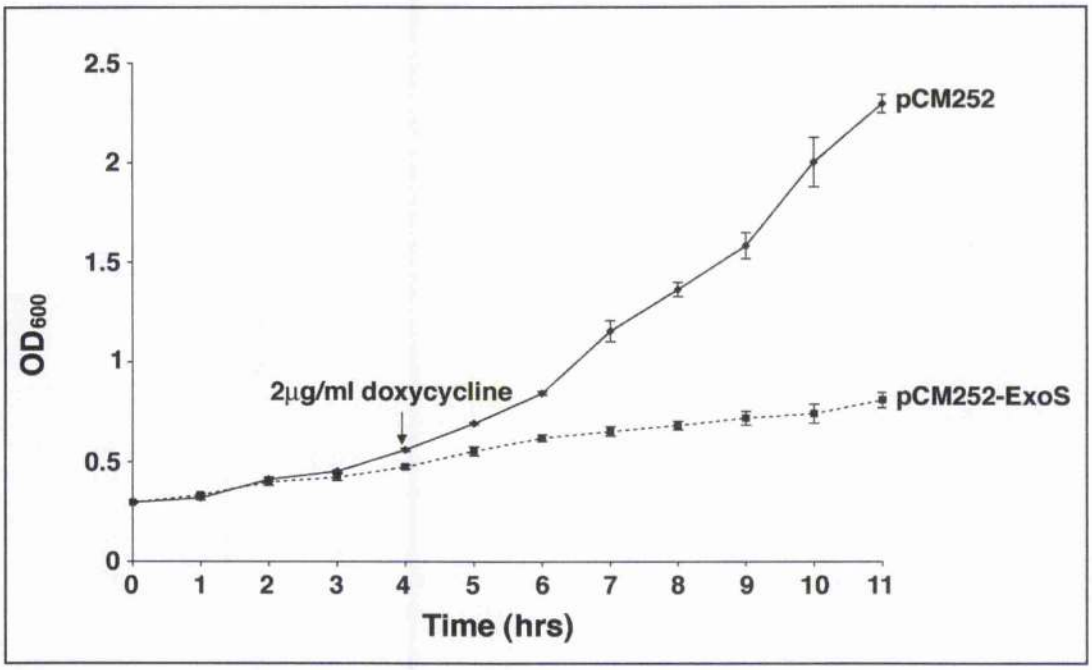


Fig. 2.5. Toxicity of ExoS in liquid culture.

Growth curves of INVSc1 with pCM242 integrated into the *LEU2* containing either the empty pCM252 vector or pCM252-ExoS. O/N cultures were diluted to an OD_{600} of approximately 0.3 and after 4 hours of growth ExoS expression was induced by the addition of 2 μg/ml doxycycline. The experiment was performed in triplicate. Error bars represent standard deviation of the mean.

2.3.3. ExoS alters the actin cytoskeleton of *S. cerevisiae* and the ADPRT domain is responsible

ExoS is known to affect the actin cytoskeleton in mammalian cells but there is some confusion over which domain of ExoS is responsible for this phenotype. In order to determine whether ExoS also disrupts the actin cytoskeleton of *S. cerevisiae* and to try to dissect the domain responsible, we induced ExoS and its mutants in INVSc1 and stained the yeast with phalloidin, to detect filamentous actin.

When the actin cytoskeleton of INVSc1+pCM242+pCM252 was examined, all the normal actin distributions were observed (Fig. 2.6.A and Fig. 2.6.B, pCM252). During G_1, the *S. cerevisiae* showed a random distribution of cortical patches and actin cables (Fig. 2.6.A, a). As a yeast cell committed to a new cell cycle, a bud site was selected where cortical patches accumulated and actin cables converged (Fig. 2.6.A, b). As the bud emerged, cortical patches clustered at its tip and actin cables extend from the mother cell into the bud (Fig. 2.6.A, c). The cortical patches were then randomly redistributed in the bud, while actin cables in the mother still extended to the bud neck (fig. 2.6.A, d). When bud growth was completed, the cortical patches and actin cables redistributed randomly in the mother and bud while a cytokinetic F-actin ring assembled at the bud neck (Fig. 2.6.A, e). During cytokinesis, the F-actin ring contracted and disassembled and the cortical patches and actin cables repolarised to the former bud neck (Fig. 2.6.A, f).

When ExoS was induced in INVSc1+pCM242+pCM252-ExoS by the addition of doxycycline, the actin cytoskeleton was dramatically disrupted (Fig. 2.6.B, ExoS). Although budding cells were still observed, the organised actin structures were absent. Many of the cortical patches appeared large and brightly stained and seemed to aggregate in a number of areas in the cell. Thick, disorganised actin cables were also observed which is unusual because actin cables are usually only visible when they are polarised. Cells were observed that had recently budded but still contained cortical patches in the mother cell and the actin cables were not polarised and travelling between the mother cell and the bud. The actin cytoskeleton of *S. cerevisiae* was so disrupted by ExoS that the only recognisable actin polarisation was the accumulation of cortical

patches in the bud. However, even though cortical patches did accumulate in the bud, they were not excluded from the mother cell as normally observed in budding cells.

Studying the actin cytoskeleton of yeast expressing the individual domain mutants of ExoS provided insight into which domain was responsible for the disruption of the actin cytoskeleton. The ExoS GAP mutant resulted in the same pattern of actin disruption as that caused by ExoS (Fig. 2.6.B, GAPM). The ADPRT mutant of ExoS, however, retained normal actin distributions (Fig. 2.6.B, ADPRTM). As expected, expression of ExoS with both the GAP and ADPRT domains mutated also did not cause any disruption to the actin cytoskeleton (Fig. 2.6.B, GAPM+ADPRTM). Therefore, the disruption of the actin cytoskeleton by ExoS was a consequence of its ADPRT activity.

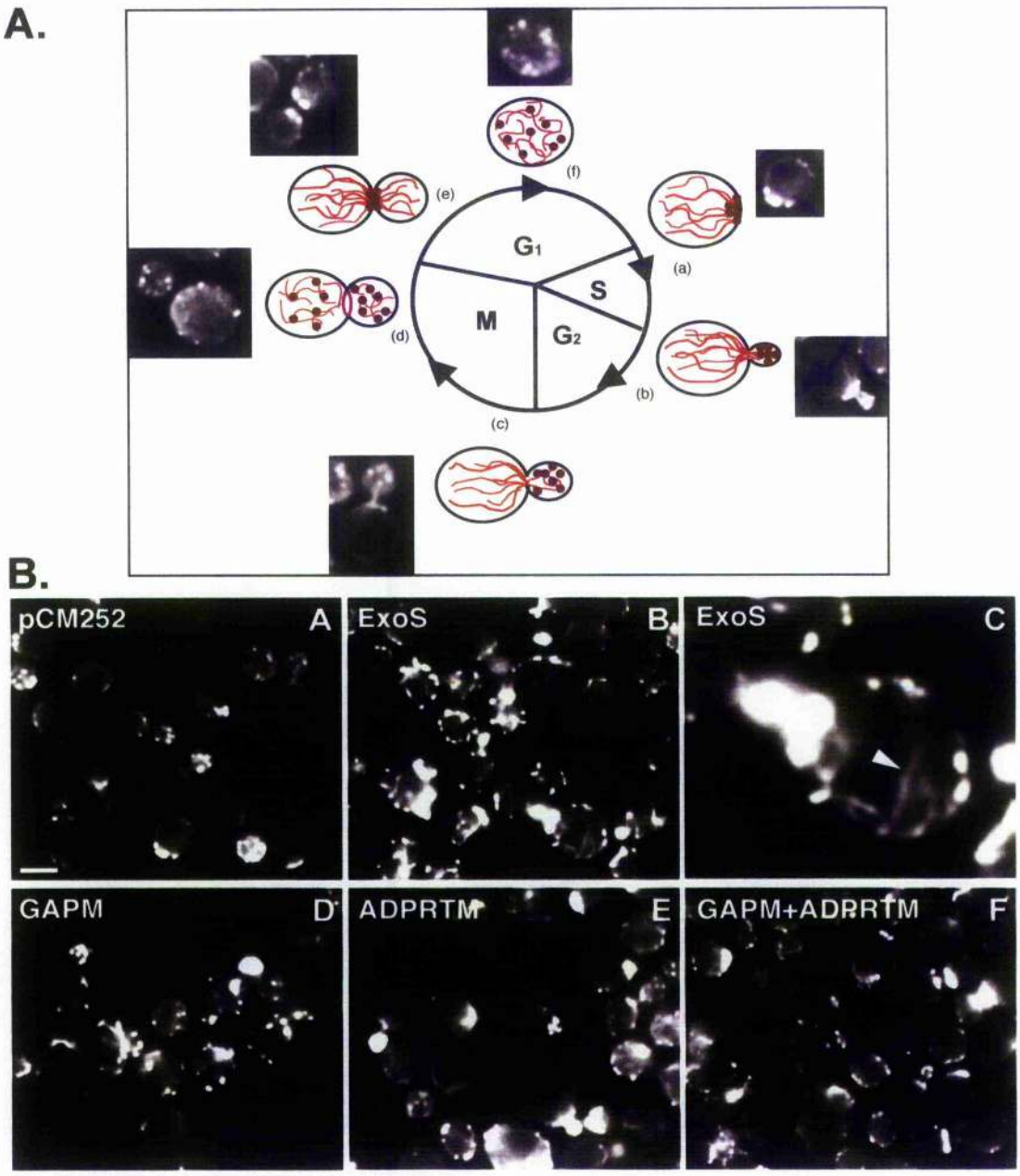


Fig. 2.6. Effect of ExoS on the actin cytoskeleton of *S. cerevisiae*.

(**A**) Polarisation of the *S. cerevisiae* actin cytoskeleton throughout the cell cycle with cortical patches (Brown), actin cables (Red) and the cytokinetic ring (Pink) indicated. Examples of the different stages are illustrated by INVSc1 transformed with pCM242 and the empty pCM252 vector. (**B**) Immunofluorescent staining of the actin cytoskeleton with phalloidin in INVSc1 containing pCM242 and either (A) the empty pCM252 vector or this vector encoding (B+C) ExoS, (D) the GAP domain mutant, (E) the ADPRT domain mutant or (F) the GAP and ADPRT mutant of ExoS. Image C illustrates an enlargement of the image in the white box in image B. The arrow points to a disorganised, thick actin cable.

2.3.4. ExoS prevents DNA replication in *S. cerevisiae* and the ADPRT domain is responsible

It has been demonstrated that ExoS inhibits DNA synthesis in mammalian cells, so we set out to determine whether ExoS also inhibited DNA synthesis in yeast. We used α-factor arrest to assess the effect of ExoS and the GAP and ADPRT domains on DNA replication in *S. cerevisiae*.

In order to utilise the α-factor mating pheromone for synchronisation of *S. cerevisiae* we needed to use a mating-type *a* haploid strain. To use the tetracycline-inducible activator-repressor system this strain must also have pCM242 integrated into its chromosome. The haploid strain BMA64-1A was used instead of BY4741 (used in the galactose system above) because integration of pCM242 required an intact but mutated *LEU2* locus and the *LEU2* locus of BY4741 is completely deleted. To ensure that ExoS was toxic to BMA64-1A, growth of BMA64-1A+pCM242+pCM252-ExoS on agar containing varying concentrations of doxycycline was assessed (Fig. 2.7). As observed in the diploid INVSc1 strain, ExoS was highly toxic to BMA64-1A. Maximal cell death resulted from activation of ExoS by as little as 0.5 μg/ml of doxycycline.

BMA64-1A+pCM242 cells containing pCM252, pCM252-ExoS, pCM252-ExoS_GAPM, pCM252-ExoS_ADPRTM or pCM252-ExoS_G+AM were synchronised with α-factor and then ExoS expression was induced by the addition of doxycycline. The cells were released from α-factor arrest and their DNA content assessed by staining with propidium iodide at various time points and subjecting to flow cytometry analysis. When BMA64-1A+pCM242+pCM252 yeast cells were released from α-factor arrest, as anticipated they had a 1N complement of DNA straight after release (Fig. 2.8, pCM252), which correlates with an arrest in G_1. 120 minutes after release from α-factor arrest, the majority of cells had replicated their DNA and had a DNA complement of 2N. After 180 minutes, most cells had undergone cell division and had a 1N complement of DNA. In contrast, when BMA64-1A+pCM242+ pCM252-ExoS was released from α-factor arrest, most cells failed to replicate their DNA and reach a 2N DNA complement even after 180 minutes (Fig. 2.8, ExoS). Expression of the GAP mutant of ExoS caused the same inhibition of DNA synthesis as the wild type ExoS (Fig. 2.8, GAPM). When ExoS with a mutated ADPRT domain in either the presence

of absence of a mutated GAP domain was expressed in BMA64-1A+pCM242 (Fig. 2.8, ADPRTM and G+AM), DNA replication proceeded as normal. Therefore, ExoS inhibited DNA replication during S phase in *S. cerevisiae* and the ADPRT domain was responsible for this phenotype.

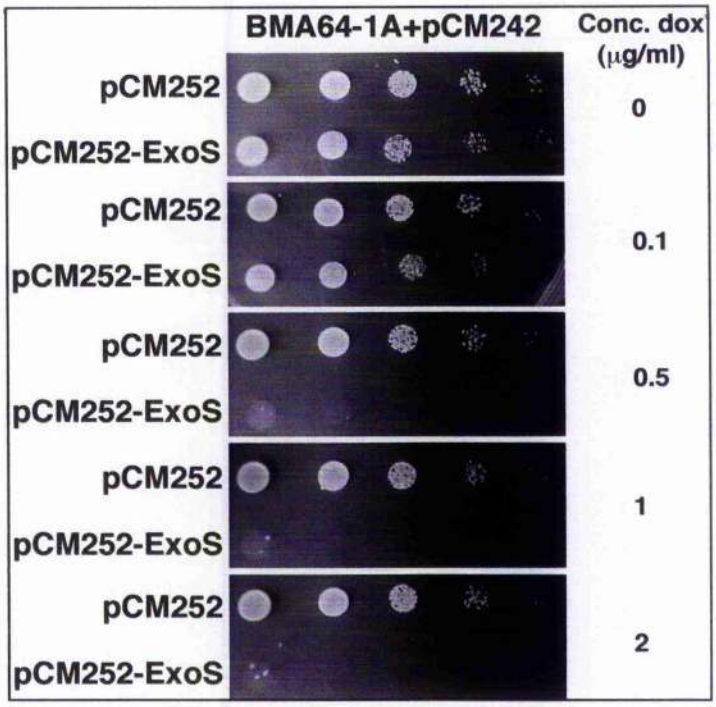


Fig. 2.7. Toxicity of ExoS in BMA64-1A.

BMA64-1A with pCM242 integrated into its *leu2* locus was transformed with the empty vector pCM252 or this vector encoding ExoS. Toxicity of each construct to BMA64-1A was assessed by spotting ten-fold serial dilutions of culture onto agar containing varying concentrations of doxycycline and assessing growth after three days.

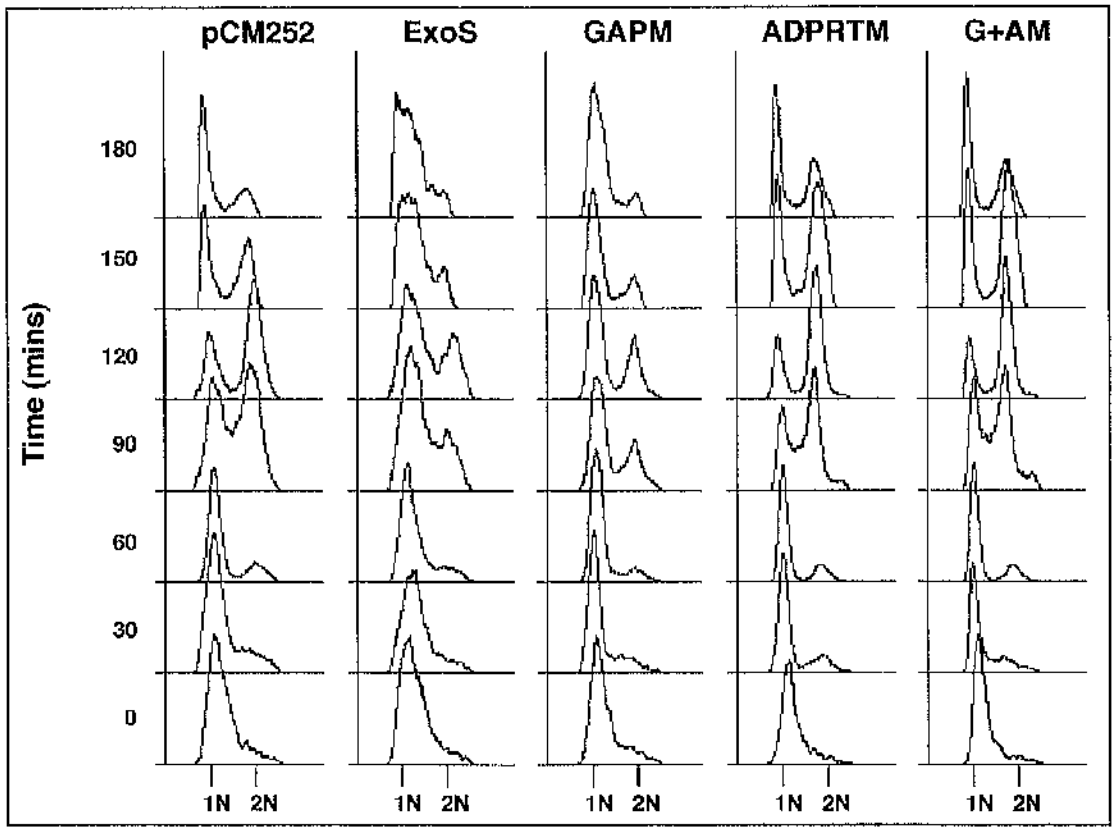


Fig. 2.8. Effect of ExoS on DNA synthesis in *S. cerevisiae*.

BMA64-1A containing pCM242 and either the pCM252 empty vector (pCM252) or pCM252 encoding ExoS (exoS), the GAP mutant of ExoS (GAPM), the ADPRT mutant of ExoS (ADPRTM) or the GAP and ADPRT mutant of ExoS (G+AM) were arrested at G_1 with α-factor, induced with doxycycline and released from α-factor arrest. At various time points the cells were fixed and stained with propidium iodide and subjected to flow cytometry analysis. 1N and 2N indicate the DNA complement of the cells.

2.3.5. ExoS increases the number of mating projections formed by α-factor arrest and prevents normal bud growth after release from α-factor arrest.

As we have previously demonstrated, ExoS disrupts the actin cytoskeleton of *S. cerevisiae* and the ADPRT domain is responsible for this phenotype. In order to gain a better understanding as to how ExoS disrupts the actin cytoskeleton of yeast, we examined the effect of ExoS following release of cells from growth arrest with α-factor.

BMA64-1A+pCM242+pCM252 and BMA64-1A+pCM252+pCM252-ExoS were arrested in G_1 using α-factor and ExoS expression was induced by the addition of doxycycline. The cells were released from α-factor arrest and their actin cytoskeleton was observed at various time points by immunofluorescent staining with phalloidin (Fig. 2.9.). Immediately after release from α-factor arrest, mating projections were visible in *S. cerevisiae* both expressing and not expressing ExoS (Fig. 2.9, 0). However, the number of cells with multiple mating projections appeared higher when ExoS was expressed in the yeast. In order to quantify this observation, we counted the number of BMA64-1A+pCM242+pCM252 and BMA64-1A+pCM252+pCM252-ExoS cells with 0, 1, 2 or 3 mating projections (no cell had more than three mating projections visible) immediately after release from α-factor arrest (Fig. 2.10). Values are the average percentage of cells with 0, 1, 2 or 3 mating projections from 3 random field of view. Only mating projections extending in the plane of focus could be counted confidently, therefore the number of mating projections per cell is probably an under-estimate. However, the values provide a reliable relative measure of mating projection number. The results indicate that *S. cerevisiae* expressing ExoS do have more mating projections than yeast that do not express the toxin. There were far fewer cells with no visible mating projections when ExoS was expressed in *S. cerevisiae* compared to yeast not expressing the toxin (21.16±4.24% v. 60.07±3.23%, Fig. 2.10, 0). Similar numbers of cells possessed one mating projection in yeast expressing and not expressing ExoS (49.42±8.01% v. 39.05±3.21%, Fig. 2.10, 1). Many more cells expressing ExoS had two mating projections compared to yeast not expressing the toxin (27.35±5.50% v. 0.88±0.89%, Fig. 2.10, 2). There were no *S. cerevisiae* cells not expressing ExoS with three mating projections, whereas a small number of cells expressing ExoS had three mating projections (2.07±0.39%, Fig. 2.10, 3). A chi-squared test comparing the

number of mating projections produced by yeast expressing or not expressing ExoS revealed that the values were significantly different ($p<0.001$).

In addition to enhancing the number of mating projections per cell during α-factor arrest, ExoS also disrupted the actin cytoskeleton and bud formation in released cells. Upon release from α-factor arrest, the actin cytoskeleton did polarise in yeast expressing ExoS and if anything, this polarisation was more pronounced than in *S. cerevisiae* not expressing the toxin (Fig. 2.9, 30). However, bud formation did not proceed as normal. Yeast cells that did not express ExoS exhibited small round buds containing cortical patches attached to large mother cells with actin fibres travelling between the mother and bud (Fig. 2.9, pCM252 60-120). In contrast, *S. cerevisiae* expressing ExoS, exhibited a strange bud morphology (Fig. 2.9, pCM252-ExoS 60-120). After the very marked immediate polarisation, buds appeared to form at the end of elongated projections. These buds did not appear to be able to detach from the mother cell and as a consequence large buds attached to the mother cells by elongated necks were observed.

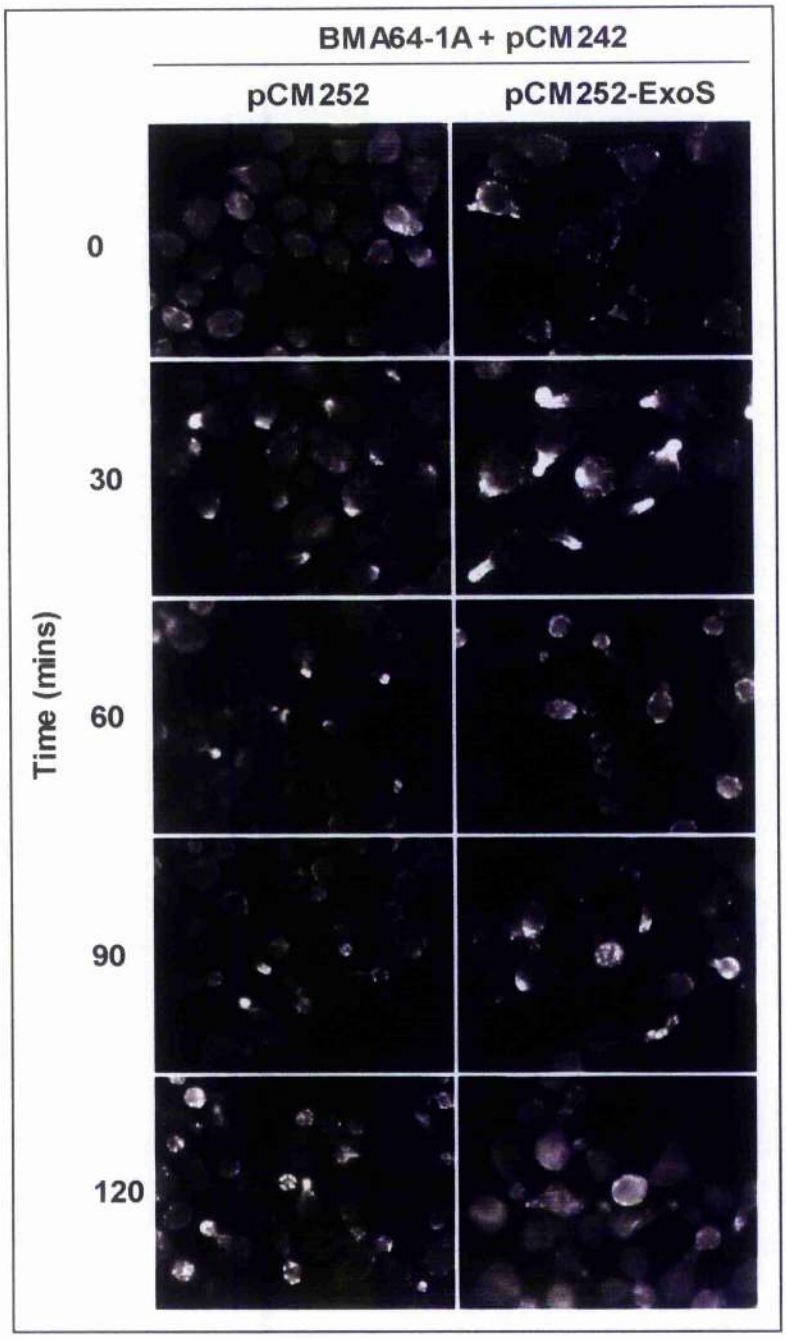


Fig. 2.9. Effect of ExoS on the actin cytoskeleton of *S. cerevisiae* after α-factor arrest.

BMA64-1A containing pCM242 and either the pCM252 empty vector or pCM252 encoding ExoS were arrested at G_1 with α-factor, induced with doxycycline and released from α-factor arrest. At various time points the cells were fixed and immunofluorescent staining of the actin cytoskeleton with phalloidin was carried out.

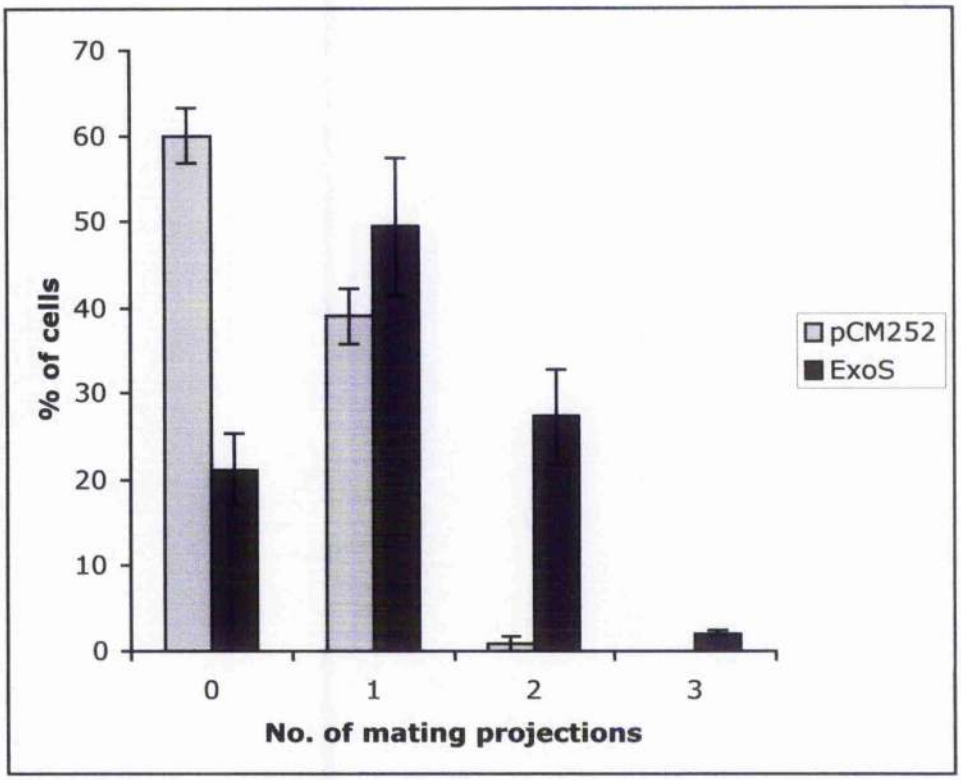


Fig. 2.10. Effect of ExoS on the number of mating projections.

A graph showing the percentage of BMA64-1A+pCM242 containing pCM252 or pCM252-ExoS cells with 0, 1, 2 or 3 mating projections after 3 h α-factor arrest and 1 h ExoS induction. Values are the average of three fields of view and each field of view contained at least 50 cells. The error bars represent standard deviation of the mean.

2.4. DISCUSSION

In this chapter, we have developed a *S. cerevisiae* model in which to study the pseudomonal TTS toxin, ExoS. We have used this model to show that ExoS is very toxic to yeast and that although both the GAP and ADPRT domains are toxic, the ADPRT domain accounts for the extreme toxicity of ExoS. We have demonstrated that ExoS disrupts the actin cytoskeleton and inhibits DNA synthesis in yeast and that the ADPRT domain is responsible for these phenotypes. We have also shown that ExoS increases the number of mating projections formed when *S. cerevisiae* is arrested with α-factor and that ExoS interferes with the normal bud development after release from α-factor.

In developing the yeast model to study ExoS, we began by controlling ExoS expression using the *GAL1* promoter system. Other groups have successfully used this system to study the effects of TTS toxins on *S. cerevisiae*. For example, the *P. aeruginosa* TTS toxin genes *exoT* and *exoU* have been successfully cloned downstream of the *GAL1* promoter and expressed upon galactose induction in yeast ([413, 437, 439] and Chapter 3). However, in our case the galactose inducible system was inadequate because of the extreme cytotoxicity of the ADPRT domain of ExoS. Constructs expressing ExoS with a mutated ADPRT domain were successfully transformed into yeast. However, when we tried to transform *S. cerevisiae* with a plasmid expressing ExoS with an active ADPRT domain we obtained no transformants. It is evident that the ADPRT domain of ExoS was so cytotoxic to *S. cerevisiae* that even when its expression was repressed by glucose, enough leaky expression from the *GAL1* promoter resulted to prevent the transformation of yeast with this plasmid. We tried to overcome this problem by cloning *exoS* into a low copy number plasmid (pYC2/NT), but again we were unable to transform *S. cerevisiae* with this construct under glucose repressing conditions. The only transformants we generated contained mutations of the *exoS* gene that either truncated the protein or mutated the ADPRT domain in such a way that probably resulted in the abrogation of its enzymatic activity.

In an attempt to overcome this problem of leaky expression from the *GAL1* promoter in glucose repressing conditions, we investigated the use of another inducible expression system, the tetracycline-regulated activator-repressor dual system [494]. This system

(described in section 2.3.2 and Fig. 2.3.) uses a strong repressor of the $tetO_7$ promoter to ensure that there is a complete switching-off of the regulated genes in the absence of tetracycline. It has been demonstrated that the *tetR*-Ssn6 repressor decreases expression of a *lacZ* control gene under the control of the $tetO_7$ promoter to virtually undetectable levels (≤0.10 Miller units of β-galactosidase activity) [494]. This is compared to the *GAL1* promoter system, which only represses β-galactosidase activity to 2 Miller units when repressed by growth on glucose [494]. In addition to its tight repression, the tetracycline-regulated activator-repressor dual system is also capable of inducing expression of regulated genes to high levels. For example, maximal *lacZ* expression levels from this system are 10-fold higher than with the *MET3*-based promoter system (a system that is induced by the removal of methionine) and 70% of that achieved by the *GAL1*-based promoter system [494, 495]. Controlling gene expression by the addition (or removal) of tetracycline has another advantage over the *GAL*-based and *MET3*-based systems. The *GAL*- and *MET3*-based systems require a nutrient change to confer regulation, either a switch from glucose to galactose or the removal of methionine, and these are likely to have pleiotrophic effects on the yeasts metabolism. Thus, it is hard to confirm which phenotypes are due to the regulated gene and which are the consequence of the nutrient change. In contrast, doxycycline has no effect on the growth rate, cell morphology or global gene expression of *S. cerevisiae* [496], so any phenotypic change will be the result of the regulated gene. We decided to use the tetracycline-inducible version of the tetracycline-regulated activator-repressor dual system as this enables rapid gene induction without the requirement for a media change. It has been demonstrated that this system can induce detectable levels of protein expression by 30 min after antibiotic addition [494].

Using the tetracycline-inducible activator-repressor dual system we were able to successfully transform *S. cerevisiae* with constructs containing *exoS* and the GAP and ADPRT mutants of *exoS*. Using this system we confirmed the implication from the *GAL1*-promoter system, that ExoS is very toxic to yeast and that the ADPRT domain is responsible for this extreme toxicity. We also confirmed that, as shown with the *GAL1*-promoter system, the GAP domain is toxic to *S. cerevisiae* but not as toxic as the ADPRT domain. That the ADPRT domain is more toxic than the GAP domain of ExoS is in agreement with what has been shown using mammalian tissue culture cells. The ADPRT domain causes death of mammalian epithelial cells and macrophages

whereas the GAP domain is not toxic when expressed or injected into these cells [378, 394, 395].

The ability of the GAP domain to elicit cytotoxicity in *S. cerevisiae,* but not mammalian cells, is reminiscent of the effects of other TTS GAP toxins on these cells. The *Yersinia* TTS GAP toxin, YopE, and the GAP domain of the *P. aeruginosa* toxin, ExoT, are also cytotoxic to yeast but not mammalian cells [261, 413, 484]. This difference is probably due to the different types of assays used to measure cytotoxicity in mammalian and yeast cells. In the mammalian cell systems, toxicity has been measured as a decrease in reporter gene expression, an increase in trypan blue uptake or an increase in release of the cytoplasmic protein lactate dehydrogenase [378, 394], whereas cytotoxicity in yeast is measured as an inhibition of growth. Thus, although unable to directly destroy a cell, the GAP domains of ExoS, ExoT and YopE may cause cytotoxicity by inhibiting cell growth. It has been proposed that the high susceptibility of yeast cells to YopE expression is the result of disruption of the actin cytoskeleton triggering a morphogeneis checkpoint in the cell-cycle that leads to an arrest in nuclear division [484]. Although we observed no disruption of the actin cytoskeleton by the GAP domain of ExoS (see below), its ability to inhibit growth suggests that it does affect the yeast in some way and therefore may also trigger a cell cycle arrest checkpoint.

The extreme toxicity of the ADPRT domain to yeast that we observed, combined with the ability of this domain to destroy mammalian cells suggests that ADPRT cytotoxicity is direct and not a result of growth inhibition. It is unclear what the mechanism of action of ADPRT cytotoxicity is. Although many potential *in vivo* targets of the ExoS ADPRT domain are known, it is not known which of these are biologically relevant and how their ADP-ribosylation results in cell death. Our results suggest however that the protein(s) whose ADP-ribosylation results in cell death is likely to be conserved from yeast to mammals. It is also unclear why the ADPRT domain of ExoS should be so cytotoxic to yeast. It is evident that the pseudomonal TTS phospholipase ExoU, which is a more potent cytotoxin to mammalian cells, is not as toxic to *S. cerevisiae* as ExoS. Whereas we were unable to transform yeast with *exoS* under the control of the *GAL1* promoter, *exoU* can be transformed into yeast using this system ([437, 439] and Chapter 3). Therefore, the small amount of leaky expression of ExoU from the *GAL1* promoter under glucose repressing conditions is not sufficient to kill the yeast, whereas the same

amount of ExoS is. It is also clear that the ADPRT domain of ExoT is not as toxic to *S. cerevisiae* as the ADPRT domain of ExoS. The ADPRT domain of ExoT is cytotoxic to yeast but *exoT* under the control of the *GAL1* promoter can be transformed into *S. cerevisiae* [413]. This difference in yeast susceptibility to the ADPRT domains of ExoS and ExoT correlates with what is observed in mammalian cells where ExoS is toxic and ExoT is not [352, 412]. It was originally thought that the ADPRT domain of ExoT possessed only about 0.2% of the activity of the ADPRT domain of ExoS and that this difference accounted for the difference in cytotoxicity [346]. However it has since been shown that ExoT is able to efficiently ADP-ribosylate Crk-I and Crk-II both *in vitro* and *in vivo* [415]. As discussed in Chapter 1, the ability of ExoT to ADP-ribosylate Crk-I and Crk-II provides a possible reason for the actin cytoskeleton disruption phenotype caused by this domain. Yeast however does not contain a Crk homologue and other targets of the ExoT ADPRT domain must therefore account for the toxicity of this protein to *S. cerevisiae*. It is possible that like the GAP domain of YopE, the ADPRT domain of ExoT is cytotoxic to yeast because it inhibits growth by disrupting actin, which triggers a morphogenesis checkpoint.

When we expressed wild type ExoS in *S. cerevisiae*, it severely disrupted the actin cytoskeleton leading to the formation of large aggregates of densely stained cortical patches and thick disorganised actin cables. Although budding cells were still present, these did not have a polarised actin cytoskeleton as cortical patches were still present in the mother cell and actin cables did not run between the mother cell and bud. Analysis of the GAP and ADPRT mutants revealed that the ADPRT domain was responsible for this dramatic phenotype and the GAP domain had no visible effect on the actin structures in *S. cerevisiae*.

The ability of the ADPRT domain of ExoS to disrupt the actin cytoskeleton in *S. cerevisiae* parallels what is observed in mammalian cell culture systems where the ADPRT domain has been shown to cause cell morphology changes in both epithelial and macrophage cell lines [378, 395]. Although it is unclear what the targets of the ADPRT domain are that result in this cytoskeletal disruption, a number of hypothesis have been proposed. The ability of ExoS to ADP-ribosylate the Rho GTPases Rac and Cdc42 *in vivo* [372, 373] may provide a direct link between ExoS expression and actin disruption because these proteins play a direct role in the regulation of actin cytoskeletal

rearrangements. Also ExoS ADP-ribosylates the Ezrin/Radixin/Moesin (ERM) family of proteins *in vivo* [376]. The ERM family of proteins contribute to actin dynamics and therefore may explain the disruption of the actin cytoskeleton by the ADPRT domain of ExoS. Finally the ADP-ribosylation of Ras and RalA by ExoS may be responsible for the cytoskeletal changes as these proteins are known to indirectly affect the morphology of eukaryotic cells [497].

Although it is not clear how the ADPRT domain of ExoS leads to the observed disruption of the actin structures in *S. cerevisiae*, a clue to its possible mechanism of action comes from the yeast V159N actin mutant [498]. When actin with a valine 159 to asparagine mutation is expressed in *S. cerevisiae* as the sole source of actin the resulting phenotype is very similar to that which we observed after expression of the ADPRT domain of ExoS. Belmont and Drubin [498] described the yeast expressing V159A actin as having " a dramatically altered actin cytoskeleton...the cortical patches are much brighter and many cells appear to have more pronounced cables." They also observe cells with " additional patches in both the mother and bud," " depolarised actin or actin structures that are not clearly identifiable as cortical patches or cables" and " excess cables and these are not properly organised. These cables frequently extend at right angles to the mother-bud axis, rather than running along the mother-bud axis as they do in wild type strains." The V159N actin mutation results in these phenotypes because it forms actin filaments that are exceptionally stable due to their slow depolymerisation. ATP-bound actin monomers polymerise to form filamentous actin, the ATP is then hydrolysed and the release of inorganic phosphate leads to a conformational change that destabilises the actin filament and promotes disassembly [499]. The V159N mutation results in actin that depolymerises slowly because the filamentous actin fails to undergo a conformation change after inorganic phosphate release [500]. The similarity of the V159N actin and ExoS ADPRT domain induced actin disruption suggests that the ADPRT domain of ExoS may also stabilise filamentous actin in some way.

Expression of the *Salmonella* TTS SipA protein in *S. cerevisiae* also results in a phenotype that is reminiscent of the V159N actin mutation phenotype and our ExoS ADPRT domain induced actin disruption [484]. SipA is able to inhibit actin depolymerisation both *in vitro* and in *S. cerevisiae* and it is thought to do this directly by

binding to the actin [268, 484]. ExoS does not bind actin, therefore if it does stabilise filamentous actin it must do so indirectly. One possible mechanism for actin stabilisation by ExoS is through the ADP-ribosylation and activation of Rho. In mammalian cells, Rho is able to activate the proteins mDia and ROCK [497]. Activated mDia binds and activates profilin that enhances actin polymerisation. Activated ROCK phosphorylates LIM kinase that in turn phosphorylates cofilin. Cofilin acts to depolymerise actin but when it is phosphorylated by LIM kinase, its actin-depolymerising activity is inhibited. Therefore, activated Rho can both enhance actin polymerisation and inhibit actin depolymerisation, which in mammalian cells results in stress fibre formation [497]. Yeast also contains profilin and cofilin that accelerate actin polymerisation and increase the rate of actin filament depolymerisation respectively. If the V159N actin mutant is expressed in a profilin or cofilin mutant background, the resulting *S. cerevisiae* mutant is not viable indicating that actin turnover is required for yeast survival [498]. Thus, if ExoS was able to ADP-ribosylate and activate Rho in *S. cerevisiae*, the resulting activation of profilin and inactivation of cofilin may lead in the observed actin disruption phenotype and cell death. ExoS is able to ADP-ribosylate the Rho GTPases Rho Rac and Cdc42 *in vitro* and Rac and Cdc42 in mammalian cells [372, 373]. Although there is little evidence that ExoS ADP-ribosylates Rho in mammalian cells, this may be different in *S. cerevisiae*. Although in the majority of instances ADP-ribosylation by ExoS results in inactivation of the target protein, it has been demonstrated that ADP-ribosylation of Rac1 by ExoS leads to its activation [373, 406]. ExoS preferentially ADP-ribosylates either arginine 66 or 68 in the Switch II domain of Rac1 and this appears to interfere with the GAP-mediated inactivation of Rac1 [406]. Therefore, if ExoS ADP-ribosylation of Rho occurs in *S. cerevisiae* and this leads to its activation, the resulting activation of profilin and inactivation of cofilin might account for the ability of the ExoS ADPRT domain to disrupt the actin cytoskeleton in yeast.

In our experiment, the GAP domain of ExoS did not disrupt the actin cytoskeleton of *S. cerevisiae*. The role of the ExoS GAP domain in mammalian cells in actin disruption is controversial. In the epithelial cell line HT-29, the GAP domain of ExoS does not appear to have any effect on cell morphology whereas in the macrophage cell line J774A.1, the GAP domain was found to exert an antiphagocytic function [378, 395]. This difference in activity of the GAP domain in different cell lines may be due to

differential localisation of the Rho GTPases. For example in the epithelial cell line HT-29, Rac1 resides primarily within the cytosol and appears to be ADP-ribosylated and activated at this location [406]. In contrast, Rac1 in the J774A.1 macrophage is more localised to the plasma membrane and at this location it appears to be inactivated by the GAP domain of ExoS [406]. The *Yersinia* TTS GAP toxin, YopE, does disrupt the actin cytoskeleton of *S. cerevisiae* and this is believed to trigger a morphogenesis checkpoint that results in cell-cycle inhibition and account for the toxicity of this protein towards yeast [484]. The observation that ExoS did not lead to actin disruption in our experiment suggests that the GAP activities of ExoS and YopE are not functionally interchangeable. Although the *in vitro* GAP activity of ExoS and YopE are biochemically indistinguishable they may be a difference in their *in vivo* targets. It has been demonstrated that the GAP domain of ExoS targets RhoA, Rac1 and Cdc42 *in vivo* but YopE appears to only act as a GAP for RhoA and Rac1 *in vivo* [262, 405, 501]. This difference in *in vivo* targets may be caused by differential localisation of the two toxins or differences in their catalytic domains. ExoS and YopE localise to the same perinuclear region in mammalian cells and their membrane localisation domains are functional interchangeable suggesting that a difference in the catalytic domain of ExoS and YopE dictates their *in vivo* substrate specificity [502]. Although we did not observe any change in the actin cytoskeleton of *S. cerevisiae* upon induction of ExoS GAP expression, the ability of the GAP domain to inhibit yeast growth suggests that the GAP domain is active in yeast. It is possible that the GAP domain of ExoS disrupted the actin cytoskeleton in yeast in a way that was too subtle to be detected by our assay or that it acts in a different way to inhibit growth. In addition to being involved in regulating the actin cytoskeleton, the Rho GTPases also have a role in modulating gene transcription and it may be this activity that explains the cytotoxic action of the GAP domain of ExoS [497].

Our results also showed that ExoS expression led to the formation of more mating projections after α-factor treatment and the disruption of normal bud formation after α-factor release. Although we did not test which domain of ExoS caused these phenotypes, the ability of the ADPRT to disrupt the actin cytoskeleton in vegetatively growing yeast suggests that this domain is responsible. As described in the introduction to this chapter, Cdc42 is the central player in polarising growth in *S. cerevisiae* and if ExoS was able to ADP-ribosylate and activate this Rho GTPase, this may account for

the phenotypes we observed after α-factor treatment. When *MATa* yeast are treated with a high concentration of the α-factor pheromone they initiate and terminate growth of mating projections with regular periodicity [503]. It has been demonstrated that the regulators of Cdc42 activity control the initiation of mating projection formation [504]. Mutation of Cdc24, the GEF for Cdc42, results in a longer mating projection initiation period and mutation of Bem3, which is a GAP for Cdc42, leads to a shorter mating projection period [504]. Therefore, it is apparent that Cdc42 activation by its GEF, Cdc24, initiates mating projection formation and this initiation is inhibited by the inactivation of Cdc42 by its GAP, Bem3. As detailed above, ExoS has been shown to ADP-ribosylate Rac1 and Cdc42 *in vivo* and ADP-ribosylation of Rac1 by ExoS activates this Rho GTPase. One could therefore speculate that ExoS ADP-ribosylates and activates Cdc42 in *S. cerevisiae* leading to an increase in the initiation of α-factor induced mating projection formation.

If ExoS was able to activate Cdc42 by ADP-ribosylation, this may also provide an explanation for the strange bud growth observed after α-factor release in yeast expressing ExoS. ExoS expression resulted in many *S. cerevisiae* cells with large buds and elongated necks. Although we have been unable to discover in the literature any yeast mutants that have a similar phenotype, it is slightly reminiscent of the filamentous growth that results during cell stress [488]. During filamentous growth, Cdc42 does not redistribute over the bud surface during G_2-M but remains localised at the tip of the bud and directs further apical growth. Also constitutive activation of Cdc42 or loss of its GAPs, Bem3 and Rga1, locks Cdc42 into a polarised distribution and hyperpolarises growth [505, 506]. Therefore if ExoS activated Cdc42 by ADP-ribosylation this may lead to the strange bud growth phenotypes we observed. There is however, no evidence that ADP-ribosylation activates Cdc42 and the ability of the ADPRT domain of ExoS to interfere with filopodium formation (a Cdc42 controlled process) in macrophages, suggests that ADP-ribosylation actually inactivates Cdc42 [378]. Thus, how the ADPRT domain of ExoS causes more mating projections and disrupts bud formation remains unclear.

When we released *S. cerevisiae* from α-factor arrest, yeast not expressing an active ADPRT domain of ExoS were able to exit G_1 and proceed with DNA synthesis in S phase. However, when ExoS with an active ADPRT domain was expressed in yeast, no

DNA synthesis was observed. This finding is consistent with what has been observed in mammalian cells where ExoS inhibits DNA synthesis and the ADPRT domain was shown to be responsible for this phenotype [351, 395]. It is not clear which target(s) of the ExoS ADPRT domain is responsible for this inhibition of DNA synthesis. Ras is integral to signal transduction pathways that affect DNA synthesis so may be the target [497]. GTP bound Ras binds and activates its effector, Raf. Raf is a protein kinase that induces gene expression through the MAPK cascade and this drives the cell cycle. Therefore, inhibition of Ras by ADP-ribosylation may result in the observed inhibition of DNA synthesis. It has been demonstrated that ADP-ribosylation of Ras by ExoS is not required for the cytotoxicity of this protein [368]. Therefore, if inhibition of DNA synthesis is the trigger for ExoS induced cell death, another protein that is involved in inducing DNA synthesis must be inactivated by ExoS ADP-ribosylation. It is known that Ral is involved in signalling processes that affect cell proliferation, Rap1 can induce DNA synthesis and that Rho/Rac/Cdc42 proteins are required for G_1 cell cycle progression [497]. As Rap1, Rap2, RalA, Rac and Cdc42 have all been shown to be *in vivo* targets of the ADPRT domain of ExoS [372, 373], inhibition of any of these proteins by ADP-ribosylation may also lead to the observed inhibition of DNA synthesis.

To summarise, in this chapter we have demonstrated that *S. cerevisiae* is a useful model in which to study the effects of ExoS as the effects of this toxin on cell viability, the actin cytoskeleton and DNA synthesis mirrors that observed in mammalian cells. The extreme toxicity of the ADPRT domain of ExoS towards yeast led us to use the tetracycline-inducible activator-repressor dual system to study the effects of this toxin, the first time this system has been used to study toxin expression in *S. cerevisiae*. In addition to the stronger repression of transcription afforded by this system, we believe that it offers further advantages as it does not require a nutrient change for regulation, it is rapidly induced and expression levels can be regulated by doxycycline concentrations. It remains unclear how the ExoS ADPRT domain acts to kill cells, disrupt the actin cytoskeleton and inhibit DNA synthesis but *S. cerevisiae* may provide a useful system in which to examine these questions due to the ease at which this simple eukaryote can be manipulated. The striking actin disruption phenotype and the similarity of this to the V159N actin mutant phenotype is particularly interesting and might provide further insight into the molecular mechanism of action of ExoS.

CHAPTER 3: SCREENING THE *SACCHAROMYCES CEREVISIAE* DELETION LIBRARY FOR MUTANTS RESISTANT TO EXOENZYME S, Y OR U.

3.1. INTRODUCTION

In the introduction to Chapter 2 the advantages of using *Saccharomyces cerevisiae* as a model in which to study bacterial toxins were highlighted. Our results concerning the ability of ExoS to elicit cytotoxicity towards yeast and the parallels between the effect of this toxin in both *S. cerevisiae* and mammalian cells, confirms the use of this simple eukaryote in studying the TTS toxins of *Pseudomonas aeruginosa*. In this chapter we aimed to develop the *S. cerevisiae* model to screen for eukaryotic genes required for action of the *P. aeruginosa* TTS toxins: ExoS, ExoY and ExoU.

As previously discussed, although many potential eukaryotic targets of ExoS have been identified, its precise mechanism of action remains unknown. Likewise, a great deal remains to be discovered about the other TTS toxins of *P. aeruginosa*. ExoY is known to exhibit adenylate cyclase activity that results in increased intracellular cAMP levels and causes actin disruption and cell rounding in target cells [465, 473]. However, it is unclear how high cAMP levels leads to actin disruption and whether there are any other consequences of the ExoY-induced increase in cAMP concentrations. For ExoU, it has been demonstrated that its cytotoxic nature is due to its phospholipase activity [439]. It is believed that ExoU kills cells by destroying the plasma membrane of target cells although it remains possible that the phospholipase activity of ExoU results in cell death by a different mechanism.

In addition to the uncertainties of the precise mechanisms of action of the pseudomonal TTS toxins, the identities of some of their eukaryotic cofactors are still unknown. As discussed in Chapter 1, all the TTS toxins of *P. aeruginosa* require a eukaryotic cofactor to limit their catalytic activities to their eukaryotic host. *In vitro* the ADPRT activity of ExoS and ExoT, the adenylate cyclase activity of ExoY and the phospholipase activity of ExoU are all dependent on factors that can be supplied by mammalian or yeast cell supernatants [385, 439, 465]. It has been demonstrated that members of the 14-3-3 protein family are absolutely required for the ADPRT activity of

ExoS and ExoT [386, 415]. However, the factors required for stimulation of the adenylate cyclase activity of ExoY by 500-fold or absolutely required for the phospholipase activity of ExoU remain unknown.

As briefly mentioned in the introduction of Chapter 2, one of the advantages of using *S. cerevisiae* as a model to study bacterial toxins is the availability of a yeast deletion library [507]. By screening such a library for deletion mutants that are resistant to the cytotoxic effects of the pseudomonal TTS toxins, one might gain further insight into the molecular sights of action of the toxins within eukaryotic cells. This library screen might also be useful in identifying the eukaryotic cofactors required for the activity of ExoY and ExoU. If the eukaryotic cofactor for ExoY or ExoU is a non-redundant, non-essential protein, its deletion will make the yeast resistant to the action of ExoY or ExoU.

The *S. cerevisiae* deletion library is commercially available and the result of the *Saccharomyces* Genome Deletion Project consortium. Sequencing of the *S. cerevisiae* genome revealed the presence of approximately 6200 ORFs and the aim of the consortium was to generate as complete a set as possible of yeast deletion strains. Four different mutant collections were generated; haploids of both mating types, homozygous diploids for non-essential genes, and heterozygous diploids, which contain deletions in the essential and non-essential ORFs. To date, the *Saccharomyces* Genome Deletion Project consortium has succeeded in disrupting 96% of the ORFs [507].

The method used in the construction of the deletion library was a PCR-based gene deletion strategy that generated a start- to stop- codon deletion of each ORF. Each ORF was disrupted by a deletion cassette containing a *KanMX4* module and two unique tag sequences that allow identification of the deleted ORF (Fig. 3.1, A). The deletion cassettes were constructed by amplifying the *KanMX4* gene from pFA6-kanMX4 with unique 74 bp UPTAG and 74 bp DOWNTAG primers (Fig. 3.1, A, Round 1 PCR). The unique 74 bp UPTAG primer consists of (5' to 3'): 18 bp of genomic sequence that flanks the 5' end of the targeted ORF directly proximal to the start codon, 18 bp of sequence common to all gene deletions (U1), a 20 bp unique sequence tag (TAG1) and 18 bp of sequence homologous to the 5' end of the *KanMX4* cassette (U2). The unique DOWNTAG primer consists of (5' to 3''): 18 bp of genomic sequence that flanks the 3'

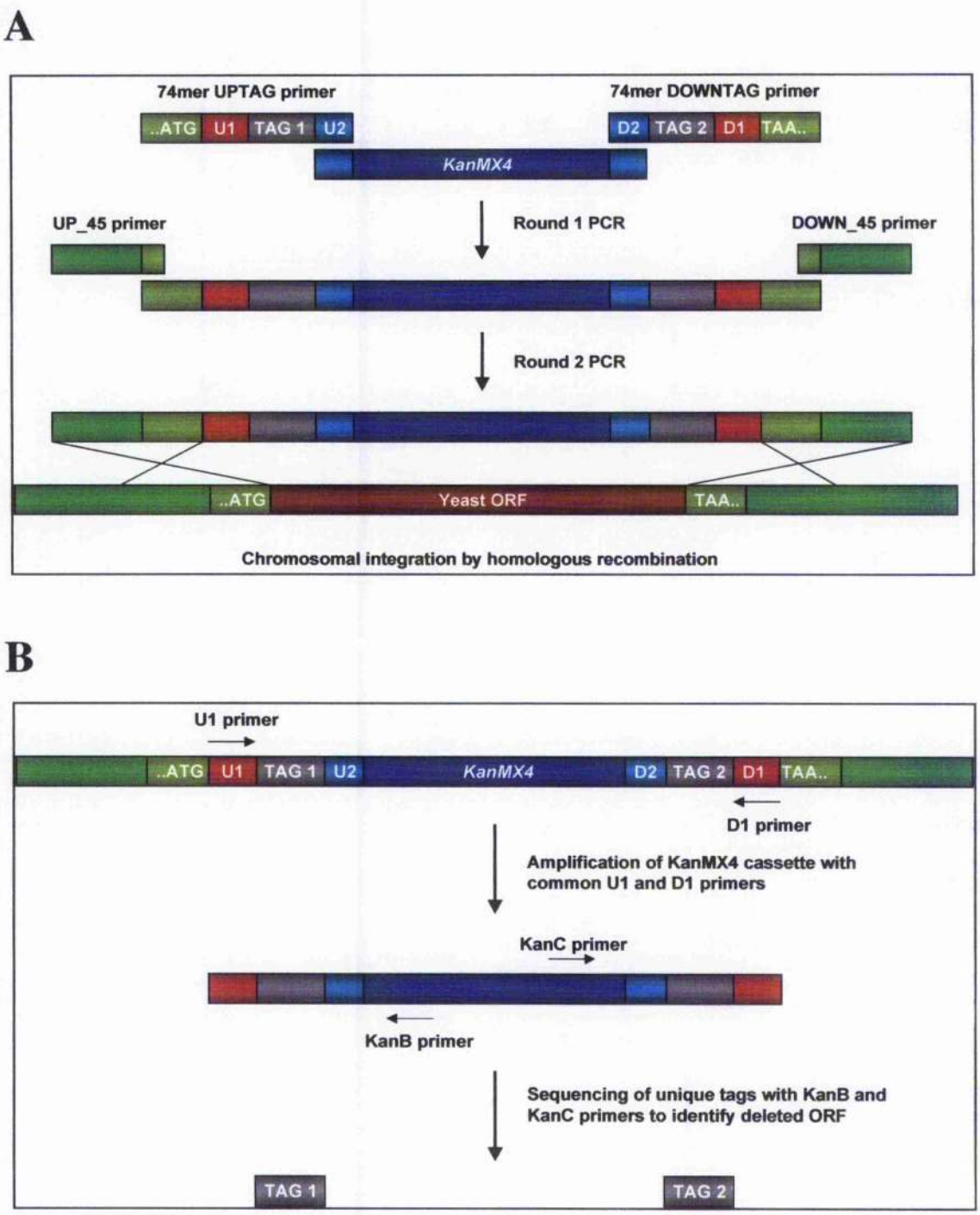


Fig. 3.1. Construction and identification of *S. cerevisiae* deletion mutants

(A) Schematic diagram detailing the generation of the *S. cerevisiae* deletion mutants using a PCR-based gene deletion strategy that generated a start- to stop- codon deletion of each ORF. **(B)** Schematic representation of the strategy used to identify the *S. cerevisiae* deletion mutant. Details of the approach are described in the text.

end of the targeted ORF directly distal to the stop codon, 17 bp of sequence common to all gene deletions (D1), a 20 bp unique sequence tag (TAG2) and 19 bp of sequence homologous to the 3' end of the *KanMX4* cassette (D2). A second round of PCR amplification was then carried out using UP_45 and DOWN_45 primers (Fig. 3.1, A, Round 2 PCR). These primers were designed to be complementary to the 45 bp directly upstream and downstream of the targeted ORF including the start and stop codon respectively. The gene disruption cassette was transformed into the diploid yeast cells, BY4743, and colonies were selected on G418 containing agar plates. Thus, only colonies that had successfully integrated the *KanMX4* cassette into the chromosome by homologous recombination were able to grow. The resulting transformants were sporulated and haploids of both mating types, *MAT*a and *MAT*α, were recovered from the tetrads. If the dissection of a tetrad resulted in two viable and two dead colonies, the deleted gene was deemed "essential." The success of the replacement of the ORF with the gene deletion cassette was confirmed by a number of PCR amplification reactions. The homozygous diploid deletion strain was then constructed by mating of the confirmed haploid deletion strains.

The presence of the unique sequence tags in the gene deletion cassette allows identification of mutants with a particular phenotype, for example resistance to a bacterial toxin. The deletion cassette is amplified from the selected strains using the common U1 and D1 primers and the unique tags (TAG1 and TAG2) are sequenced using the KanB and KanC primers (Fig. 3.1, B). The tag sequences are then run through a programme maintained by the *Saccharomyces* Genome Deletion Project to identify the deleted ORF (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html).

There are a number of advantages of using this *S. cerevisiae* deletion library over classical random mutagenesis for large-scale screens. The first is that each mutant phenotype reflects a complete loss of function of the gene. Secondly, the deleted gene responsible for the mutant phenotype can be rapidly identified because it has been " bar-coded" during the construction of the deletion strain. Finally, as all of the ORFs have been systematically deleted, one can ensure genome saturation in contrast to random mutagenesis where some genes elude deletion. To date, the *S. cerevisiae* library has been used for a number of genome wide screens. During the construction of the *S.*

cerevisiae deletion library, all the essential genes for this simple eukaryote were identified [507]. Out of the 5916 genes deleted, 1105 (18.7%) were demonstrated to be essential for growth on rich medium. The deletion library has since been used to show which genes are necessary for optimal growth under a variety conditions including: rich medium, high salt, sorbitol, galactose, pH 8, minimal medium and nystatin treatment [507]. The library has also been used to identify genes required for mitochondrial function, sporulation and meiosis [508, 509]. A heterozygous diploid library of strains that contains a deletion in one copy of its essential and non-essential ORFs has been used to test for drug targets [510]. If a drug targets a specific protein it follows that if the amount of this protein is decreased, due to the presence of just one gene encoding for it, the yeast may be more sensitive to the toxic effects of the drug. The *S. cerevisiae* deletion library has also recently been used to identify potential targets of the *P. aeruginosa* toxin pyocyanin [511]. Yeast is sensitive to the oxidative stress caused by the increased levels of H_2O_2 and O_2^- triggered by pyocyanin and the yeast deletion library was screened for mutants that were either more sensitive or more resistant to this toxin. 50 genes were identified that exhibited altered sensitivity to pyocyanin including multiple V-ATPase mutants that showed increased susceptibility to pyocyanin. It was subsequently shown that pyocyanin inactivates human V-ATPases in lung epithelial cells, confirming that these pyocyanin targets are conserved from yeast to humans.

The work described in this chapter describes how we used the *S. cerevisiae* homozygous diploid deletion library to screen for eukaryotic genes required for the action of the *P. aeruginosa* TTS toxins ExoS, ExoY and ExoU. Before embarking on the screen we established the sensitivity of *S. cerevisiae* to the pseudomonal TTS toxins. We had already shown in Chapter 2 that ExoS was toxic to *S. cerevisiae*, so we confirmed that ExoU was toxic and illustrated for the first time that yeast are also susceptible to ExoY. After an initial whole library screen, the deletion mutants isolated as being resistant to the toxins were re-tested. Most of the identified deletion mutants were shown to be false positives and were probably initially isolated due to a mutation in the pseudomonal toxin gene. Yeast mutants that did confer resistance to the TTS toxins were strains with either the *GAL3* or *GAL4* genes deleted or strains lacking the gene encoding the transcription elongation factor, Spt4.

3.2. METHODS

3.2.1. Materials

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Griener Bio-One (Kremsmuenster, Austria) supplied the plasticware unless otherwise stated. The primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA) and DNA sequencing was performed by the Dundee University Sequencing Service (Dundee, UK).

3.2.2. *S. cerevisiae* strains

In addition to the INVSc1 and BY4741 strains used previously (Section 2.2.2), yeast deletion clones generated by the *Saccharomyces* Genome Deletion Project were used. The Yeast Deletion Homozygous Diploid Pool (Invitrogen, Carlsbad, CA, USA) contains deletion mutants in the BY4743 background (*MATa/MATα; his3Δ1/ his3Δ1; leu2Δ0/ leu2Δ0; met15Δ0/ met15Δ0; ura3Δ0/ ura3Δ0*) and the individual deletion mutants used (Euroscarf, Frankfurt, Germany) were in the BY4741 background.

3.2.3. Maintenance and growth of *S. cerevisiae*

Maintenance and growth of *S. cerevisiae* was identical to that described previously (Section 2.2.3) but in addition the yeast deletion clones were grown in the presence of 200 μg/ml Geneticin® (G418) to maintain selection on the *KanMX4* deletion cassettes.

3.2.4. Genomic DNA extraction from *S. cerevisiae*

Genomic DNA was extracted from 1.5 ml of overnight culture of *S. cerevisiae* using the YeaStar Genomic DNA Kit™ (Zymo Research, Orange, CA, USA) according to Protocol I of the manufacturers instructions. In summary, the cells were centrifuged at 500 x g for 2 min and the supernatant was removed. The pellet was resuspended in 120 μl YD Digestion Buffer and 5 μl RNascA-Zymolyase™ and incubated at 37°C for 60 min. 120 μl YD Lysis Buffer was added and the mixture was vortexed hard for 15 s. The lysed yeast were mixed for 1 min with 250 μl chloroform before being centrifuged

at 16100 x g for 2 min. The supernatant was loaded onto a Zymo-spin III column and centrifuged at 16100 x g for 1 min. The column was washed twice with 300 µl DNA Wash Buffer and the DNA was eluted by adding 60 µl DDW to the column membrane, incubating for 1 min then centrifuging at 16100 x g for 10 s.

3.2.5. Construction of plasmids

This study utilised the galactose-inducible expression vector pYES2/NT (Invitrogen) and the constitutive expression vector pAD4M (kindly supplied by Dr. K. Haynes, Imperial College, London). pYES2/NT contains the *URA3* gene and pAD4M contains the *LEU2* gene, therefore *S. cerevisiae* transformed with these plasmids were selected for on SD media lacking uracil and leucine respectively.

The pYES2/NT-ExoS plasmid generated previously (Chapter 2) was used and pYES2/NT-ExoY and pYES2/NT-ExoU were made. The *exoY* gene was amplified from PA01 genomic DNA using the same PCR reaction mixture and conditions as described for the amplification of *exoS* (Section 2.2.5) with the following changes: the primers ExoY-Fw and ExoY-Rev (see Table 3.1) were used and the PCR amplification cycle involved 35 cycles with an annealing temperature of 62°C and an extension time of 1.5 min. The amplified *exoY* gene was then cloned into the *EcoR*I and *Not*I sites of pYES2/NTC. The *exoU* gene was amplified from PA103 genomic DNA using the same PCR reaction mixture and conditions as described for the amplification of *exoS* (Section 2.2.5) with the following changes: the primers ExoU-Fw and ExoU-Rev (see Table 3.1) were used and the PCR amplification cycle involved 35 cycles with an annealing temperature of 62°C and an extension time of 2 min. The amplified *exoU* gene was then cloned into the *BamH*I and *Not*I sites of pYES2/NTC. The sequence of the *exoY* gene was confirmed by sequencing with the T7prom and CYC1R (Table 2.2) primers and the sequence of the *exoU* gene was confirmed by sequencing with the T7prom and CYC1R primers in addition to ExoU-Int1-4 primers (Table 3.1).

The *SPT4* gene and the YGR064W ORF were amplified from 1 µl INVSc1 genomic DNA (prepared as described in section 3.2.4) using 2.5 U of *PfuTurbo*® Hotstart DNA polymerase (Stratagene, La Jolla, CA. USA), 1 x cloned *Pfu* DNA polymerase reaction buffer (Stratagene), 25 mM of each dNTP, 1 pmole/µl Spt4-Fw and 1 pmole/µl Spt4-

Rev primers or 1 pmole/µl YGR064W-Fw and 1 pmole/µl YGR064W-Rev primers (Table 3.1) in a total volume of 100 µl. A touchdown PCR amplification programme was employed to decrease non-specific priming and comprised of: an initial denaturing cycle of 94°C for 5 min, 31 cycles of a denaturing step of 94°C for 30 s, an annealing step for 30 s and an extension step at 72°C for 30 s. In the first 6 cycles the annealing temperature was 60°C, over the next 9 cycles the annealing temperature dropped 1°C a cycle to 51°C and the final 16 cycles had an annealing temperature of 50°C. The amplification reaction was then completed with a final extension cycle of 72°C for 10 min. After cloning *SPT4* and YGR064W into the *Sal*I and *Sac*I sites of pAD4M, their sequences were confirmed by sequencing with the ADH1prom primer (Table 3.1).

Table 3.1. Primers

PRIMER	SEQUENCE (5'-3')
ExoY-Fw	CGGAATTCATGCGTATCGACGGTCATCGT
ExoY-Rev	TCGCGGCCGCTCAGACCTTACGTTGGAAAAAGTC
ExoU-Fw	CGGGATCCATGCATATCCAATCGTTGGGG
ExoU-Rev	TCGCGGCCGCTCATGTGAACTCCTTATTCCGCCA
ExoU-Int1	GCGTTTCAGCAGTCCCCAAGG
ExoU-Int2	GGCGTTCAAGACCCTTTC
ExoU-Int3	CGGTTGAGTGCTTACATTCC
ExoU-Int4	GCGAGCAAACCGTTGTGG
Spt4-Fw	GCAGTCGACCGAACGAGGTACAGTGTAAGAGATGTC
Spt4-Rev	TAGAGCTCCGGAAGGTTTTACTCAACTTGACTGC
YGR064W-Fw	GCAGTCGACCAGCTATACTATGATCTACGCTCAGCC
YGR064W_Rev	TAGAGCTCGGTCCTCGTAGTCCAATTTACGTG
ADH1prom	CCTTCATTCACGCACACTACTC
U1	GATGTCCACGAGGTCTCT
D1	CGGTGTCGGTCTCGTAG
KanB	CTGCAGCGAGGAGCCGTAAT
KanC3	CCTCGACATCATCTGCCCAGAT

3.2.6. Growth assay

INVSc1 and BY4741 were transformed with pYES2/NT-ExoY and pYES2/NT-ExoU using the Frozen-EZ Yeast Transformation II™ Kit (Zymo Research) as described in section 2.2.7. The effect of ExoY and ExoU on the growth of *S. cerevisiae* was then assessed on SD-uracil+glucose and SD-uracil+galactose/raffinose agar plates as described in section 2.2.10.

The effect of the ExoS GAP domain, ExoY or ExoU on *S. cerevisiae* deletion mutants identified in the *Saccharomyces* deletion mutant library screen (see below) was determined by transforming the individual deletion mutants with the relevant pYES2/NT plasmid and assessing their growth on SD-uracil+glucose+G418 and SD-uracil+galactose/raffinose+G418 agar plates as described in section 2.2.10.

To determine whether deletion of the *SPT4* gene or the YGR064W ORF accounted for the resistance of the YGR064W *S. cerevisiae* deletion strain to the exoenzymes, this strain was transformed sequentially with pYES2/NT-ExoS and pAD4M-Spt4 or pAD4M-YGR064W using the Frozen-EZ Yeast Transformation II™ Kit (Zymo Research) as described in section 2.2.7. Growth was then assessed on SD-uracil-leucine+glucose+G418 and SD-uracil-leucine+galactose/raffinose+G418 agar plates as described in section 2.2.10.

3.2.7. S. *cerevisiae* deletion library screen

The Yeast Deletion Homozygous Diploid Pool (Invitrogen) was transformed with pYES2/NT-ExoS, -ExoY, or -ExoU using a scaled up protocol for the Frozen-EZ Yeast Transformation II™ Kit (Zymo Research). A 0.5 ml aliquot of the *S. cerevisiae* deletion library was defrosted and mixed with 4.5 ml YPD media containing 200 µg/ml G418 and grown with shaking at 30°C for 4 h. The yeast were pelleted at 500 x g for 4 min at room temperature, washed in 5 ml EZ1 solution and resuspended in 0.5 ml EZ2 solution. 50 µl of the pYES2/NT-ExoS, -ExoY or ExoU plasmid and 5 ml EZ-3 solution was mixed with the cells and the reaction was incubated for 2 h at 30°C with occasional mixing. A 100 µl aliquot of the transformation reaction was plated on a 20 ml SD-uracil+glucose+G418 agar plate and the remaining yeast suspension was split between three 100 ml SD-uracil+galactose/raffinose+G418 agar plates. The plates were incubated at 30°C for up to 14 days.

3.2.8. Identification of *S. cerevisiae* deletion mutants

S. cerevisiae deletion mutants able to grow in the presence of ExoS, ExoY, or ExoU were re-streaked on SD-uracil+galactose/raffinose+G418 agar plates and grown at 30°C for 2 days. The genomic DNA from these mutants was purified from an overnight

culture as described in section 3.2.4. The gene deletion cassette was amplified from 2 µl genomic DNA using 5 U Taq DNA polymerase, 1 x PCR buffer, 25 mM of each dNTP, 1 µM U1 and 1 µM D1 primers (Table 3.1) in a total volume of 100 µl. The mixture was subjected to one denaturing cycle of 5 min at 94°C and then 35 cycles using the following conditions: a 30 s denaturing step at 94°C, followed by a 45 s annealing step at 56°C and a 2 min extension step at 72°C, and completed by a final extension cycle of 10 min at 72°C. The amplified gene deletion cassette was purified by precipitating with polyethylene glycol (PEG) to remove the primers. The 100 µl PCR amplification reaction was mixed with 100 µl 26% PEG 8000, 6.5 mM $MgCl_2$, 0.6 M NaAc, pH 6.5 and incubated at room temperature for 10 min. The mixture was centrifuged at 16100 x g for 10 min and the pellet was washed twice in 100% ethanol. The pellet was then air dried for 15 min and resuspended in 20 µl DDW. The unique sequence tags in the gene deletion cassette were sequenced using the KanB and KanC3 primers (Table 3.1) and the identity of the mutant determined by searching the *Saccharomyces* Genome Deletion Project database for the unique sequence tags.

3.3 RESULTS

3.3.1. Use of a galactose inducible expression system to assess the toxicity of ExoY and ExoU in *S. cerevisiae*

In order to utilise the *S. cerevisiae* deletion library to identify deletion mutants able to grow in the presence of ExoS, ExoY or ExoU, we needed to express the toxins using the galactose inducible expression plasmid pYES2/NT. We were unable to use the tetracycline inducible expression plasmid pCM252 because the selection for this plasmid is the ability it confers on yeast to be able to grow in the absence of tryptophan. The *S. cerevisiae* deletion library was constructed in the diploid strain BY4743 and this strain is not a tryptophan auxotroph, thus pCM252 transformants cannot be selected. Even though ExoS is highly toxic to yeast and the pYES2/NT-ExoS plasmid cannot be transformed into wild type yeast even under glucose repressing conditions (see Chapter 2), this construct could be utilised in the library screen because *S. cerevisiae* mutants able to grow in the presence of ExoS were being screened for.

The *exoY* gene was amplified from PA01 genomic DNA and ligated in-frame into pYES2/NTC. Sequence analysis revealed that there were four nucleotide changes between our *exoY* gene and the database sequence. These changes occurred in *exoY* from a number of separate amplification reactions confirming that they were the result of allelic differences and not errors during amplification. Two of the nucleotide changes were silent (531 A-G and 1080 C-T). The other two nucleotide changes (472 G-C and 958 G-C) resulted in substitution of a valine with a leucine at amino acid residues 158 and 320 respectively.

The *exoU* gene was amplified from PA103 and ligated in frame into pYES2/NTC. Sequence analysis of amplified *exoU* revealed that, compared to the database sequence, the cysteine at position 1340 is substituted with a thymidine. This substitution resulted in a change of the amino acid residue 447 from a proline to a leucine. The occurrence of this nucleotide change in *exoU* from a number of different amplification reactions confirmed that it was an allelic change.

A pre-requisite for the *S. cerevisiae* deletion screen was that the toxins ExoS, ExoY and ExoU exhibited toxicity to wild type yeast. We had already established that ExoS is extremely cytotoxic to *S. cerevisiae* (see Chapter 2) but we needed to determine whether ExoY and ExoU were also toxic to yeast.

When ExoY expression was induced in INVSc1 or BY4741 by growing the yeast transformed with pYES2/NT-ExoY on agar containing galactose and raffinose, yeast cell death occurred (Fig. 3.2, ExoY Gal/Raf). Growth of the yeast strains containing pYES2/NT-ExoY on glucose was also slightly inhibited compared to the yeast transformed with the empty pYES2/NT vector (Fig. 3.2, ExoY Glu). These results established that ExoY was cytotoxic to *S. cerevisiae* and that the small amount of leaky expression from the *GAL1* promoter under glucose repressing conditions is sufficient to cause a small amount of cell death. The ability to transform yeast with the pYES2/NT-ExoY construct indicated that ExoY was not as toxic to *S. cerevisiae* as ExoS.

When INVSc1 or BY4741 containing pYES2/NT-ExoU were spotted onto solid media supplemented with galactose and raffinose they were unable to grow (Fig. 3.2, ExoU Gal/Raf). Growth of these strains containing pYES2/NT-ExoU was also inhibited to a lesser degree on glucose containing solid media (Fig. 3.2, ExoU Glu). Therefore, ExoU was toxic to *S. cerevisiae* although not as toxic as ExoS.

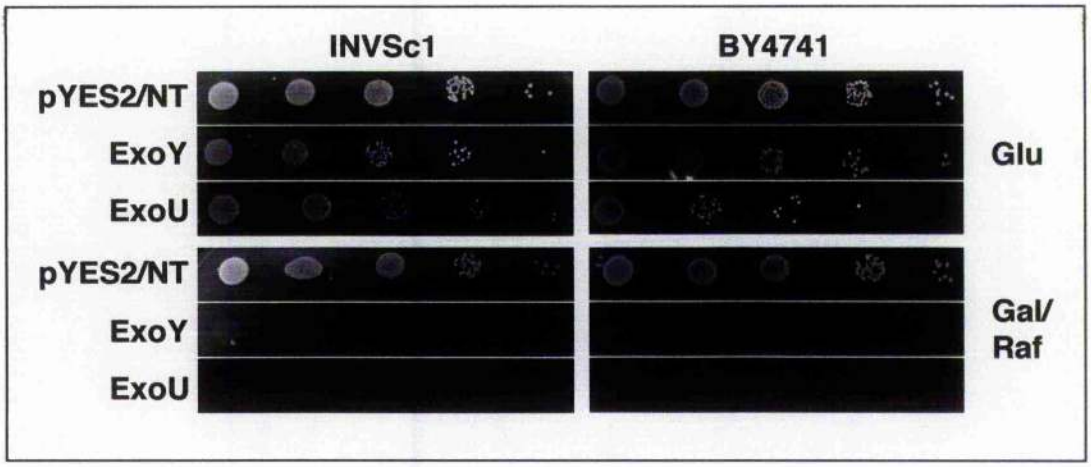


Fig. 3.2. Toxicity of ExoY and ExoU in *S. cerevisiae*.

A diploid and haploid strain of *S. cerevisiae* (INVSc1 and BY47471 respectively) were transformed with the empty pYES2/NT vector (pYES2/NT), this vector encoding ExoY and this vector encoding ExoU. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

3.3.2. Use of the *S. cerevisiae* deletion library to screen for ExoS resistant mutants

In order to identify any yeast deletion mutants able to grow in the presence of ExoS, the *S. cerevisiae* deletion library was transformed with pYES2/NT-ExoS and the transformants were plated onto galactose containing agar. Any colonies that appeared were selected and their genomic DNA isolated. Amplification of the *KanMX* cassette and sequencing of the unique tags was carried out to reveal the identity of the mutants (Fig. 3.1, B). At each stage in the identification process some of the mutants failed to be identified; for example, amplification of the *KanMX* cassette or sequencing of the unique tag did not always work. Table 3.2 summarises the information on the colonies that grew after transformation with pYES2/NT-ExoS. Information on the number of days after which the colony appeared, the success of subsequent growth of the colony and the success of the amplification of the *KanMX* cassette and sequencing of the unique tag is listed. For deletion mutants that were successfully identified, the deleted ORF and the gene, if known, is recorded.

The *S. cerevisiae* deletion library screen for mutants able to grow in the presence of ExoS identified 18 possible candidate mutants. To test whether these deletion mutants were really resistant to the cytotoxic effects of ExoS, the individual deletion mutants were transformed with pYES2/NT-ExoS to assess their growth. The only deletion mutant strain that we were able to transform with pYES2/NT-ExoS was the strain with the YGR064W ORF deleted (see section 3.3.5 and Fig. 3.6). All the other deletion mutants failed to be transformed with pYES2/NT-ExoS, suggesting that they were not resistant to the effects of the ADPRT domain of ExoS even when its expression was repressed by glucose. To determine whether these deletion mutants were resistant to the GAP activity of ExoS, we transformed them with pYES2/NT-ExoS_ADPRTM and grew them on galactose containing media. All the deletion mutants, except the mutant with YGR064W deleted (see section 3.3.5), exhibited the same sensitivity to pYES2/NT-ExoS_ADPRTM when induced by galactose as the wild type BY4741 strain did (Fig. 3.3, representative results).

The library coverage for a screen such as this can be calculated by counting the number of colonies from a proportion of the transformation reaction that grow on non-inducing, glucose containing media. We previously demonstrated that ExoS is so cytotoxic to

yeast that it is impossible to grow *S. cerevisiae* containing *exoS* under the control of the *GAL1* promoter on glucose. Therefore it was not possible to calculate the coverage of the library for this screen.

Table 3.2. *S. cerevisiae* mutants identified in ExoS screen.

	Day	Growth	Amplification	Sequencing	ORF	Gene
1	3	Y	Y	Y	YNL285W	UNKNOWN
2	3	Y	Y	Y	YML131W	UNKNOWN
3	3	Y	N			
4	3	Y	N			
5	3	Y	N			
6	3	Y	Y	N		
7	3	Y	Y	Y	YMR224C	*MRE11*
8	3	Y	Y	Y	YKR078W	UNKNOWN
9	3	Y	N			
10	3	Y	N			
11	3	Y	Y	N		
12	3	Y	Y	Y	YDL206W	UNKNOWN
13	3	Y	Y	Y	YOL058W	*ARG1*
14	3	Y	N			
15	3	Y	N			
16	3	Y	Y	Y	YGR057C	*LST7*
17	3	Y	Y	Y	YOR123C	*LEO1*
18	3	Y	Y	N		
19	3	Y	Y	Y	YJL058C	*BIT1*
20	3	Y	Y	Y	YGL049C	*TIF4632*
21	3	Y	Y	Y	YGR064W	UNKNOWN
22	5	Y	Y	Y	YPL145C	*KES1*
23	5	Y	Y	Y	YKL106W	*AAT1*
24	5	Y	Y	Y	YNL111C	*CYB5*
25	5	Y	N			
26	5	Y	Y	Y	YHR126C	UNKNOWN
27	5	Y	N			
28	5	Y	Y	Y	YLR363C	*NMD4*
29	10	Y	Y	Y	YGR210C	UNKNOWN
30	10	Y	Y	Y	YOL124C	*TRM11*

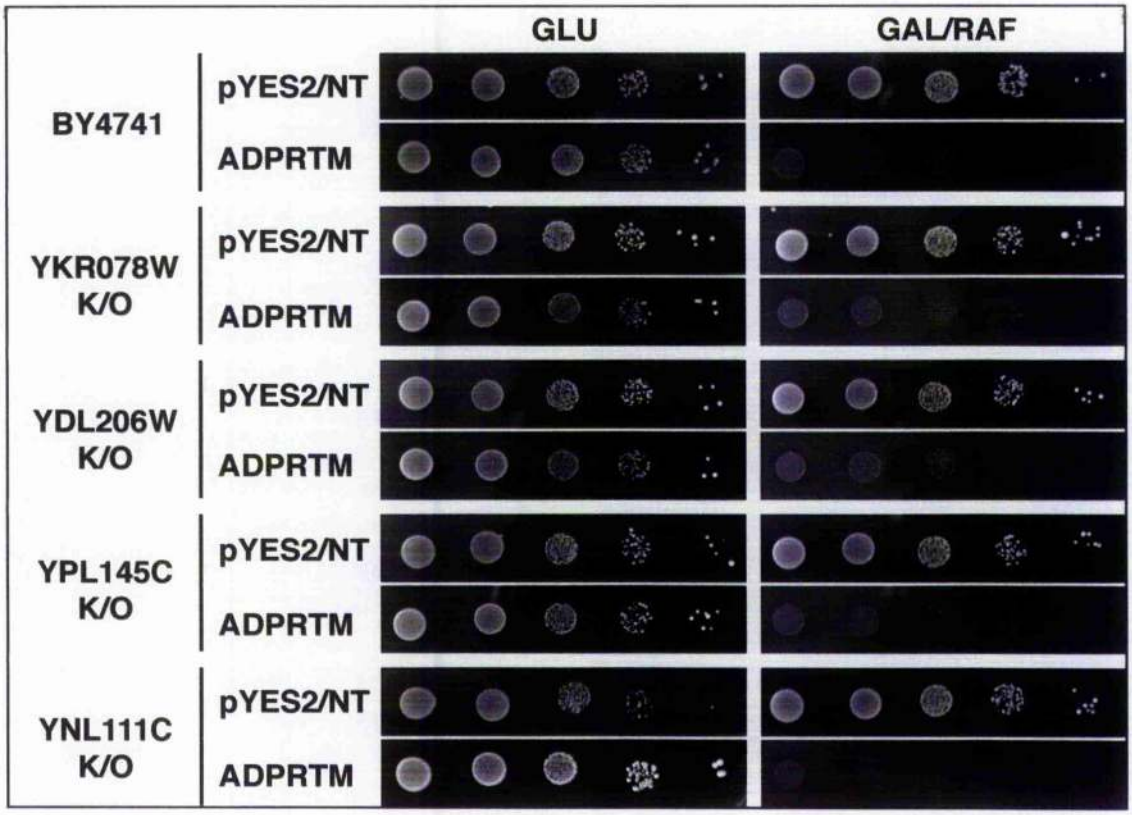


Fig. 3.3. Toxicity of the GAP domain of ExoS in a number of *S. cerevisiae* deletion mutants.

Wild type BY4741 and BY47441 with the YKR078W, YDL206W, YPL145C (*KES1*), or YNL111C (*CYB5*) ORFs deleted were transformed with the empty pYES2/NT vector (pYES2/NT) and this vector encoding the ADPRT mutant of ExoS. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

3.3.3. Use of the *S. cerevisiae* deletion library to screen for ExoY resistant mutants

The *S. cerevisiae* deletion library screen for mutants resistant to ExoY was carried out in an identical way to the ExoS screen. The results for this screen are summarised in Table 3.3. From the initial 30 colonies that grew, the identity of 21 of them was determined. Mutants with deletions in the *GAL3* or *GAL4* genes accounted for 7 of the identified mutants. It is likely that these mutants that have a non-functional galactose system were able to grow because they were not capable of using the galactose to induce expression of ExoY. The mutant with YGR064W deleted, which was also identified in the ExoS library screen, appeared 4 times in this screen. YGR064W overlaps a gene on the opposite DNA strand called *SPT4* and the mutant with this gene deleted was also identified twice in this screen. The final 8 deletion mutants identified were unique.

Individual strains of the 8 unique deletion mutants and the YGR064W deletion mutant were transformed with pYES2/NT-ExoY and their growth assessed when ExoY was induced by galactose. Again, it was only the YGR064W deletion mutant that was resistant to the effects of ExoY (see section 3.3.5 and Fig. 3.6) and the other deletion mutants were as sensitive to ExoY as wild type *S. cerevisiae* (Fig. 3.4).

An aliquot of 100 µl (out of a total of 5550 µl) of the transformation reaction of the *S. cerevisiae* deletion library transformed with pYES2/NT-ExoY was plated onto glucose containing agar. After 4 days (which is when the last colony from the library screen was picked) there were 234 colonies growing on the non-inducing plate. This corresponds to 12753 colonies being plated onto the library screen plates. There were 4741 individual clones in the *S. cerevisiae* deletion library. Therefore, this screen achieved a 2.69x coverage of the library. The recovery of a number of mutants (e.g. *GAL3*, *GAL4*, YGR064W and *SPT4*) more than once in this screen also confirms a good coverage.

Table 3.3. *S. cerevisiae* mutants identified in ExoY screen.

	Day	Growth	Amplification	Sequencing	ORF	Gene
1	3	Y	Y	Y	YGL096W	*TOS8*
2	3	Y	Y	N		
3	3	Y	Y	Y	YEL020C	UNKNOWN
4	3	Y	Y	Y	YCR010C	*ADY2/ATO1*
5	3	Y	Y	N		
6	3	Y	Y	Y	YNL323W	*BRE3/LEM3/ ROS3*
7	3	Y	Y	Y	YKL162C	UNKNOWN
8	3	Y	N			
9	4	Y	Y	N		
10	4	Y	Y	N		
11	4	Y	Y	Y	YPL248C	*GAL4*
12	4	Y	Y	Y	YHR127W	UNKNOWN
13	4	Y	Y	Y	YGR064W	UNKNOWN
14	4	Y	Y	N		
15	4	Y	Y	Y	YDR009W	*GAL3*
16	4	Y	Y	Y	YDR009W	*GAL3*
17	4	Y	Y	Y	YPL248C	*GAL4*
18	4	Y	Y	Y	YDR004W	*RAD57*
19	4	Y	Y	N		
20	4	Y	Y	Y	YGR063C	*SPT4*
21	4	N				
22	4	Y	Y	Y	YGR063C	*SPT4*
23	4	Y	Y	Y	YKL001C	*MET14*
24	4	Y	Y	Y	YGR064W	UNKNOWN
25	4	Y	Y	Y	YDR009W	*GAL3*
26	4	Y	Y	Y	YDR009W	*GAL3*
27	4	N				
28	4	Y	Y	Y	YGR064W	UNKNOWN
29	4	Y	Y	Y	YDR009W	*GAL3*
30	4	Y	Y	Y	YGR064W	UNKNOWN

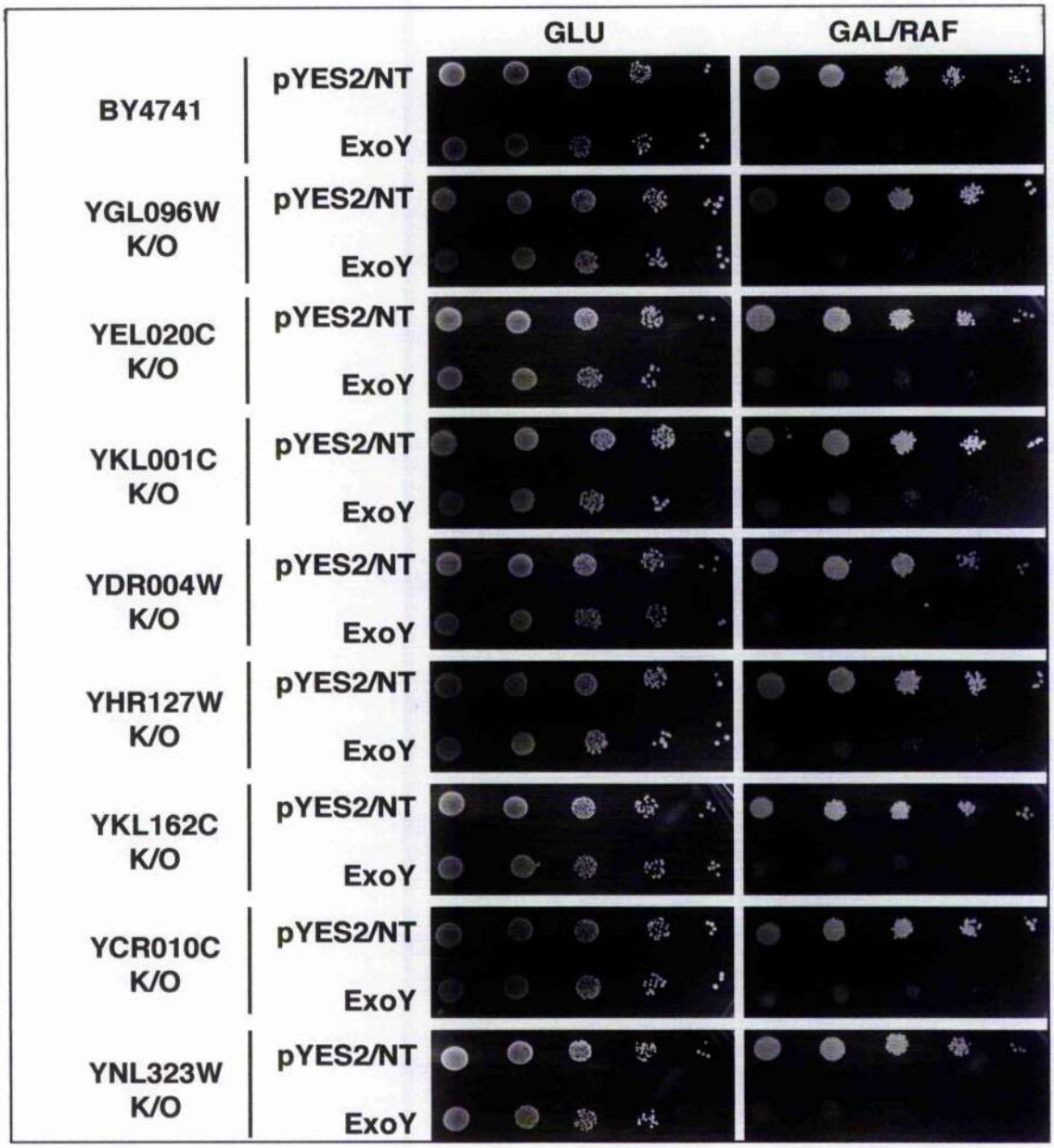


Fig. 3.4. Toxicity of the ExoY in a number of *S. cerevisiae* deletion mutants.
Wild type BY4741 and BY47441 with the YGL096W (*TOS8*), YEL020C, YKL001C (*MET14*), YDR004W (*RAD57*), YHR127W, YKL162C, YCR010C (*ADY2/ATO1*) or YNL323W (*BRE3/LEM3/ROS3*) ORFs deleted were transformed with the empty pYES2/NT vector (pYES2/NT) and this vector encoding ExoY. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

3.3.4. Use of the *S. cerevisiae* deletion library to screen for ExoU resistant mutants

Transforming the *S. cerevisiae* deletion library with pYES2/NT-ExoU yielded 10 colonies that were able to grow on the initial galactose plate. Of these 10 colonies, 8 were identified (Table 3.4, 1-10). Two of the mutants had the *GAL3* gene deleted and the other 6 represent unique deletion mutants. The library coverage for this screen was not very high. Only 65 colonies grew on the non-inducing glucose plate from 100 µl of transformation reaction, which correlates with a coverage of only 0.75x. In an attempt to increase the coverage, we repeated the library screen and achieved a coverage of 1.03x. This second library screen identified a further 5 colonies able to grow on galactose, of which 3 were identified (Table 3.4, A-E). Two of the mutants had a deletion in the *GAL3* gene and one had a novel deletion.

The 7 deletion mutants identified, that did not have the *GAL3* gene deleted, were examined individually to determine whether they were resistant to the cytotoxic effects of ExoU. When ExoU was induced in the deletion mutants by growth on media containing galactose, the cell death observed was the same level as that seen in the wild type *S. cerevisiae* (Fig. 3.5). Therefore none of the mutants identified in the screen are resistant to ExoU except for the *GAL3* mutant that is unable to synthesise ExoU.

Table 3.4. *S. cerevisiae* mutants identified in ExoU screen.

	Day	Growth	Amplification	Sequencing	ORF	Gene
1	4	Y	Y	Y	YOR088W	*YVC1*
2	4	Y	Y	Y	YPR062W	*FCY1*
3	4	Y	Y	Y	YGL096W	*TOS8*
4	7	Y	Y	Y	YJL051W	UNKNOWN
5	7	Y	Y	Y	YGR182C	UNKNOWN
6	8	Y	Y	Y	YDR009W	*GAL3*
7	8	Y	Y	Y	YDR009W	*GAL3*
8	8	Y	Y	Y	YLR461W	*PAU4*
9	11	N				
10	11	N				
A	7	Y	Y	N		
B	7	Y	Y	Y	YBR162W-A	*YSY6*
C	14	Y	Y	Y	YDR009W	*GAL3*
D	14	Y	N			
E	14	Y	Y	Y	YDR009W	*GAL3*

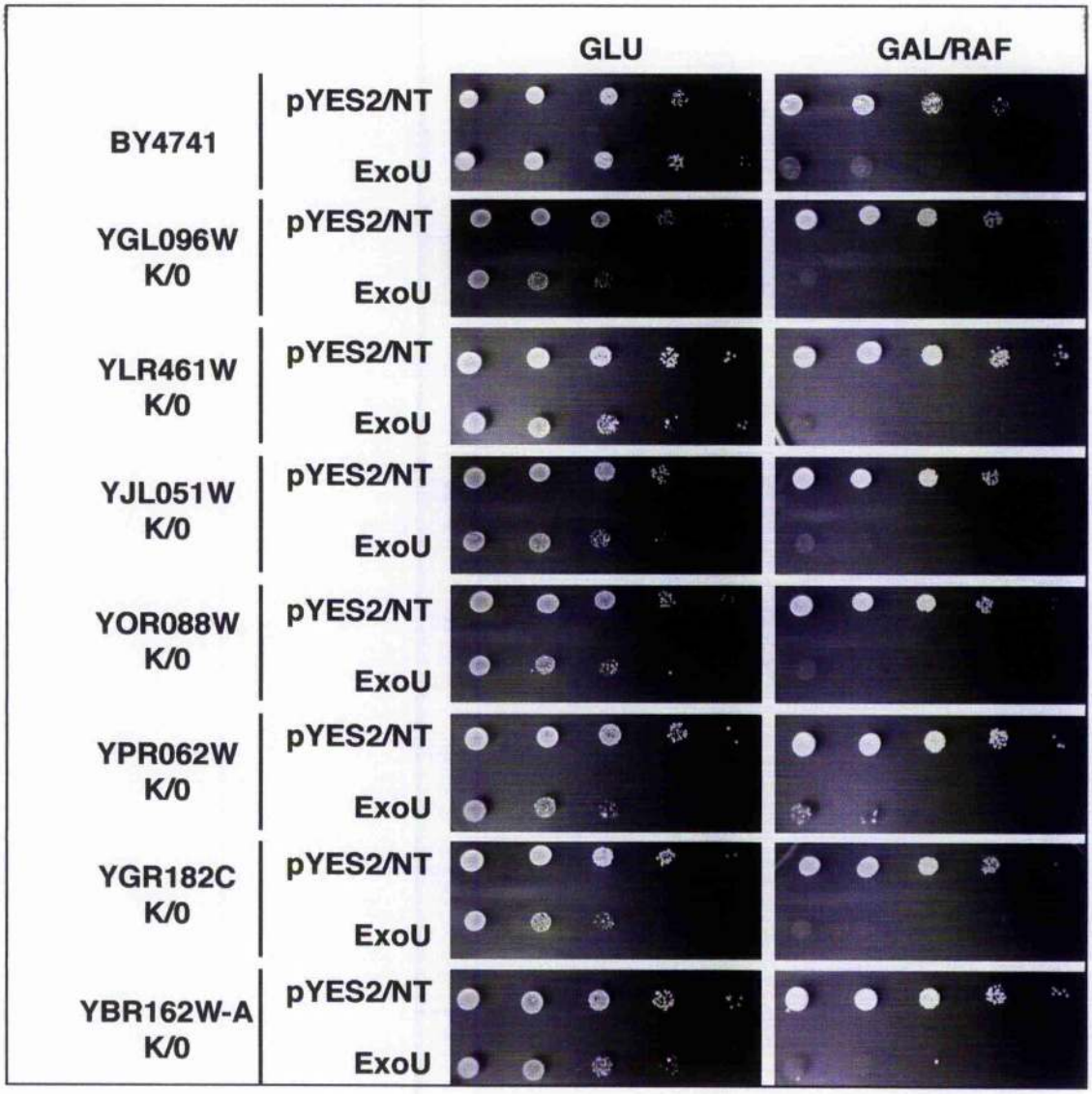


Fig. 3.5. Toxicity of the ExoU in a number of *S. cerevisiae* deletion mutants.
Wild type BY4741 and BY47441 with the YGL096W (TOS8), YLR461W (PAU4), YJL051W, YOR088W (YVC1), YPR062W (FCY1), YGR182C or YBR162W-A (YSY6) ORFs deleted were transformed with the empty pYES2/NT vector (pYES2/NT) and this vector encoding ExoU. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

3.3.5. The *S. cerevisiae SPT4* deletion mutant is resistant to the effects of ExoS, ExoY and ExoU

The *S. cerevisiae* deletion library screens for mutants able to grow in the presence of ExoS and ExoY both identified a strain with a deletion in the YGR064W ORF. The *SPT4* deletion mutant was also identified in the ExoY screen. YGR064W is a putative ORF that completely overlaps *SPT4* on the opposite DNA strand therefore deleting YGR064W would also cause *SPT4* to be deleted.

The screens suggested that deletion of the YGR064W/*SPT4* locus rendered *S. cerevisiae* resistant to the toxic affects of ExoS and ExoY. This was confirmed by transforming the YGR064W deletion mutant with pYES2/NT-ExoS or pYES2/NT-ExoY and growing the transformants on media containing galactose. The YGR064W mutant containing pYES2/NT-ExoS or pYES2/NT-ExoY grew equally well on glucose or galactose and raffinose containing agar and as well as the strain containing the empty pYES2/NT vector (Fig. 3.6, ExoS and ExoY).

The ExoU library screen did not identify the YGR064W/*SPT4* locus as being important in resisting the cytotoxicity of ExoU but the low library coverage of this screen may have caused it to be missed. We therefore examined the consequence of ExoU expression in the YGR064W deletion mutant. The YGR064W mutant was transformed with pYES2/NT-ExoU and varying dilutions were spotted on agar containing either glucose or galactose and raffinose. The YGR064W mutant containing pYES2/NT-ExoU grew equally well on glucose or galactose and raffinose and as well as the strain containing the empty pYES2/NT vector (Fig. 3.6, ExoU). Thus, deletion of the YGR064W/*SPT4* locus also renders *S. cerevisiae* resistant to ExoU.

In order to determine whether the deletion of the YGR064W putative ORF or *SPT4* was responsible for conferring resistance to ExoS, ExoY and ExoU, we attempted to complement the YGR064W deletion strain with constitutively expressed YGR064W or Spt4 to see which restored the ExoS sensitive phenotype to the strain. The YGR064W deletion mutant was transformed with pYES2/NT-ExoS and either the empty pAD4M vector or this vector expressing YGR064W or Spt4 constitutively from the *ADH1* promoter. Growth was then assessed on agar containing galactose. Complementing the

strain with YGR064W had no effect on yeast cell viability because the cells were still able to grow as well on galactose as yeast just containing pYES2/NT-ExoS (Fig. 3.7). However, when the YGR064W deletion mutant containing pYES2/NT-ExoS was complemented with constitutively expressed Spt4, some cell death did result during growth on galactose (Fig. 3.7). The ExoS sensitivity of *S. cerevisiae* was not fully restored to the YGR064W deletion mutant by Spt4 expression, but it was significantly more sensitive than the strain transformed with just the pYES2/NT-ExoS plasmid.

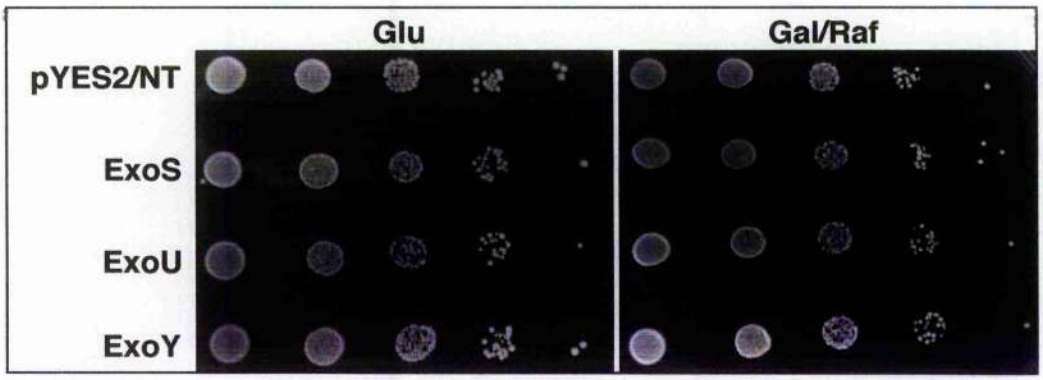


Fig. 3.6. Toxicity of ExoS, ExoU and ExoY in the YGR064W *S. cerevisiae* deletion mutant.

BY4741 with the YGR064W ORF deleted was transformed with the empty pYES2/NT vector (pYES2/NT) and this vector encoding ExoS, ExoU or ExoY. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

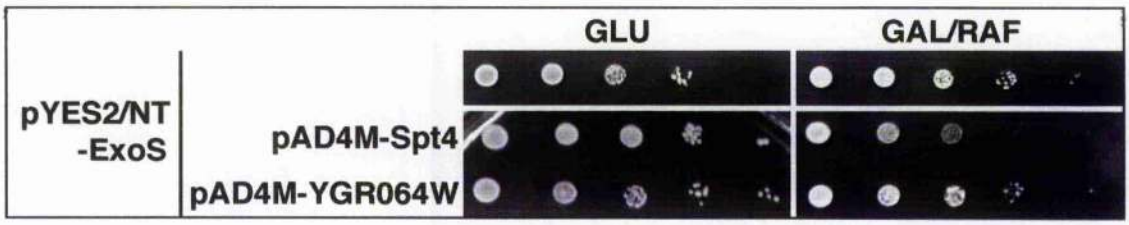


Fig. 3.7. Complementation of the toxin resistant phenotype in the YGR064W deletion mutant with Spt4 and YGR064W.

BY4741 with the YGR064W ORF deleted was transformed with only pYES2/NT-ExoS or pYES2/NT-ExoS and pAD4M-Spt4 or pAD4M-YGR064W. Growth of the transformants was assessed by spotting ten-fold serial dilutions of each culture onto repressing (Glu) agar or inducing (Gal/Raf) agar plates.

3.4. DISCUSSION

In this chapter we have demonstrated for the first time that ExoY is toxic to *S. cerevisiae* and confirmed the results of others that yeast is also sensitive to ExoU. We carried out a *S. cerevisiae* deletion library screen for mutants that were resistant to the toxic effects of ExoS, ExoY or ExoU and isolated a number of candidates. Unfortunately re-testing the possible resistant mutants revealed that only yeast with deletions in genes required for toxin expression from the *GAL1* promoter, *gal3*, *gal4*, *spt4* and YGRO64W deletion mutants (see below), were truly resistant. The other mutants that we identified in our initial screen probably arose by mutation of the toxin gene itself and therefore were not resistant when individually re-transformed with the toxin expressing plasmid.

Previous research has revealed that *S. cerevisiae* is sensitive to the pseudomonal TTS toxins ExoT and ExoU and we showed in Chapter 2 that ExoS is also extremely toxic to yeast [413, 437, 439]. We now demonstrate that the fourth *P. aeruginosa* TTS toxin, ExoY, is also toxic to *S. cerevisiae*. Like ExoU, induction of ExoY expression from the *GAL1* promoter by galactose leads to an inhibition in yeast cell growth. The ability of ExoY to be transformed and expressed in *S. cerevisiae* from the galactose inducible promoter system indicates that ExoY is not as toxic to yeast as ExoS. ExoY is an adenylate cyclase that is known to disrupt the actin cytoskeleton and cause morphological changes in mammalian cells [465, 473]. Thus, as suggested for YopE [484], ExoY may disrupt the actin cytoskeleton in yeast and arrest cell growth by triggering a morphogenesis checkpoint.

Our *S. cerevisiae* screen for mutants resistant to the toxic effects of ExoY and ExoU identified the *gal3* and *gal4* deletion mutants. Gal4 is a transcriptional activator of the *GAL1* promoter and in the absence of galactose Gal4 is bound and repressed by Gal80 [512]. In the presence of galactose, Gal3 binds to Gal80, thus freeing Gal4 to initiate transcription from the *GAL1* promoter [512]. Therefore, deletion of either the *GAL3* or *GAL4* genes would abolish the ability of galactose to induce expression of ExoY or ExoU from the *GAL1* promoter. Neither *gal3* or *gal4* deletion mutants were identified in the ExoS deletion library screen, which strengthens our observation that ExoS is so

cytotoxic that galactose induction is not required for enough of the toxin to be expressed to elicit cytotoxicity.

In addition to the *S. cerevisiae* mutants with *GAL3* or *GAL4* deleted, yeast strains that had the *SPT4* gene deleted were also resistant to the toxic effects of ExoS, ExoY and ExoU. The *SPT4* locus was deleted in both the *spt4* and YGR064W mutant strains as YGR064W overlaps *SPT4* on the opposite DNA strand. We confirmed that it was the loss of the *SPT4* gene and not the YGR064W dubious ORF that was responsible for the toxin resistance by complementation analysis. Spt4 forms a complex with Spt5, which functions with Spt6 to mediate both activation and inhibition of transcription elongation by RNA polymerase II [513]. Spt4, Spt5 and Spt6 are thought to modulate transcription elongation by altering chromatin assembly or stability. The Spt4/Spt5 complex has also been shown to play a role in pre-mRNA processing, homologous DNA recombination, transcription-coupled DNA repair, kinetochore function and gene silencing [514-516]. The resistance of a yeast strain with a deletion in a RNA polymerase II transcription elongation factor gene to the toxic effects of ExoS, ExoY, and ExoU, suggests that these toxins might not be transcribed in this mutant. Indeed, it has been demonstrated that Spt4 is required for proper transcription of long or GC-rich DNA sequences [517]. For example, the bacterial *lacZ* ORF (length: 3 kb, GC content: 56%), the *S. cerevisiae LYS2* gene (length: 3.5 kb, GC content: 40%) and the *S. cerevisiae YAT1* gene (length: 2 kb, GC content: 58%) cannot be transcribed in a *spt4 S. cerevisiae* mutant when driven from the *GAL1* promoter. In contrast, a shorter gene with an average *S. cerevisiae* GC content, the *PHO5* gene (length: 1.5 kb, GC content: 40%), can be transcribed in a *spt4 S. cerevisiae* mutant when driven from the *GAL1* promoter. Although the *exoS*, *exoY* and *exoU* genes are not long genes, 1.4 kb, 1.1 kb and 2.1 kb respectively, they are GC rich. The average GC content of a *S. cerevisiae* gene is 40%, whereas that of *exoS*, *exoY* and *exoU* is 64.1%, 59.0% and 62.8% respectively. Therefore, the resistance of the *spt4* and YGR064W mutant strains to ExoS, ExoY and ExoU is probably a consequence of the lack of expression of the toxins in these yeast. It is interesting to note that in the *spt4* mutant strain, transcription of these pseudomonal toxins must be completely abrogated as we have previously demonstrated how toxic ExoS is even when expressed at very low levels. Spt5 and Spt6 act with Spt4 to promote transcription elongation but both *SPT5* and *SPT6* are essential genes and therefore were not present in our library screen.

Apart from yeast strains with deletions in genes required for toxin expression from the *GAL1* promoter, all the other mutants that we identified in our initial screens were shown not to be resistant to ExoS, ExoY or ExoU when re-tested. We propose that the toxin gene was mutated in these initial isolates and is therefore non-toxic. Yeast transformation is known to be a slightly mutagenic process and this combined with the strong selection pressure on inactive toxin mutants would result in the false positives. There may be a number of reasons why our screen failed to identify any biologically relevant proteins that were required for pseudomonal TTS toxin cytotoxicity. With respect to identifying the eukaryotic cofactors for ExoY and ExoU, this screen would only be effective if the cofactor was a protein that was non-essential and non-redundant. If the cofactors were non-proteineceous, they would not be identified in this screen unless the formation of the non-proteineceous component required a specific, non-essential protein for its manufacture. Although the ExoY and ExoU cofactors are thought to be proteins or contain a protein component [447, 465], this may not be the case. The *S. cerevisiae* library that we screened only has strains containing deletions in non-essential genes, because by definition a homozygous diploid knockout of an essential gene would be non-viable. Therefore, if the eukaryotic cofactors for ExoY or ExoU were an essential gene, they would not be identified in our screen. Also, if more than one protein could act as a cofactor for ExoY or ExoU, they would not be identified, as each strain in the library only has a single gene deleted. This point is illustrated by the eukaryotic cofactor required for the ADPRT activity of ExoS. The ADPRT domain of ExoS requires a 14-3-3 protein for activity and in humans it has been demonstrated that at least 5 out of the 7 isoforms of the 14-3-3 proteins can activate ExoS [386, 387]. There are two genes in *S. cerevisiae* that encode 14-3-3 proteins, *BMH1* and *BMH2* and deletion of either of these genes is not lethal, although deletion of both genes is [518]. Therefore, if either *BMH1* or *BMH2* were deleted, the other gene would still be able to produce a 14-3-3 protein to activate the ADPRT activity of ExoS.

The screen that we carried out was unlikely to identify an enzymatic target of any of the pseudomonal TTS toxins because if the toxin caused yeast cell death by inhibiting the activity of its target, this target is likely to be an essential protein. However, proteins required for the activity of the toxin might have been identified. For example, if the toxin needed to be localised in order to exert its cytotoxic action, deletion of a yeast gene that was required for toxin localisation would render the deletion strain resistant to

the toxin. It has been shown that although ExoS is specifically localised to a perinuclear membrane location, this targeting is not required for its cytotoxic action [368]. Thus, if yeast proteins are involved in ExoS targeting they would not be identified in our screen. Almost nothing is known about the localisation of ExoY and ExoU and it is not known whether they require specific targeting or if eukaryotic proteins are required for this. However, the failure of our screen to identify any proteins required for ExoY or ExoU action suggests that if they do require specific localisation to exert their cytotoxic effect, a non-essential, non-redundant yeast gene is not required for this. The screen for mutants resistant to the effects of ExoS-induced toxicity is further complicated by the presence of the two catalytic domains in this toxin. As we previously demonstrated in Chapter 2, both the GAP domain and ADPRT domains of ExoS are toxic to *S. cerevisiae*. Therefore, for a yeast mutant to be resistant to ExoS cytotoxicity it must have a protein deleted that is required for both the action of the GAP and ADPRT domain. It has been shown that ExoY is able to cause morphological changes even when its adenylate cyclase activity has been abolished [473], thus it might also have more than one enzymatic domain, which would complicate our screen.

To summarise, in this chapter we have screened a *S. cerevisiae* homozygous diploid deletion library for strains resistant to the cytotoxic effects of ExoS, ExoY and ExoU. The only yeast deletion mutants we identified were resistant to galactose-induced expression because they lacked genes required for transcription of the toxin genes from the *GAL1* promoter. Although we did not identify any toxin targets or accessory factors, the identification of the *Spt4* mutant strain validates this screen for identifying deletion mutants able to resist the cytotoxic effects of pseudomonal TTS toxins. It remains a possibility that we may have missed some deletion mutants due to incomplete coverage, but our inability to identify any deletion strains able to resist the cytotoxic effects of these pseudomonal TTS toxins suggests that a non-essential, non-redundant eukaryotic protein is not required for their activity.

CHAPTER 4: STUDYING THE MODIFICATION, LOCALISATION AND TOXICITY OF EXOENZYME U IN A MAMMALIAN CELL MODEL.

4.1. INTRODUCTION

As discussed in the general introduction, the *Pseudomonas aeruginosa* TTS toxin ExoU shows homology to the phospholipase A family of enzymes and is able to hydrolyse a broad range of substrates including neutral lipids, phospholipids and lysophospholipids [439, 440, 447, 448]. ExoU is cytotoxic to a variety of cell lines and is associated with increased virulence in both mammalian infection models and humans [430, 431, 435, 437, 519]. The cytotoxic action of ExoU is dependent on its lipase activity, although it is unclear exactly how the ability of ExoU to hydrolyse lipids leads to cell death. Human PLA_2s play a role in both oncotic and apoptotic cell death [460] and it appears that ExoU-induced cell death exhibits features of necrosis (or oncosis) as opposed to apoptosis [434, 441]. It may be that ExoU causes cell death simply by punching holes in the plasma membrane of host cells. Alternatively, ExoU may target intracellular membranes leading to cell death as a consequence of the destruction of intracellular organelles. The release of free fatty acids by ExoU phospholipase activity may also trigger cell death.

In an attempt to further define how ExoU induces eukaryotic cell death it would be useful to know the sub-cellular localisation of this toxin. ExoU is injected into the cytosol of the host cell by the TTS needle and for it to hydrolyse phospholipids it must first be targeted to them. There are no regions within ExoU that are homologous to known membrane interacting domains, so it is unclear how this targeting occurs. Human $cPLA_2$ is targeted to membranes in a calcium-dependent manner via its N-terminal C2 domain [520, 521]. When the C2 Ca^{2+}-dependent lipid-binding domain is deleted, $cPLA_2$ is no longer able to associate with membranes and hydrolyse liposomal substrates. However, $cPLA_2$ lacking its C2 domain is still able to hydrolyse monomeric phospholipids. This demonstrates that the membrane targeting and catalytic domains of $cPLA_2$ are separate but that membrane targeting is required for phospholipase activity within the cell [520]. Patatin lacks a C2 membrane-binding domain but may be targeted to membranes via a hydrophobic region of 50 amino acid residues at its N-terminus

[449]. ExoU contains neither a C2 Ca^{2+}-dependent lipid-binding domain nor an obvious hydrophobic motif, suggesting that it is targeted to membranes by a different mechanism to $cPLA_2$ or patatin.

In addition to the lack of knowledge regarding where and how ExoU is targeted, it is also unclear how ExoU is activated. In an attempt to predict how ExoU is activated, it may be useful to consider how other lipases are activated. Human $cPLA_2$ requires three levels of activation; phosphorylation, calcium ions and interfacial activation. MAPK phosphorylates serine 505 of $cPLA_2$, which may be involved in maintaining the C2 and catalytic domains in the optimal orientation for catalysis [522]. Calcium ions are then required for targeting $cPLA_2$ to the membrane via the C2 domain as described above [520]. Finally, interfacial activation is required to induce a conformational change of a flexible lid domain that allows the catalytic sites of $cPLA_2$ to access the phospholipid substrates [446]. In addition to $cPLA_2$, calcium ions are also cofactors for $sPLA_2$ and ATP is known to increase $iPLA_2$ activity [523]. The mechanism of patatin activation is unknown but involves translocation of the enzyme from storage vacuoles to the cytosol. Patatin lacks both the C2 domain and flexible lid domain of $cPLA_2$ and as a consequence does not require calcium ions or interfacial activation [524]. No proteinaceous cofactors have been reported for mammalian phospholipases, but the pancreatic triglyceride lipase (PTL) requires a small protein cofactor, colipase, for efficient hydrolysis [525, 526]. Colipase binds to the C-terminal colipase-binding site of PTL and allows a lid domain to open, such that lipid substrates are accessible to the N-terminal catalytic domain containing the serine-aspartate-histidine catalytic triad. In contrast to $cPLA_2$ and $iPLA_2$, ExoU is not activated by calcium but like PTL, does require a eukaryotic cofactor for activity [439]. As discussed in the general introduction, the ExoU eukaryotic cofactor is unknown but is thought to be a large protein or protein complex that is required during hydrolysis [447]. The requirement for the cofactor during hydrolysis suggests that it is not required for modification or pre-activation of ExoU, unlike the phosphorylation of $cPLA_2$ by MAPK, but is required as a catalytic cofactor. This however does not preclude additional modifications, such as phosphorylation, being required for ExoU activation. Also, although ExoU lacks a domain homologous to the flexible lid domain of $cPLA_2$, it is possible that interfacial activation is also required for ExoU activity.

Another unknown regarding ExoU is the requirement for the C-terminus of this toxin for phospholipase activity. ExoU is a 687 amino acid protein with the phospholipase homology domain residing in the N-terminal half between amino acid residues 107 and 357 [439]. A number of studies have demonstrated that regions that lie C-terminal to the phospholipase homology domain are also required for ExoU toxicity in mammalian and yeast cells [431, 436, 438-440]. For example, deletion of just the final 20 amino acids is sufficient to abrogate ExoU toxicity in CHO cells [440]. Also, insertion of random five amino acid stretches into ExoU between residues 601 and 620 results in the loss of ExoU-induced cytotoxicity in *S. cerevisiae* [438]. These studies show that C-terminal regions that reside a long way away from the phospholipase homology domain are essential for ExoU cytotoxicty, but the reason for their requirement is unknown. It has been postulated that the C-terminus of ExoU might be required for targeting the toxin to its phospholipid substrates or for binding to the eukaryotic cofactor [449], but as yet there is no evidence supporting these hypotheses.

The work described in this chapter explains how we studied the targeting of ExoU in a mammalian cell line and determined the regions of the toxin required for its localisation. This chapter also describes how we attempted to determine how ExoU was activated and the role that the C-terminus plays in this. In Chapter 3, we tried to identify the ExoU eukaryotic cofactor by screening a *S. cerevisiae* deletion library for yeast mutants able to grow in the presence of ExoU. Unfortunately this approach failed, so this work details another approach we used to try and identify the cofactor of ExoU. We found that ExoU is localised to the plasma membrane and undergoes modification at this site by the addition of two ubiquitin molecules. A region of 5 amino acids at position 679-683 near the C-terminus of the ExoU protein is required for both membrane localisation and ubiquitinylation. Site-directed mutagenesis identified a tryptophan at position 681 as crucial for this modification. We found that the same region between residues 679-683 was also required for ExoU cell toxicity as well as *in vitro* phospholipase activity. Our attempts at identifying the eukaryotic cofactor were again unsuccessful but we did discover that the activating factor separates with the membrane-enriched particulate fraction of mammalian cells. Finally, we showed that ExoU binds specifically to particular phospholipids but the C-terminal residues shown to be required for membrane localisation did not determine this binding specificity.

4.2. METHODS

4.2.1. Materials

All chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Griener Bio-One (Kremsmuenster, Austria) supplied the plasticware unless otherwise stated. The tissue culture medium and supplements were obtained from Invitrogen (Carlsbad, CA, USA). The primers were synthesized by Operon Biotechnologies (Huntsville, AL, USA) and DNA sequencing was performed by the Dundee University Sequencing Service (Dundee, UK).

4.2.2. HeLa cells

The Human Negroid cervix epitheloid carcinoma cells (European Collection of Cell Cultures), referred to hereafter as HeLa cells, were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum, 2 mM L-Glutamine, 10 U/ml Penicillin and 10 μg/ml Streptomycin. Cells were incubated at 37°C with 5% CO_2 in a humid atmosphere and all manipulations were carried out in a sterile laminar flow hood.

4.2.3. HeLa cell passage

The HeLa cells were routinely grown in 75-cm^2 sterile tissue culture flasks and split 1:10 once they had reached 70-80% confluency. When the HeLa cells were ready to split, they were washed in 10 ml Hanks' Balanced Salt Solution lacking $MgCl_2$ and $CaCl_2$ before being detached from the surface of the flask with 1 ml pre-warmed Trypsin-EDTA for approximately 1 min at 37°C. Detachment was assisted by tapping the flask and confirmed by phase-contrast microscopy. Pre-warmed HeLa medium was added to the cells to terminate the action of the trypsin and a tenth of the trypsinised cells were transferred to a new 75-cm^2 tissue culture flask containing pre-warmed HeLa medium.

4.2.4. HeLa cell cryopreservation

For long-term storage of HeLa cells, a 90% confluent 75-cm^2 tissue culture flask of cells were trypsinised (as described in section 4.2.3) and the cells were scraped up and centrifuged at 200 x g for 5 min at 4°C. The cell pellet was resuspended in 1 ml Foetal Bovine Serum supplemented with 10% DMSO and transferred to a 1.5 ml cryovial (Starstedt, Numbrecht, Germany). The cells were cooled at approximately 1°C a minute in a freezing container (Nalgene Nunc, Abingdon, UK) containing isopropanol to a final temperature of -80°C. The HeLa cells were kept at -80°C for 24 hours before being transferred to liquid nitrogen for long-term storage.

4.2.5. HeLa cell thawing and replating

HeLa cells stored in liquid nitrogen were partially thawed at 37°C then allowed to fully thaw on ice. The cells were added to 7 ml pre-warmed HeLa media in a 25-cm^2 tissue culture flask. Once the cells in the 25-cm^2 tissue culture flask had reached 90% confluency, they were trypsined and all transferred to a 75-cm^2 tissue culture flask.

4.2.6. *Pseudomonas aeruginosa*

The *P. aeruginosa* strain used in this study was PA103Δ*exoU*Δ*exoT*::Tc, referred to hereafter as PA103ΔTΔU (kindly supplied by Prof. D. Frank, Medical College of Wisconsin, USA). PA103 is a cytotoxic lung isolate that produces significant amounts of ExoT and ExoU but fails to express ExoY and does not possess *exoS*. The PA103ΔTΔU strain therefore does not express any known TTS exoenzymes.

4.2.7. Electroporation of *P. aeruginosa*

PA103ΔTΔU was electroporated with the pUCP19-based vectors using a procedure adapted from the work of Diver et. al. [527]. To prepare the electrocompetent cells, 5 ml of Luria Bertani broth (LB; Invitrogen) was inoculated with either 30 µl glycerol stock of PA103ΔTΔU or a colony from a fresh plate and grown overnight at 37°C with shaking at 250 rpm. 0.2 ml of the overnight culture was used to inoculate 20 ml of fresh LB broth and was incubated at 37°C with shaking at 250 rpm until the OD_{600} = 0.6-0.8.

The bacteria were centrifuged at 10000 x g for 10 min at 4°C and the pellet was washed twice with 10 ml ice-cold SMH buffer (300 mM sucrose, 1 mM $MgCl_2$, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0). The bacterial pellet was resuspended in 1 ml ice-cold SMH buffer and the electrocompetent PA103ΔTΔU were frozen at -80°C in 100 µl aliquots.

For transformation, the electrocompetent PA103ΔTΔU were thawed on ice and mixed with approximately 0.5 µg of plasmid DNA. The mixture was incubated on ice for 1 min before being transferred to a 0.2 cm electroporation cuvette (Invitrogen). Electroporation was carried out using the GenePulser Xcell™ (Bio-Rad Laboratories, Hercules, CA) set to the following parameters: 800 Ω, 25 µF and 1.6 kV. Immediately after electroporation, 900 µl of ice-cold SOC medium (Invitrogen) was added to the bacteria. The cells were incubated on ice for 30 min and then at 37°C for 30 min. 200 µl of transformants were plated out onto LB agar containing 300 µg/ml carbenicillin.

4.2.8. Construction of plasmids

The mammalian expression vector pCMV-Tag2 (Stratagene, La Jolla, CA, USA) was used to constitutively express N-terminally FLAG-tagged ExoU and mutant ExoU proteins in transfected HeLa cells. The *Escherichia-Pseudomonas* shuttle vector pUCP19 was used to constitutively express ExoU or mutant ExoU and the ExoU chaperone, SpcU in PA103ΔTΔU. The mammalian expression vector pEGFP-C was used to constitutively express a C-terminally enhanced green fluorescent protein (EGFP)-tagged ExoU fragment in transfected HeLa cells. The pET-100/D plasmid (Invitrogen) was used to express His-tagged ExoU and mutant ExoU in *E. coli* upon induction with isopropyl-beta-D-thiogalactopyranoside (IPTG).

The plasmids were constructed as described in Table 4.1. Site-directed mutagenesis was performed as described in Section 2.2.6. with the extension time in the amplification reaction calculated at 1 min per kb of plasmid. Table 4.2. lists the primers used in the site-directed mutagenesis reactions and the restriction sites introduced by the mutagenesis. Possible successful site-directed mutants were selected by testing for the insertion of the restriction site as this suggested that the desired mutation was also

present. The *exoU* gene and *exoU* N-terminal deletion mutants were amplified using the same conditions as described in Section 3.2.5. using the primers listed in Table 4.3.

Table 4.1. Plasmid construction

PLASMID	CONSTRUCTION
pCMV-Tag2-ExoU	*exoU* was sub-cloned from pYES-ExoU into the *Bam*HI and *Apa*I sites of pCMV-Tag2B.
pCMV-Tag2-ExoU_S142A	The S142A mutation was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the S142A-Fw and S142A-Rev primers.
pCMV-Tag2-ExoU_S142A_K178R	The K178R mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the K178R-Fw and K178R-Rev primers.
pCMV-Tag2-ExoU_S142A_K428R	The K428R mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the K428R-Fw and K428R-Rev primers.
pCMV-Tag2-ExoU_S142A_K679A	The K679A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the K679A-Fw and K679A-Rev primers.
pCMV-Tag2-ExoU_S142A_W681A	The W681A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the W681A-Fw and W681A-Rev primers.
pCMV-Tag2-ExoU_S142A_R682A	The R682A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the R682A-Fw and R682A-Rev primers.
pCMV-Tag2-ExoU_S142A_N683A	The N683A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the N683A-Fw and N683A-Rev primers.
pCMV-Tag2-ExoU_S142A_K684A	The K684A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the K684A-Fw and K684A-Rev primers.
pCMV-Tag2-ExoU_S142A_E685A	The E685A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the E685A-Fw and E685A-Rev primers.
pCMV-Tag2-ExoU_S142A_F686A	The F686A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the F686A-Fw and F686A-Rev primers.

PLASMID	CONSTRUCTION
pCMV-Tag2-ExoU_S142A_T687A	The T687A mutation was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the T687A-Fw and T687A-Rev primers.
pCMV-Tag2-ExoU_ S142A_N1 (Δ1-9)	*exoU* lacking the N-terminal 9 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N1-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N2 (Δ1-17)	*exoU* lacking the N-terminal 17 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N2-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N3 (Δ1-25)	*exoU* lacking the N-terminal 25 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N3-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N4 (Δ1-40)	*exoU* lacking the N-terminal 40 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N4-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N5 (Δ1-56)	*exoU* lacking the N-terminal 56 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N5-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N6 (Δ1-64)	*exoU* lacking the N-terminal 64 amino acids was amplified from pCMV-Tag2B-exoU_S142A using N6-Fw and N-Rev and cloned into the *Eco*RI and *Bam*HI of pCMV-Tag2B-ExoU_S142A.
pCMV-Tag2-ExoU_ S142A_N7 (Δ1-82)	A *Sac*II(blunt)/*Apa*I fragment of *exoU* lacking the N-terminal 82 amino acids was sub-cloned from pYES-ExoU into the *Eco*RV and *Apa*I sites of pCMV-Tag2C. The S142A mutation was then introduced into ExoU by site-directed mutagenesis using the S142A-Fw and S142A-Rev primers.
pCMV-Tag2-ExoU_ S142A_N8 (Δ1-122)	The N-terminal 122 amino acids of ExoU were deleted by digesting pCMV-Tag2-ExoU_S142A with *Sma*I and re-ligating the plasmid backbone.
pCMV-Tag2-ExoU_ S142A_C1 (Δ654-687)	The C-terminal 34 amino acids of ExoU were deleted by digesting pCMV-Tag2-ExoU_S142A with *Eco*RV and *Apa*I, blunt ending and re-ligating the plasmid backbone.

PLASMID	CONSTRUCTION
pCMV-Tag2-ExoU_ S142A_C2 (Δ413-687)	The C-terminal 275 amino acids of ExoU were deleted by digesting pCMV-Tag2-ExoU_S142A with *Xho*I and *Apa*I, blunt ending and re-ligating the plasmid backbone.
pCMV-Tag2-ExoU_ S142A_C3 (Δ343-687)	The C-terminal 345 amino acids of ExoU were deleted by digesting pCMV-Tag2-ExoU_S142A with *Eco*RI and *Apa*I, blunt ending and re-ligating the plasmid backbone.
pCMV-Tag2-ExoU_ S142A_C4 (Δ663-687)	A stop codon at amino acid 663 was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the C4-Fw and C4-Rev primers.
pCMV-Tag2-ExoU_ S142A_C5 (Δ671-687)	A stop codon at amino acid 671 was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the C5-Fw and C6-Rev primers.
pCMV-Tag2-ExoU_ S142A_C6 (Δ679-687)	A stop codon at amino acid 679 was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the C6-Fw and C6-Rev primers.
pCMV-Tag2-ExoU_ S142A_C7 (Δ684-687)	A stop codon at amino acid 684 was introduced into ExoU in pCMV-Tag2-ExoU_S142A by site-directed mutagenesis using the C7-Fw and C7-Rev primers.
pCMV-Tag2-ExoU_K178R	The K178R mutation was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the K178R-Fw and K178R-Rev primers.
pCMV-Tag2-ExoU_K428R	The K428R mutation was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the K428R-Fw and K428R-Rev primers.
pCMV-Tag2-ExoU_K679A	The K679A mutation was sub-cloned from pCMV-Tag2-ExoU_S142A_K679A into the MluI and EcoRI sites of pCMV-Tag2-ExoU.
pCMV-Tag2-ExoU_W681A	The W681A mutation was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the W681A-Fw and W681A-Rev primers.
pCMV-Tag2-ExoU_ R682A	The R682A mutation was sub-cloned from pCMV-Tag2-ExoU_S142A_R682A into the *Mlu*I and *Eco*RI sites of pCMV-Tag2-ExoU.
pCMV-Tag2-ExoU_ N683A	The N683A mutation was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the N683A-Fw and N683A-Rev primers.
pCMV-Tag2-ExoU_ C4 (Δ663-687)	A stop codon at amino acid 663 was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the C4-Fw and C4-Rev primers.

PLASMID	CONSTRUCTION
pCMV-Tag2-ExoU_ C5 (Δ671-687)	A stop codon at amino acid 671 was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the C5-Fw and C6-Rev primers.
pCMV-Tag2-ExoU_ C6 (Δ679-687)	A stop codon at amino acid 679 was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the C6-Fw and C6-Rev primers.
pCMV-Tag2-ExoU_ C7 (Δ684-687)	A stop codon at amino acid 684 was introduced into ExoU in pCMV-Tag2-ExoU by site-directed mutagenesis using the C7-Fw and C7-Rev primers.
pEGFP-ExoU653-687	A fusion construct of EGFP and the C-terminal 33 amino acids of ExoU was constructed by sub-cloning the *Eco*RV/*Apa*I fragment from pCMV-Tag2-ExoU into the *Kpn*I(blunt)/*Apa*I sites of pEGFP-C3.
pUCP19-ExoU-SpcU	Kind gift from Prof. D. Frank
pUCP19-ExoU_S142A-SpcU	The S142A mutation was sub-cloned from pCMV-Tag2B-ExoU_S142A into the *Sac*II and *Eco*RV sites of pUCP19-ExoU-SpcU.
pUCP19-ExoU_K178R-SpcU	The K178R mutation was sub-cloned from pCMV-Tag2B-ExoU_K178R into the *Sac*II and *Eco*RV sites of pUCP19-ExoU-SpcU.
pUCP19-ExoU_W681A-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. The W681A mutation was introduced into ExoU by site-directed mutagenesis using the W681A-Fw and W681A-Rev primers. A fragment of ExoU_W681A -spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU-SpcU.
pUCP19-ExoU_ C6(Δ679-687)-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. A stop codon at amino acid 679 was introduced into ExoU by site-directed mutagenesis using the C6-Fw and C6-Rev primers. A fragment of exoU_C6-spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU-SpcU.

PLASMID	CONSTRUCTION
pUCP19-ExoU_ C7(Δ684-687)-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. A stop codon at amino acid 684 was introduced into ExoU by site-directed mutagenesis using the C7-Fw and C7-Rev primers. A fragment of exoU_C7-spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU-SpcU.
pUCP19-ExoU_S142A_K178R-SpcU	A fragment of *exoU* containing the S142A and K178R mutations was-sub-cloned from pCMV-Tag2-ExoU_S142A_K178R into the *Sac*II and *Eco*RV sites if pUCP19-ExoU-SpcU.
pUCP19-ExoU_S142A_W681A-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. The W681A mutation was introduced into ExoU by site-directed mutagenesis using the W681A-Fw and W681A-Rev primers. A fragment of exoU_W681A-spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU_S142A-SpcU.
pUCP19-ExoU_S142A_ C6(Δ679-687)-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. A stop codon at amino acid 679 was introduced into ExoU by site-directed mutagenesis using the C6-Fw and C6-Rev primers. A fragment of exoU_C6-spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU_S142A-SpcU.
pUCP19-ExoU_S142A_ C7(Δ684-687)-SpcU	A fragment of exoU-spcU was sub-cloned from pUCP19-ExoU-SpcU into the *Sma*I and *Hind*III sites of pUC19. A stop codon at amino acid 684 was introduced into ExoU by site-directed mutagenesis using the C7-Fw and C7-Rev primers. A fragment of exoU_C7-spcU was then sub-cloned into the *Eco*RV and *Bam*H1 sites if pUCP19-ExoU_S142A-SpcU.
pUCP19-ExoU_S142A-EGFP-SpcU	EGFP was amplified and cloned into the *Nsi*I sites of pUCP19-ExoU_S142A-SpcU.
pET-100/D-ExoU	The *exoU* gene was amplified from PA4 genomic DNA using the rExoU-Fw and rExoU-Rev primers and cloned into the pET-100/D-TOPO directional cloning vector.

PLASMID	CONSTRUCTION
pET-100/D-ExoU_S142A	The S142A mutation was introduced into ExoU in pET-100/D-ExoU by site-directed mutagenesis using the S142A-Fw and S142A-Rev primers.
pET-100/D-ExoU_K178R	The K178R mutation was introduced into ExoU in pET-100/D-ExoU by site-directed mutagenesis using the K178R-Fw and K178R-Rev primers.
pET-100/D-ExoU_W681A	The W681A mutation was sub-cloned as a *Not*I(blunt)/*Xho*I fragment from pCMV-Tag2B-ExoU_W681A into the *Sac*I(blunt)/*Xho*I sites of pET-100/D-ExoU.
pET-100/D-ExoU_C6(Δ679-687)	A stop codon at amino acid 679 was introduced into ExoU in pET-100/D-ExoU by site-directed mutagenesis using the C6-Fw and C6-Rev primers.
pET-100/D-ExoU_ C7(Δ684-687)	The stop codon at amino acid 684 was sub-cloned as a *Not*I(blunt)/*Xho*I fragment from pCMV-Tag2B-ExoU_C7(Δ684-687) into the *Sac*I(blunt)/*Xho*I sites of pET-100/D-ExoU.

Table 4.2. Site-directed mutagenesis primers

PRIMER	SEQUENCE (5'-3')	R.S.
S142A-Fw	GTCCGGTTCGGCCGCTGGCGGCA	*Eag*I
S142A-Rev	TGCCGCCAGCGGCCGAACCGGAC	*Eag*I
K178R-Fw	CTCGATAGCTCGAACAGGAAGCTTAAGCTGTTCCAA CACA	*Hind*III
K178R-Rev	TGTGTTGGAACAGCTTAAGCTTCCTGTTCGAGCTATC GAG	*Hind*III
K428R-Fw	ACCGTTGTGGTGCCGTTACGTAGCGAGCGCGGTGAT TTC	*Sna*BI
K428R-Rev	GAAATCACCGCGCTCGCTACGTAACGGCACCACAAC GGT	*Sna*BI
K679A-Fw	CTACCGTTGAGATGGCTGCAGCTTGGCGGAATAAGG AGTT	*Pst*I
K679A-Rev	AACTCCTTATTCCGCCAAGCTGCAGCCATCTCAACGG TAG	*Pst*I
W681A-Fw	CCGTTGAGATGGCCAAGGCTGCGCGGAATAAGGAGT TC	*Msc*I
W681A-Rev	GAACTCCTTATTCCGCGCAGCCTTGGCCATCTCAACG G	*Msc*I
W681K-Fw	CTACCGTTGAGATGGCCAAGGCTAAGCGGAATAAGG AGTTC	*Msc*I
W681K-Rev	GAACTCCTTATTCCGCTTAGCCTTGGCCATCTCAACG GTAG	*Msc*I
W681E-Fw	CTACCGTTGAGATGGCCAAGGCTGAGCGGAATAAGG AGTTC	*Msc*I

PRIMER	SEQUENCE	R.S.
W681E-Rev	GAACTCCTTATTCCGCTCAGCCTTGGCCATCTCAACG GTAG	*Msc*I
R682A-Fw	TTGAGATGGCTAAGGCCTGGGCGAATAAGGAGTTCA CATGAG	*Stu*I
R682A-Rev	CTCATGTGAACTCCTTATTCGCCCAGGCCTTAGCCAT CTCAA	*Stu*I
N683A-Fw	ATGGCTAAGGCTTGGCGCGCTAAGGAGTTCACATGA GC	*Bss*HII
N683A-Rev	GCTCATGTGAACTCCTTAGCGCGCCAAGCCTTAGCC AT	*Bss*HII
K684A-Fw	CTAAGGCTTGGCGGAATGCGGAATTCACATGAGCGG CC	*Eco*RI
K684A-Rev	GGCCGCTCATGTGAATTCCGCATTCCGCCAAGCCTTA G	*Eco*RI
E685A-Fw	GCTTGGCGGAATAAGGCCTTCACATGAGCGGC	*Stu*I
E685A-Rev	GCCGCTCATGTGAAGGCCTTATTCCGCCAAGC	*Stu*I
F686A-Fw	GCTTGGCGGAATAAGGAGGCTACGTAAGCGGCCGCT CGAGTC	*Sna*BI
F686A-Rev	GACTCGAGCGGCCGCTTACGTAGCCTCCTTATTCCGC CAAGC	*Sna*BI
T687A-Fw	GGCGGAATAAGGAATTCGCATGAGCGGCCGCT	*Eco*RI
T687A-Rev	AGCGGCCGCTCATGCGAATTCCTTATTCCGCC	*Eco*RI
C4-Fw	ACAACTACTCGGCACGAGGTTAACTGCGTTTCGGCA AACC	*Hpa*I
C4-Rev	GGTTTGCCGAAACGCAGTTAACCTCGTGCCGAGTAG TTGT	*Hpa*I
C5-Fw	CTTCCTGCGTTTCGGCAAACCCCTTTAAAGCACTACC GTTGAGATGGCTA	*Dra*I
C5-Rev	TAGCCATCTCAACGGTAGTGCTTTAAAGGGGTTTGCC GAAACGCAGGAAG	*Dra*I
C6-Fw	CTACCGTTGAGATGGCTTAAGCTTGGCGGAATAAGG AGTT	*Hind*III
C6-Rev	AACTCCTTATTCCGCCAAGCTTAAGCCATCTCAACGG TAG	*Hind*III
C7-Fw	ATGGCTAAGGCTTGGCGGAATTGAATTCTCACATGA GCGGCCGCTC	*Eco*RI
C7-Rev	GAGCGGCCGCTCATGTGAGAATTCAATTCCGCCAAG CCTTAGCCAT	*Eco*RI

Table 4.3. PCR amplification primers

PRIMER	SEQUENCE (5'-3')
N1-Fw	TCGGATCCGCCTCCTCGCTGAATCAGG
N2-Fw	TCGGATCCGTCGAAACCCCGTCGCAG
N3-Fw	TCGGATCCCATAAGTCCGCCAGCTTGC
N4-Fw	TCGGATCCGGGGTTGCCCTAAAGAGC
N5-Fw	TCGGATCCGAAAGCGTTAGTGACGTGCG
N6-Fw	TCGGATCCAGCAGTCCCCAAGGGCAAG
N-Rev	TCTTGTCGATCATCTCAGGGACC
rExoU-Fw	CACCATGCATATCCAATCGTTGGGG
RExoU-Rev	TCATGTGAACTCCTTATTCCGCCA

The plasmids were sequenced to confirm they were correct. In addition to sequencing with the internal ExoU primers, ExoU-Int1-5 (Table 3.1.), genes in pCMV-Tag2 were sequenced with the T7 prom and T3 prom primers, genes in pUCP19 were sequenced with the M13F and M13R primers, genes in pEGFP-C were sequenced with the EGFPseq primer and genes in pET-100/D were sequenced with the T7 prom and T7 term primers (Table 3.1. and Table 4.4.).

Table 4.4. Sequencing primers

PRIMER	SEQUENCE (5'-3')
T3 prom	AATTAACCCTCACTAAAGGG
T7 term	TATGCTAGTTATTGCTCAG
M13 F	GTAAAACGACGGCCAGTG
M13 R	GGAAACAGCTATGACCATG
EGFP-Cseq	CATGGTCCTGCTGGAGTTCGTG

4.2.9. Transfection of HeLa cells

HeLa cells were transfected with plasmid DNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturers instructions. The DNA and Lipofectamine™ 2000 were diluted separately in OptiMEM® 1 GlutaMAX™ I using the volumes listed in Table 4.5, mixed gently and incubated at room temperature for 5 minutes. The diluted DNA and Lipofectamine™ 2000 were then mixed and incubated at room temperature for 20 minutes before being added to 90-95% confluent HeLa cells in the appropriate volume of HeLa media as shown in Table 4.5. HeLa cells were incubated with the transfection reagents at 37°C with 5% CO_2 for at least 12 hours.

Table 4.5. Volumes for HeLa cell transfection

Culture vessel	Vol. of HeLa media	DNA (μg) in media vol. (μl)	Lipofectamine™ 2000 (μl) in media vol. (μl)
T75 flask	12.5 ml	30 μg in 1.75 ml	56.25 μl in 1.75 ml
6-well plate	2 ml	4.0 μg in 250 μl	7.5 μl in 250 μl
24-well plate	1 ml	1.6 μg in 100 μl	3 μl in 100 μl
2-well slide	1 ml	1.6 μg in 100 μl	3 μl in 100 μl

4.2.10. Infection of HeLa cells with *P. aeruginosa*

PA103ΔTΔU were prepared for infecting HeLa cells by inoculating 10 ml LB medium with 30 μl glycerol stock of the bacteria. The LB medium was supplemented with 300 μg/ml carbenicillin when PA103ΔTΔU contained a pUCP19-based plasmid to maintain selection on the plasmid. The culture was incubated at 37°C with shaking at 225 rpm overnight. 12 ml of fresh LB medium (supplemented with 300 μg/ml carbenicillin if a pUCP19-based plasmid was present) was inoculated with 1 ml of the overnight culture and grown at 37°C with shaking at 225 rpm until OD_{600} = 0.4. The culture was centrifuged at 3200 x g for 10 min and the bacterial pellet was washed twice in sterile PBS. The bacteria were resuspended in sterile PBS to obtain a suspension containing approximately 5 x 10^5 CFU/μl (volume of PBS added = (OD_{600}/0.4) x 3.6).

The infection of HeLa cells required the cells to have reached approximately 90% confluency and to be in HeLa media lacking penicillin and streptomycin. 2.5 x 10^6 CFU of *P. aeruginosa* were used to infect HeLa cells in a 2-well slide or a 24-well plate and 5 x 10^6 CFU of *P. aeruginosa* were used to infect HeLa cells in a 6-well plate. The HeLa cells inoculated with PA103ΔTΔU were incubated at 37°C with 5% CO_2 for 3 - 4 hours and then washed three times in ice-cold PBS to remove the extracellular bacteria.

4.2.11. Immunoblotting

To detect proteins in transfected HeLa cells, cells from a 6-well plate were lysed directly into 2 x sodium dodecyl sulfate (SDS) gel-loading buffer (100 mM Tris.Cl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Samples prepared by immunoprecipitation, membrane-cytosol fractionation or protein purification (see below) were diluted 1:1 in 2 x SDS gel-loading buffer. The samples were heated to 95°C for 5 min before the proteins were separated on 4-12% SDS-

polyacrylamide gel electrophoresis (PAGE) gels (Invitrogen) and electrophoretically transferred to Hybond™-P polyvinylidene difluoride transfer membranes (Amersham Biosciences, Little Chalfont, UK). Either the biotinylated Magic Mark XP Standard (Invitrogen) or the See Blue Plus 2 Pre-stained Standard (Invitrogen) were used as size markers. Non-specific binding to membranes was blocked by immersing the membranes in 5% Marvel dried skimmed milk (Premier International Foods, Spalding, UK) in PBS and incubating for 1 h at room temperature or overnight at 4°C. The membranes were incubated at room temperature for 1 h with the primary antibody in PBS containing 5% Marvel dried skimmed milk according to the dilutions in Table 4.6.

Table 4.6. Primary antibodies used in immunoblotting and immunofluorescence

Antigen	Type	Animal	Dilution for Immuno-blotting	Dilution for immuno-fluoresence	Supplier
FLAG tag	Monoclonal	Mouse	1/1000	1/1000	Sigma-Aldrich
ExoU	Polyclonal	Rabbit	1/500	1/500	
Ubiquitin	Monoclonal	Mouse	1/1000		Sigma-Aldrich
GAPDH	Polyclonal	Rabbit	1/1000		Abcam[a]
Calnexin	Polyclonal	Rabbit	1/2000		Stressgen Bioreagents[b]
52kDa Ro/SSA	Monoclonal	Mouse	1/100		Santa Cruz Biotechnology[c]
CD98	Polyclonal	Goat	1/200		Santa Cruz Biotechnology
6x His tag	Polyclonal	Rabbit	1/1000		Abcam

[a] Cambridge, UK

[b] Victoria, BC, Canada

[c] Santa Cruz, CA, USA

The primary antibody was washed off the membranes by two rinses, a 15 min wash and two 5 min washes in Tris-buffered saline (TBS; 10 mM Tris.Cl pH 7.6, 150 mM NaCl) + 0.1% Tween® 20. The membranes were then incubated at room temperature for 1 h with 1.5 μg/ml of the appropriate biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) in PBS containing 5% Marvel dried skimmed milk before being washed as above. The membranes were then incubated at room temperature for 1 h with a 1/3000 dilution of horseradish peroxidase (HRP)-conjugated streptavidin (Biosorce, Camarillo, CA, USA) in PBS containing 5% Marvel dried skimmed milk. The HRP-conjugated streptavidin was washed off the membranes by two rinses, a 10 min wash and four 5 min washes in TBS + 0.1% Tween 20. All blocking, antibody

incubation and washing steps were carried out on an orbital shaker to ensure uniform coverage of the membrane. The ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) were used according to the manufacturers instructions to detect immunoreactive protein bands. The chemifluorescent signal was detected on Hyperfilm ECL film (Amersham Biosciences).

After use, membranes were stored wet wrapped in Saran Film (Dow Chemical Company, Edegen, Belgium) at 4°C and could be stripped of bound antibodies and reprobed with different antibodies. The membranes were stripped by submerging in stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and incubating at 50°C for 30 min with gentle agitation. The membranes were then washed twice for 10 min in large volumes of PBS containing 0.1% Tween® 20 at room temperature. After blocking the membranes in 5% Marvel dried skimmed milk in PBS for 1 h at room temperature, they were ready to be reprobed.

4.2.12. Immunofluorescence staining

HeLa cells in 2-well slides transfected with a pCMV-Tag2-based plasmids or infected with PA103ΔTΔU containing a pUCP19-based plasmids were immunostained with an anti-FLAG or anti-ExoU antibody respectively. HeLa cells were first rinsed with PBS then fixed with 0.5 ml 1% paraformaldehyde in PBS for 30 min at room temperature. The fixed cells were rinsed with PBS and permeabilised with 0.2% Triton-X 100 for 20 min at room temperature. The permeabilised cells were rinsed in PBS and blocked in 10% Normal Goat Serum (NGS) in PBS for 1 h at room temperature or overnight at 4°C. HeLa cells were then incubated for 1 h at room temperature with the anti-FLAG antibody or anti-ExoU serum in 10% NGS in PBS at the concentrations indicated in Table 4.6. Unbound primary antibody was removed by washing the cells with PBS for 5 min, three times. The cells were then incubated with 2 μg/ml AlexaFluor® 488 goat anti-mouse or goat-anti-rabbit IgG (Invitrogen) in 10% NGS in PBS for 1 h at room temperature. The unbound secondary antibody was washed off as described above and the nucleus of the cells were stained with 0.33 μg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 10 min at room temperature. After a final round of washing, the cells were mounted in Vectashield (Vector Laboratories) and viewed using a Zeiss Axiovert S100 microscope with a Zeiss Plan-NEOFLUAR 63x lens (Carl Zeiss Ltd.,

Welwyn Garden City, UK). For each view, 5 images were taken at 0.5 micron intervals and Openlab software (Improvision) was used to deconvolve the images.

4.2.13. Preparation of ExoU and possible ExoU binding proteins for mass spectroscopy analysis

To analyse the composition of ExoU and modified ExoU, a 75-cm^2 flask of HeLa cells was transfected with pCMV-Tag2-ExoU_S142A and 20 h after transfection the HeLa cell lysate was immunoprecipitated with Anti-FLAG M2 Affinity Gel as described in section 4.2.14. The proteins were then separated by 4-12% SDS-PAGE and the proteins were fixed and stained by incubating the gel with 0.25% Comassie Brilliant Blue R250 in 30% methanol and 10% acetic acid for 15 min at room temperature on an orbital shaker. Excess stain was removed by rinsing the gel twice in destain (30% methanol and 10% acetic acid) and then washing in destain for 1 h on an orbital shaker. The ExoU and modified ExoU bands were cut out of the gel and analysed by mass spectrometry as described in section 4.2.15.

To determine the molecular weights of the complete ExoU and modified ExoU proteins, a 75-cm^2 flask of HeLa cells was transfected with pCMV-Tag2-ExoU_S142A and 20 h after transfection the HeLa cell lysate was immunoprecipitated with Anti-FLAG M2 Affinity Gel as described in section 4.2.14. The protein mixture was then analysed by mass spectrometry as described in section 4.2.15.

In order to identify any proteins that coimmunoprecipitate with ExoU, 4 x 75-cm^2 flasks of HeLa cells were transfected with pCMV-Tag2 or pCMV-Tag2-ExoU_S142A. ExoU was immunoprecipitated from the HeLa cell lysates as described in section 4.2.14. and the protein profiles of the two eluted samples were compared by 4-12% SDS-PAGE and commassie blue staining as described above. Any protein bands present in the sample immunoprecipitated from HeLa cells transfected with pCMV-Tag2-ExoU_S142A that were not present in the sample immunoprecipitated from HeLa cells transfected with pCMV-Tag2 (except for the ExoU and modified ExoU protein bands) were cut out and analysed by mass spectroscopy as described in section 5.2.15.

4.2.14. Immunoprecipitation of ExoU

The HeLa cells were washed twice with ice-cold PBS and lysed by incubating with lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 containing 10 µl protease inhibitor cocktail per ml) on ice with agitation for 30 mins. The cells were then scraped up using a cell scraper, transferred to a 1.5 ml test tube and centrifuged at 16100 x g for 10 minutes at 4°C. The post-nuclear supernatant was stored at -80°C unless used immediately.

The Anti-FLAG M2 Affinity Gel was prepared by pelleting the gel suspension at 16100 x g for 1 min at 4°C and washing the packed gel twice in ice-cold TBS. Immunoprecipitation of FLAG-tagged ExoU was achieved by incubating the HeLa cell lysate with the packed gel for 3 hours at 4°C with rotation. The gel was then washed three times with ice-cold TBS to remove unbound proteins.

Proteins were eluted from the Anti-FLAG M2 Affinity Gel either directly into 2 x SDS gel loading buffer or by using a 3x FLAG peptide solution. To elute with 2 x SDS gel loading buffer, this was added to the agarose and heated to 100°C for 5 mins. The sample was centrifuged at 5000 x g for 30 sec and the supernatant was transferred to a fresh tube. In addition to containing the FLAG-tagged proteins and any additional protein that bound to the FLAG-tagged protein, the sample generated from this elutuion method also contained the light and heavy chains of the FLAG M2 antibody. To prevent elution of the FLAG M2 antibody, a 3x FLAG peptide solution was used to elute just the FLAG-tagged protein and any bound proteins. A 3x FLAG peptide stock solution was made (5 µg/µl 3x FLAG peptide in 100 mM Tris HCl, pH 7.5, 200 mM NaCl). For elution, 1.5 µl of the 3x FLAG peptide stock solution was added to 50 µl TBS (150 ng/µl final concentration) and this was incubated with the packed gel for 30 min at 4°C with rotation. The gel was then pelleted by centrifugation at 16100 x g for 1 min at 4°C and the supernatant was transferred to a fresh tube. The supernatants were stored at -20°C unless used immediately.

4.2.15. Mass Spectrometry

Prof. D. Oxley (Babraham Institute, Cambridge, UK) carried out the mass spectrometry analysis. To analyse the composition of ExoU and the modified ExoU protein and the potential ExoU binding protein bands, the coomassie-stained bands were destained, reduced, carbamidomethylated and digested overnight with 10 ng/µl modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 30°C. The resulting peptide mixtures were separated by reverse-phase liquid chromatography (column: 0.1 x 100 mm, Vydac C18, 5 µm particle size), with an acetonitrile gradient (0 – 30% over 30 min) containing 0.1% formic acid, at a flow rate of 500 nL/min. The column was coupled to a nanospray ion source (Protana Engineering, Denmark) fitted to a quadrupole-TOF mass spectrometer (Qstar Pulsar I; Applied Biosystems/MDS Sciex, Canada). The instrument was operated in information dependent acquisition mode, with an acquisition cycle consisting of a 0.5 sec TOF scan over the m/z range 350 – 1500 followed by 2 sec MS/MS scans (triggered by 2+ or 3+ ions), recorded over the m/z range 100-1700. Proteins were identified by database searching of the mass spectral data using Mascot software (Matrix Science, London, UK). Mascot was also used to determine ubiquitinylation sites using the Lysine GlyGly "variable modification" parameter. All identified ubiquitnylation sites were verified by manual interpretation of the corresponding MS/MS spectra. All the unassigned MS/MS spectra of significant intensity were also manually interpreted.

The molecular weights of the complete ExoU and modified ExoU proteins were also measured using mass spectrometry. The solution containing ExoU and modified ExoU was desalted and the FLAG tags were removed from the proteins before being analysed by mass spectrometry. The mass-to-charge ratio of the parental ExoU and modified ExoU ions provided the molecular mass of the two entities and the difference in mass between them.

4.2.16. Stability of ExoU and ubiquitinylated ExoU

HeLa cells in a 6-well plate were transfected with pCMV-Tag2-ExoU_S142A (see section 4.2.9). 12 hours after transfection, protein synthesis was inhibited by incubating the HeLa cells with 25 µg/ml cyclohexamide at 37°C with 5% CO_2. At 0, 4, 8, 12 and

24 hours after addition of cyclohexamide, the HeLa cells were washed twice in PBS and lysed in 200 µl 2 x SDS gel-loading buffer. The samples were subjected to immunoblotting with the anti-FLAG antibody (see section 4.2.14). The resulting photographic film was scanned and densitometric analysis was performed using the NIH 1.61 analysis program (National Institute of Health, Betheseda, MD, USA).

4.2.17. Membrane/cytosol fractionation

HeLa cells in a single well of a 6-well plate were transfected with pCMV-Tag2-ExoU_S142A (see section 4.2.9). After 16 hours of transfection, the cells were washed in ice-cold PBS and lysed in 0.5 ml ice-cold lysis buffer (20 mM Tris.Cl, 10 mM EDTA) supplemented with 5 µl/ml protease inhibitor cocktail. The cells were scraped up with a cell scraper and sonicated twice for 10 sec on ice. The sonicated cells were centrifuged at 1500 x g for 5 min at 4°C to remove the nuclei and large cellular debris. The supernatant were transferred to pre-chilled 13 x 51 mm polyallomer centrifuge tube (Beckman Instruments, Palo Alto, CA, USA) and centrifuged at 100000 x g for 1 h in a pre-chilled 4°C 55Ti rotor in a XL-90 ultracentrifuge (Beckman Instruments). The supernatant, which contains the cytosolic fraction, was mixed with an equal volume of SDS gel-loading buffer and stored at -20°C until required. The pellet was washed by resuspending in 500 µl lysis buffer and centrifuging at 100000 x g for 1 h. The supernatant was discarded and the pellet was resuspended in 50 µl SDS gel-loading buffer by vigorous vortexing and heating at 95°C for 5 min. The resuspended pellet was stored at -20°C until required.

The cytosolic and membrane-enriched samples were heated to 95°C for 5 min and subjected to SDS-PAGE. The proteins were transferred to a membrane that was immunoblotted with anti-FLAG, anti-Calnexin, and anti-GAPDH antibodies as described in section 4.2.11.

4.2.18. Luciferase assay

HeLa cells in a 24-well plate were transfected with the pGL2-control plasmid (Promega), which constitutively expresses the firefly (*Photinus pyralis*) luciferase in mammalian cells, and a pCMV-Tag2-ExoU or mutant ExoU plasmid. 24 hours after

transfection, the HeLa cell media was replaced with fresh media and the cells were incubated for a further 24 hours. The Luciferase Assay Kit (Stratagene) was used according to manufacturers instructions to measure the amount of luciferase produced in the cells transfected with different ExoU and ExoU mutant constructs. Luciferase catalyzes the following chemiluminescent oxidation-reduction reaction:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + \text{light (560 nm)} + \text{AMP} + \text{PPi} + CO_2$$

Therefore the amount of light produced is a measure of the amount of luciferase present, which correlates with the number of viable cells. To perform the assay, the HeLa cells were washed twice in PBS and the cells in each well were lysed with 75 µl 1 x Cell Lysis Buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) for 15 min with occasional swirling. The cells were scraped and transferred to a microcentrifuge tube on ice. The cells were vortexed for 15 s and centrifuged at 16100 x g for 2 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and allowed to reach room temperature. A 20 µl aliquot of the HeLa cell supernatant was added to 100 µl of room temperature Luciferase Substrate – Assay Buffer Mixture in a 5 ml polystyrene round bottom tube. Immediately after addition of the HeLa cell supernatant, the light produced from the reaction was measured over a 10 s period in a Lumat LB 9507 luminometer (Berthold Technologies).

4.2.19. Lactate dehydrogenase assay

HeLa cells were grown to 90% confluency in a 24-well plate. Two hours before infection, the normal HeLa media was replaced with Dulbecco's Modified Eagle's Medium minus phenol red supplemented with 1% Foetal Bovine Serum and 2 mM L-Glutamine to reduce background absorbance and remove the antibiotic selective pressure. The HeLa cells were infected with *P. aeruginosa* as described in section 4.2.10. After 4 h of infection, the HeLa cells in the 24-well plates were centrifuged at 250 x g for 4 min. The HeLa cell culture supernatant were diluted 1 in 10 in Dulbecco's Modified Eagle's Medium minus phenol red supplemented with 1% Foetal Bovine Serum and 2 mM L-Glutamine and 50 µl aliquots were transferred to a 96 well plate.

The amount of lactate dehydrogenase (LDH) released by HeLa cell lysis after *P. aeruginosa* infection was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturers instructions. This assay works by coupling the amount of LDH in the supernatant to the enzymatic conversion of a tetrazolium salt (INT) into a red formazan product:

$$\text{NAD}^+ + \text{lactate} \xrightarrow{\textbf{LDH}} \text{pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{INT} \xrightarrow{\textbf{Diaphorase}} \text{NAD}^+ + \text{formazan (red)}$$

Briefly, 50 µl of the reconstituted Substrate Mix was added to the HeLa cell culture supernatant and incubated for 30 min at room temperature in the dark. 50 µl of Stop Solution (1 M acetic acid) was added to stop the reaction and the absorbance was read at 490 nm. The amount of LDH released by unifected cells and the amount of LDH released by cells completely lysed by the addition of 100 µl of 10X Lysis Solution (9% (v/v) Triton X-100), was determined. The % of cell death caused by each pseudomonal strain was then calculated as follows:
% Cell Death = 100 x (LDH release – LDH release by uninfected cells) / (1.1 x LDH released by lysed cells - LDH release by uninfected cells).

4.2.20. Purification of recombinant ExoU and mutant ExoU

BL21 Star (DE3) One Shot Chemically Competent Cells (Invitrogen) were transformed with 1 µl pET-100/D-ExoU or ExoU mutant according to the manufacturers instructions. The entire transformation mix was added to 10 ml of LB broth supplemented with 100 µg/ml ampicillin and grown overnight at 37°C with shaking at 250 rpm. 5 ml of the overnight culture was used to inoculate 500 ml of LB broth supplemented with 100 µg/ml ampicillin and the culture was incubated at 30°C with shaking at 250 rpm until the OD_{600} = 0.5-0.8. His-tagged ExoU or mutant ExoU expression was induced by adding IPTG (Invitrogen) to a final concentration of 1 mM and incubating for a further 2 h at 30°C with shaking at 250 rpm. The culture was centrifuged at 9950 x g for 10 min at 4°C to pellet the bacteria and this bacterial pellet was stored at -20°C until required. To prepare cleared bacterial lysate, the bacterial

pellet was thawed on ice for 15 min and resuspended in 5 ml 1 x Ni-NTA Bind Buffer (Novagen) by vigorous vortexing. Lysozyme was added to the resuspended bacteria at a final concentration of 0.5 mg/ml and the mixture was incubated at 30°C for 10 min. The viscosity of the lysate was reduced by sonicating at 80% power for 1 min in 10 s bursts on ice. The bacterial lysates were then centrifuged at 9950 x g for 20 min at 4°C and the supernatants were removed and stored on ice. Ni-NTA His•Bind® Resin (Novagen) was used to purify the His-tagged proteins with all the steps being carried out at 4°C. The resin was prepared by mixing 1 ml of the 50% slurry of Ni-NTA His•Bind® Resin with 4 ml of 1 x Bind Buffer, allowing the resin to settle by gravity and removing the supernatant. The cleared bacterial lysate was then added to the resin and mixed on a rotating wheel for 1 h. The slurry was poured into a small column and the resin was allowed to settle while the lysate ran through it. The resin was washed twice with 4 ml 1 x Ni-NTA Wash Buffer (Novagen) and the His-tagged ExoU or mutant ExoU was eluted with 4 x 0.5 ml aliquots of Ni-NTA Elution Buffer (Novagen). The eluted fractions were analysed for purity by SDS-PAGE and the amount of protein was quantified using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturers instructions. The eluted fractions were stored at 4°C.

4.2.21. Phospholipase activity assay

The cPLA$_2$ Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to determine the phospholipase activity of the ExoU and mutant ExoU proteins prepared as described in 4.2.20. This assay works by incubating ExoU with the synthetic substrate, arachidonoyl thio-PC and measuring the release of free thiol by hydrolysis of the arachidonyl thioester bond at the *sn*-2 position as a result of PLA$_2$ activity. The free thiol then reacts with 5,5'-dithio-*bis*-(2-Nitrobenzoic Acid) (DNTB) to produce the coloured compound 5-Thio-2-Nitrobenzoic Acid.

HeLa cell lysate was prepared by adding 0.5 ml ice-cold lysis buffer (50 mM HEPES pH 7.4, 1 mM EDTA) to a confluent 75-cm^2 flask of HeLa cells and scraping the cells into a microcentrifuge tube. The cells were sonicated and centrifuged at 1000 x g for 15 min at 4°C and the supernatant was kept on ice or at -80°C for long-term storage.

The assay was performed by mixing 5 μg ExoU or mutant ExoU with 9 μl HeLa lysate, 5 μl Assay Buffer and 200 μl arachidonoyl thio-PC Substrate Solution and incubating the reaction for 1 h at room temperature. 10 μl DTNB/EGTA was added to develop the reaction and the absorbance at 405 nm was read after 30 min. A non-enzymatic control (blank) was carried out where 15 μl Assay Buffer was incubated with the Substrate Solution. The PLA_2 activity of HeLa extract without ExoU and ExoU without HeLa extract were also determined.

The PLA_2 activity of ExoU and mutant ExoU was calculated as follows:

$$\Delta A_{405}/\text{min} = \frac{A_{405}(\text{sample})-A_{405}(\text{blank})}{90\ \text{minutes}^{a}}$$

$$PLA_2\ \text{Activity} = 1000 \times \frac{\Delta A_{405}/\text{min} \times 0.225\ \text{ml}}{10.0\ \text{mM}^{-1b} \times 0.005\ \text{g}} = \text{nmoles/min/g}$$

[a]The change in A_{405} is calculated over 90 min because ExoU activity is not dependent on the presence of Ca^{2+}, therefore the addition of EGTA does not stop the reaction and the hydrolysis of the arachidonyl thioester bond continues during the DTNB incubation.
[b]The extinction coefficient for DTNB at 405 nm adjusted for the pathlength of the solution in the well (0.784 cm) is 10.0 mM^{-1}.

The ability of various phospholipids to activate ExoU was examined by replacing the HeLa extract in the above PLA_2 assay with 10 μM of the following phospholipids:

- 1,2-Diacyl-sn-glycero-3-phospho-L-serine
- D-*myo*-Phospatidylinositol D (+)-*sn*-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho linked (Echelon Biosciences Incorporated, Bryce Canyon, UT, USA)
- D-*myo*-Phospatidylinositol 3-phosphate D (+)-*sn*-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho linked (Echelon Biosciences Incorporated)
- D-*myo*-Phospatidylinositol 4-phosphate D (+)-*sn*-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho linked (Echelon Biosciences Incorporated)
- D-*myo*-Phospatidylinositol 5-phosphate D (+)-*sn*-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho linked (Echelon Biosciences Incorporated)
- D-*myo*-Phospatidylinositol 3,4,5-trisphosphate D (+)-*sn*-1,2-di-*O*-octanoylglyceryl, 3-*O*-phospho linked (Echelon Biosciences Incorporated).

4.2.22. Pull-down of ExoU by phosphoinositide-coated agarose beads

HeLa cell lysate was prepared from HeLa cells transfected with pCMV-Tag2-ExoU_S142A in a 6-well plate. The cells were washed twice with ice-cold PBS and incubated with 200 µl ice-cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100 containing 10 µl protease inhibitor cocktail per ml) for 30 min on ice on an orbital shaker. The cells were scraped, transferred to a microcentrifuge tube and centrifuged at 16100 x g for 10 min at 4°C. The supernatant was then kept on ice or stored at -80°C until required.

The phosphoinositide-coated agarose beads (Echelon Bioscience Incorporated) were prepared by centrifuging 50 µl of a 50% slurry of each type of beads at 100 x g for 1 min at 4°C and removing the supernatant. The beads were incubated with the HeLa cell extract for 3 h with rotation at 4°C. The beads were then washed three times with 10x excess of ice-cold lysis buffer (minus the protease inhibitor cocktail). Proteins were eluted from the beads by adding 25 µl of 2 x SDS gel-loading sample buffer and heating to 95°C for 5 min. The beads were centrifuged at 16100 x g for 1 min and the supernatant was subjected to SDS-PAGE and immunoblotting with the anti-FLAG antibody as described in section 4.2.11.

4.2.23. Binding of ExoU to PIP strips

The ability of ExoU and mutant ExoU to bind to phospholipids immobilised on a nitrocellulose membrane was assessed using PIP Strips™ (Echelon Biosciences Incorporated). The membranes were blocked by incubating in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween-20) + 3% fatty acid free bovine serum albumin (BSA) and gently agitating for 1 h at room temperature. The membranes were then incubated for 3 h at room temperature with TBS-T + 3% BSA containing approximately 2.5 µg/ml ExoU or mutant ExoU prepared as described in section 4.2.20. Unbound ExoU or mutant ExoU was washed from the membrane by three 10 min incubations in TBS-T + 3% BSA. Bound ExoU was then detected by immunoblotting with an anti-6x His tag antibody as described in section 5.2.11 but with all incubation and washing steps carried out in TBS-T + 3% BSA.

4.3. RESULTS

4.3.1. ExoU is modified to a higher molecular weight form in HeLa cells

In order to study the expression and localisation of ExoU within mammalian cells, HeLa cells were transfected with a construct encoding wild type FLAG-tagged ExoU (pCMV-Tag2-ExoU). ExoU was not detected by immunoblotting at any time point between 12 and 36 hours following transfection (Fig. 4.1A). This observation is consistent with the findings of other investigators who have also failed to detect active ExoU expression in a number of different eukaryotic cell lines by immunoblotting or immunofluorescence. It has been shown that ExoU is so cytotoxic to eukaryotic cells, that as few as 300-600 toxin molecules are required to kill a cell. This potent cytotoxicity would explain the absence of detectable ExoU, because any cell expressing the toxin at an appreciable level will be killed.

Mutation of the active site serine residue at position 142 of ExoU renders the protein non-toxic as it is no longer able to act as a phospholipase. When HeLa cells were transfected with a construct encoding the FLAG-tagged ExoU S142A mutant (pCMV-Tag2-ExoU_S142A), a protein was observed migrating at the expected molecular weight of about 85 kDa (Fig. 4.1A, ExoU). Interestingly, there was an additional FLAG-tagged protein band migrating at about 20 kDa higher than the native ExoU band (Fig. 4.1A, ExoU*). The amount of this higher molecular weight band increased over time between 12 and 36 hours. This protein band must represent a covalently modified form of ExoU because any non-covalent modification would be destroyed by the denaturing conditions of the experiment.

During pseudomonal infection, the TTS toxins are secreted fully formed into the cytoplasm of the eukaryotic cell. In the transfection study described above, ExoU was transcribed and translated within the eukaryotic cell so it was important to establish that the observed modification was not an artefact of this non-physiological expression of ExoU.

When HeLa cells were infected with a strain of *P. aeruginosa* lacking all known TTS exoenzymes (PA103ΔTΔU), and the lysate immunoblotted with ExoU anti-serum, as

expected there was no ExoU detected (Fig. 4.1B). When this strain was complemented with a plasmid (pUCP19-exoU_S142A-spcU) expressing the ExoU S142A mutant and the cognate chaperone of ExoU, SpcU, both ExoU and modified ExoU proteins were observed (Fig. 4.1B, ExoU and ExoU*).

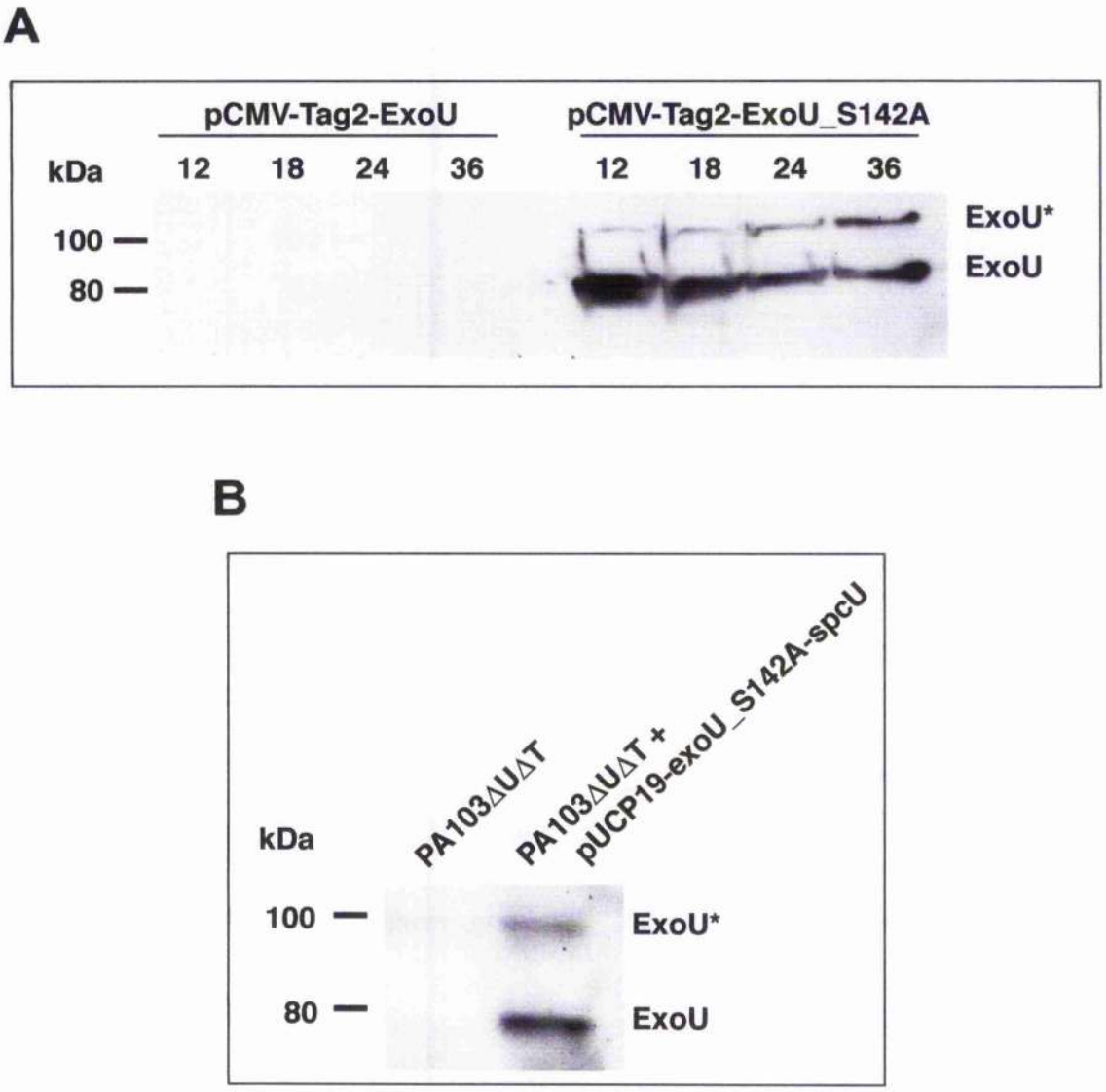


Fig. 4.1. Expression of ExoU in HeLa cells after transfection and infection.

A) HeLa cells were transfected with constructs encoding FLAG-tagged ExoU (pCMV_Tag2-ExoU) and FLAG-tagged lipase-inactive ExoU (pCMV_Tag2_ExoU_S142A). 12, 18, 24, and 36 hours post-transfection, HeLa cell lysates were prepared and immunoblotted with an anti-FLAG antibody. **B)** HeLa cells were infected with a Pseudomonal strain lacking all TTS toxins (PA103ΔTΔU) and this strain complemented with a construct encoding lipase-inative ExoU and its cognate chaperone, SpcU (PA103ΔTΔU + pUCP19-exoU_S142A-spcU). Three hours after infection, Hela lysates were prepared and immunoblotted with ExoU anti-serum. Native ExoU (ExoU) and modified ExoU (ExoU*) protein bands are indicated.

4.3.2. The C-terminus is required for ExoU modification

In order to determine whether the N-terminus of ExoU is required for this observed modification, a number of FLAG-tagged N-terminal deletion mutants of ExoU S142A were generated in the pCMV-Tag2 vector (Fig. 4.2A). Immunoblot analysis of lysates from HeLa cells transfected with the N-terminal deletion mutants revealed that deletion of up to the N-terminal 82 amino acids of ExoU did not abolish ExoU modification (Fig. 4.2B, N1-N7). When the N-terminal 122 amino acids of ExoU were deleted it was difficult to determine whether ExoU had been modified or not (Fig. 4.2B, N8). The larger the N-terminal deletion, the closer the modified and unmodified ExoU proteins migrated during SDS-PAGE; therefore, the modified ExoU band in the N8 deletion mutant may be obscured by the unmodified ExoU band.

To assess the importance of the C-terminus in ExoU modification, a number of C-terminal deletion mutants of FLAG-tagged ExoU S142A were constructed (Fig. 4.3A). The ability of these C-terminal deletion mutants to be modified was then assessed by Western blot analysis. The C-terminal deletion mutants C1 · · C6 were not modified when transfected into HeLa cells as shown by the absence of higher molecular weight protein bands in these samples (Fig. 4.3B, C1 – C6). The C-terminal deletion mutant C6 lacked only the last 9 amino acids of ExoU and yet was not capable of being modified to a higher molecular weight form. This indicates that the last 9 amino acids of ExoU, residues 679 to 687, are essential for ExoU modification. The C7 deletion mutant has a stop codon at residue 684 and this mutant was modified when transfected into HeLa cells (Fig. 4.3B, C7). This narrows down the region important for ExoU modification to the 5 amino acids between positions 679 and 683.

The C-terminal 9 amino acids of ExoU S142A (Fig. 4.4A) were individually mutated to alanine residues (apart from the alanine residue at position 680). All the alanine mutants retained their ability to be modified to a higher molecular weight form when transfected into HeLa cells apart from the W681A mutant (Fig. 4.4B). When the tryptophan residue at position 681 was substituted with an alanine, the level of modified ExoU observed was greatly reduced. Although this Western blot does not show a band for modified ExoU in the W681A mutant sample (Fig. 4.4B, W681A), when the blot was exposed to photographic film for a lot longer, a faint band was detectable.

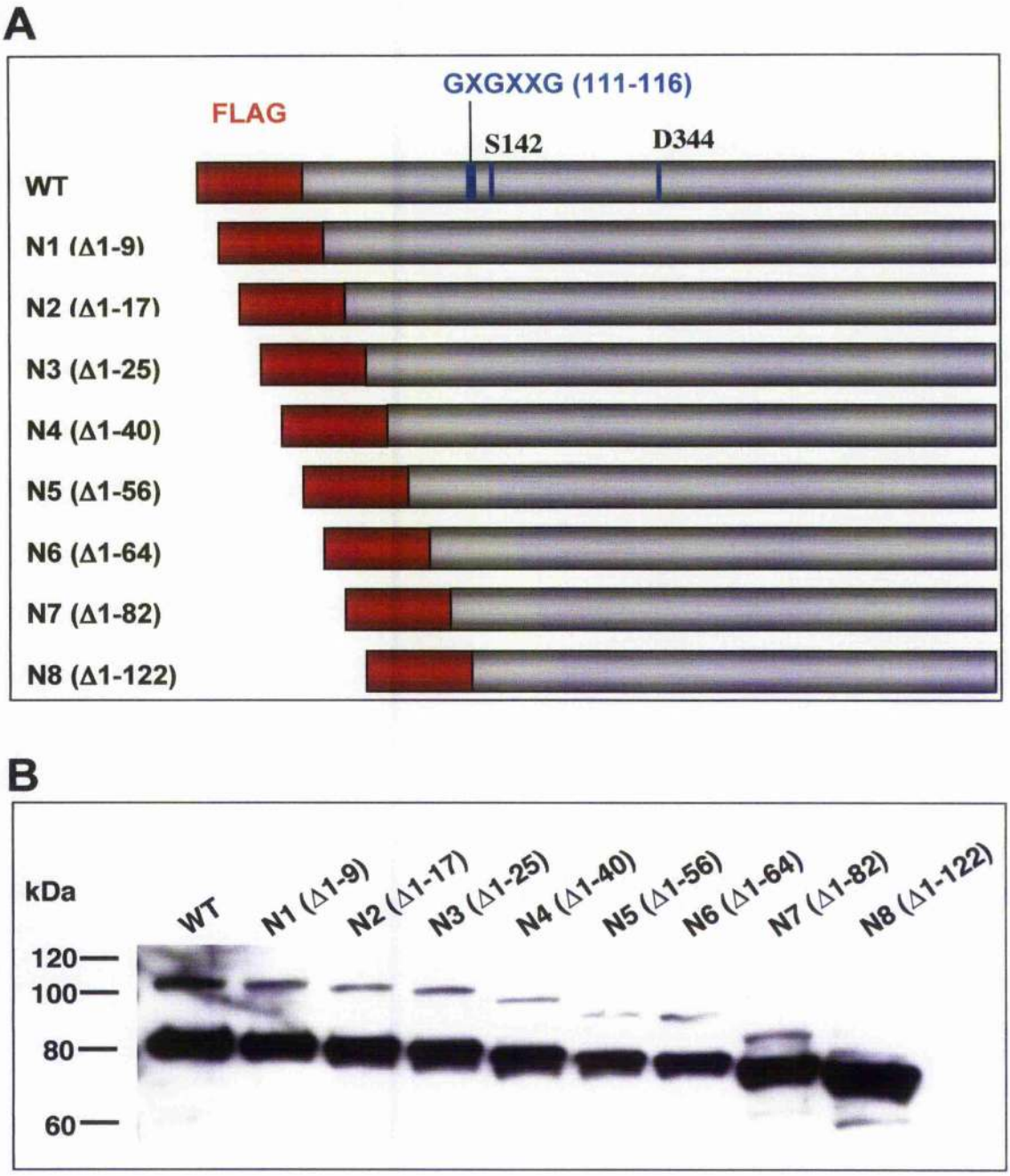


Fig. 4.2. Modification of N-terminal deletion mutants of ExoU.

A) A number of ExoU N-terminal deletion mutants (N1 - N8) were generated in pCMV-Tag2-ExoU_S142A. The size of the truncations (not to scale) are indicated in relation to the important catalytic residues of ExoU; these are shown as GXGXXG (111-116), S142 amd D344. The FLAG tag is shown in red. **B)** HeLa cells were transfected with pCMV-Tag2-ExoU_S142A (WT) and the N-terminal deletion mutants (N1 – N8) and the lysates were immunoblotted with an anti-FLAG antibody.

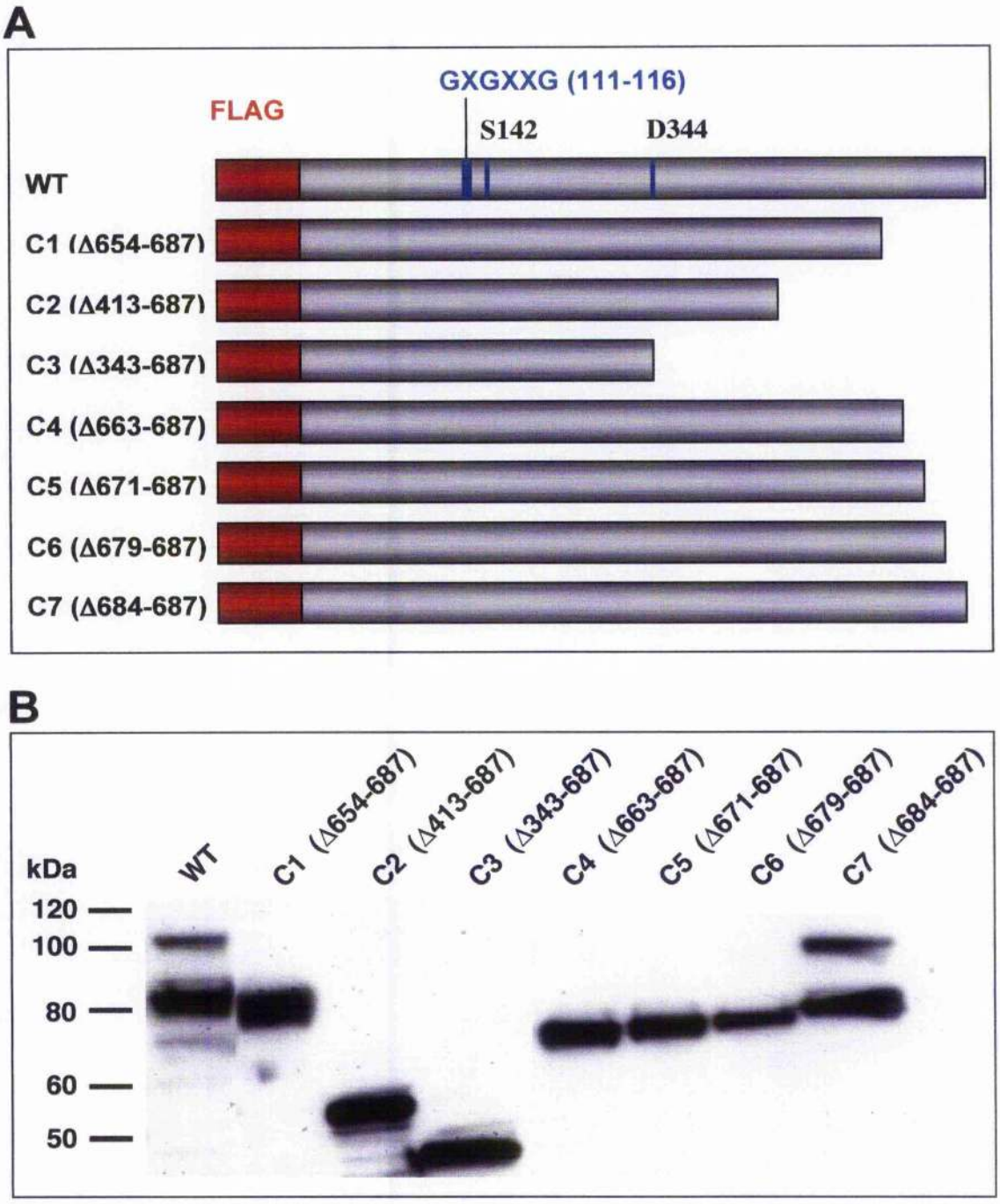


Fig. 4.3. Modification of C-terminal deletion mutants of ExoU.

A) A number of ExoU C-terminal deletion mutants (C1–C7) were generated in pCMV-Tag2-ExoU_S142A. The size of the truncations (not to scale) are indicated in relation to the important catalytic residues of ExoU; these are shown as GXGXXG (111-116), S142 amd D344. The FLAG tag is shown in red. **B)** HeLa cells were transfected with pCMV-Tag2-ExoU_S142A (WT) and the C-terminal deletion mutants and the lysates were immunoblotted with an anti-FLAG antibody.

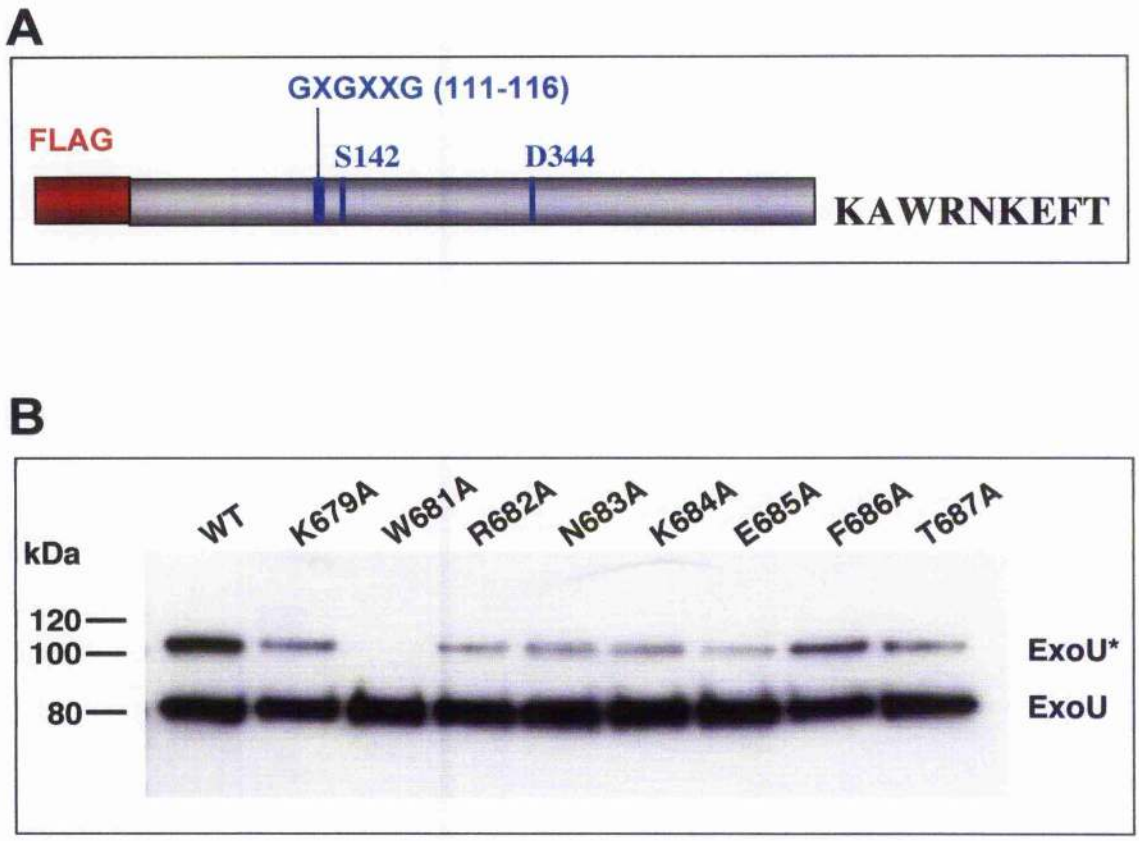


Fig. 4.4. Modification of C-terminal alanine substitution mutants of ExoU.

A) The final 9 amino acids of ExoU in pCMV-Tag2-ExoU_S142A, shown to the right of the FLAG-tagged ExoU molecule, were individually mutated to alanine. **B)** HeLa cells were transfected with pCMV-Tag2-ExoU_S142A (WT) and the C-terminal alanine substitution mutants and the lysates were immunoblotted with an anti-FLAG antibody. Native ExoU (ExoU) and modified ExoU (ExoU*) protein bands are indicated.

4.3.3. ExoU is diubiquitinated at lysine residue 178

We have demonstrated that ExoU is modified to a higher molecular weight form after transfection and infection of HeLa cells. This modification is dependent on the C-terminal region between amino acids 679 and 683, with the tryptophan at residue 681 being particularly important. It is unlikely that this tryptophan is the modified residue because a small amount of modification does occur in the W681A mutant. To determine what this modification was, the modified ExoU protein was analysed by mass spectrometry.

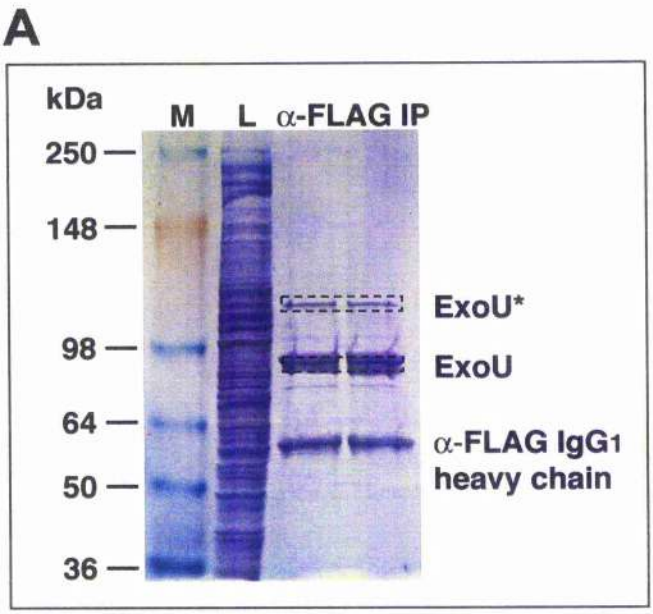
The modified ExoU protein was prepared by immunoprecipitating the lysate of HeLa cells transfected with pCMV-Tag2-ExoU_S142A with anti-FLAG M2 affinity gel and subjecting the 2 x SDS gel loading buffer eluted material to SDS-PAGE (Fig. 4.5A). The gel was stained with commassie blue and the modified ExoU band (Fig. 4.5A, ExoU*) was cut out of the gel. The gel band was destained, reduced and alkylated and then the protein was digested with trypsin. The trypsin-digested peptides were purified by reverse phase liquid chromatography before being analysed by tandem mass spectrometry. Analysis of the peptide mass spectral data using a bacterial database confirmed the protein to be ExoU from *P. aeruginosa*. Using a human protein database, numerous peptides from ubiquitin were also identified (Fig. 4.5B, red amino acids). Ubiquitin is a 76 amino acid protein whose C-terminal glycine carboxy group can form an isopeptide bond with the ε-amino group of a lysine residue in a target protein. Further analysis of the peptide mass spectral data from the modified ExoU protein revealed that ubiquitin was conjugated to lysine residue 178 of ExoU. Similar analysis of the unmodified ExoU protein showed that it was not modified by ubiquitin. Mass spectrometry also revealed that serine residue 30 is probably phosphorylated in both the unmodified and ubiquitinated toxins to a small degree.

The molecular weights of the complete unmodified and modified forms of immunoprecipitated ExoU were then determined by mass spectrometry. The molecular weight of the unmodified ExoU was measured at 75,493.0 Da, compared to a theoretical value of 75,489.0 Da. The theoretical value takes into account both the S142A mutation and the P447L variant (section 3.3.1), as well as N-terminal acetylation (the ExoU N-terminal peptide with an acetyl group was observed during the mass spectrometry

analysis). The consistent 4 Da discrepancy between the measured and theoretical molecular weights for unmodified ExoU is slightly higher than the estimated error of ± 1-2 Da. This difference in molecular weights may be accounted for by multiple deaminations, amino acid substitutions or residual salt in the sample. The measured molecular weight of the modified ExoU was 17,094.4 Da higher than that of the unmodified ExoU, which could only be accounted for by the addition of two ubiquitin residues per molecule of ExoU (theoretical increase 17.093.6 Da).

Ubiquitin itself can be ubiquitinated at any one of its 7 lysine residues resulting in a polyubiquitin chain. The presence of one ubiquitination site in ExoU and the observation that the increase in molecular weight is accounted for by the addition of two ubiquitin moieties per ExoU molecule, suggests that ExoU is diubiquitinated at lysine residue 178. Further analysis of the mass spectral data obtained from the tryptic digest of modified ExoU showed that two different ubiquitin linkages could be identified, involving lysines at positions 48 and 63. However, the lysine 63 linked form was present at much higher abundance than the lysine 48 linked form.

To confirm that the modified ExoU band represents ExoU diubiquitinated at lysine residue 178, we examined whether mutation of this lysine to an arginine abolished ExoU modification. HeLa cells transfected with the empty pCMV-Tag2 vector and immunoprecipitated with anti-FLAG affinity gel, as expected did not have any protein bands when immunoblotted with anti-ubiquitin or anti-FLAG antibodies (Fig. 4.6, pCMV-Tag2). Western blot analysis of immunoprecipitaed HeLa cell lysates from cells transfected with pCMV-Tag2-ExoU_S142A revealed that the modified band of ExoU contained ubiquitin (Fig. 4.6, ExoU_S142A). When HeLa cells were transfected with the K178R mutant of ExoU S142A and the lysate was immunoprecipitated with anti-FLAG agarose beads, immunoblotting with an anti-ubiquitin antibody revealed no bands and when the sample was immunoblotted with an anti-FLAG antibody there was no higher molecular weight form of ExoU (Fig. 4.6, ExoU_S142A_K178R). These results confirm that the presence of the higher molecular weight band when HeLa cells are transfected or infected with ExoU is a consequence of ubiquitination of lysine residue 178 of ExoU.



B

Ubiquitin:

MQIFVKTLTG KTITLEVEPS DTIENVKAKI

QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN

Fig. 4.5. Immunprecipitation and mass spectrometry analysis of modified ExoU.
A) HeLa cells were transfected with pCMV-Tag2B-ExoU_S142A and the lysate was immunoprecipitated with anti-FLAG agarose beads. A size marker (M), total lysate of HeLa cells transfected with pCMV-Tag2B-ExoU_S142A (L) and the proteins immunoprecipitated from the lysate with anti-FLAG agarose beads (α-FLAG IP) were subjected to SDS-PAGE and the gel was coomassie stained. ExoU, modified ExoU (ExoU*) and the α-FLAG IgG_1 heavy chain protein bands are identified. The dashed boxes indicate the portions of gel excised and subjected to mass spectroscopy analysis. **B)** Tandem mass spectrometry analysis revealed that the modified ExoU band contained ExoU and ubiquitin. The ubiquitin peptides identified by mass spectrometry are indicated in red.

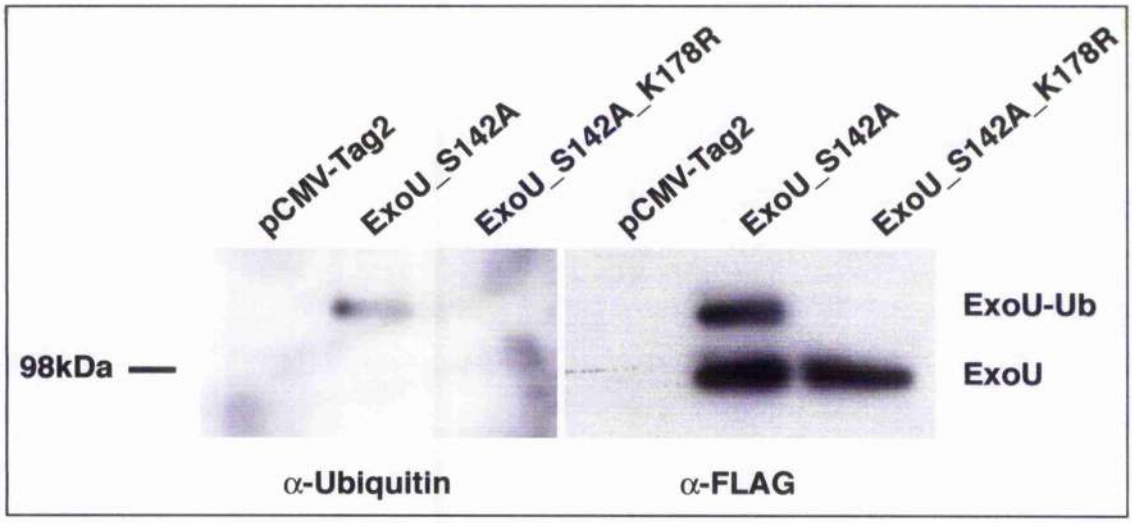


Fig. 4.6. Ubiquitination of lysine residue 178 in ExoU.

HeLa cells were transfected with an empty vector control (pCMV-Tag2), this vector encoding lipase-inactive ExoU (ExoU_S142A) or pCMV-Tag2 encoding lipase-inactive ExoU containing the K178R substitution mutation (ExoU_S142A_K178R). Lysates were immunopreciptated with α-FLAG agarose beads and eluted proteins were immunoblotted with α-ubiquitin then α-FLAG antibodies. ExoU and ubiquitinated ExoU (ExoU-Ub) protein bands are indicated.

4.3.4. Co-immunoprecipitation and mass spectrometry analysis failed to identify the eukaryotic cofactor for ExoU.

The immunoprecipitation reaction we carried out in order to purify the unmodified and modified ExoU protein bands (section 4.3.3) could also be utilised to identify potential cofactors of ExoU. Any protein that bound to ExoU would co-immunoprecipitate with FLAG-tagged ExoU and potentially be visible on an SDS polyacrylamide gel. When we carried out the initial immunoprecipitation, there were a few faint protein bands on the SDS polyacrylamide gel in addition to the ExoU, diubiquitinated ExoU and FLAG antibody IgG_1 heavy chain bands (Fig. 4.5A). We therefore scaled up our transfection and immunoprecipitation reaction 4-fold in an attempt to increase the abundance of any potential ExoU binding proteins. We also eluted using the 3 x FLAG peptide to avoid complication of our analysis by the IgG_1 heavy and light chains of the FLAG antibody. Proteins immunoprecipitated from HeLa cells transfected with pCMV-Tag2 or pCMV-Tag2-ExoU_S142A were subjected to SDS-PAGE and the gel was stained with commassie blue. The negative control immunoprecitation reaction, in which HeLa cells were transfected with the empty pCMV-Tag2 vector, yielded only one faint band on the SDS polyacrylamide gel at about 60 kDa that probably corresponds to the FLAG antibody IgG_1 heavy chain (Fig. 4.7). The immunoprecipitated sample from HeLa cells transfected with pCMV-Tag2-ExoU_S142A exhibited a number of bands. In addition to the ExoU, diubiquitinated ExoU and FLAG antibody IgG_1 heavy chain protein bands, we observed a number of faint bands (Fig. 4.7, 1 - 7) that could represent interacting proteins. Bands 1 – 7 were excised from the gel and analysed by mass spectrometry. In addition to ExoU, ubiquitin and keratins (a common contaminant in mass spectrometry analysis) four minor proteins were identified. The CD98 cell-surface antigen heavy chain was identified in bands 1 and 2. A mitochondrial 60 kDa heat shock protein was found in band 5. Analysis of band 6 identified the 52 kDa Ro/SSA protein and alpha enolase. Of these proteins, the 52 kDa Ro/SSA protein was the most abundant.

We decided to investigate whether CD98 and 52 kDa Ro/SSA do co-immunoprecipitate with ExoU. As a positive control for CD98, HeLa cell lysate was immunoblotted with an anti-CD98 antibody and CD98 was detected (Fig. 4.8A, HeLa lysate). When anti-FLAG M2 affinity gel was used for immunoprecipitation of HeLa cell lysates transfected with pCMV-Tag2 or pCMV-Tag2-ExoU_S142A, the 2 x SDS gel loading

buffer eluted samples did not contain CD98 (Fig. 4.8A, α-FLAG IP). This indicates that the CD98 cell-surface antigen heavy chain did not interact with ExoU and its identification in the initial co-immunoprecipitation was spurious.

Western blot analysis of HeLa cell lysate with an anti-52 kDa Ro/SSA antibody identified the 52 kDa Ro/SSA protein (Fig. 4.8B, HeLa lysate). When HeLa cell lysates of cells transfected with pCMV-Tag2 or pCMV-Tag2-ExoU_S142A were immunoprecipitated with anti-FLAG affinity gel and eluted with 2 x SDS gel loading buffer, two protein bands of just over 50 kDa were visible in both samples after immunoblotting with an anti-52 kDa Ro/SSA antibody (Fig. 4.8B, α-FLAG IP). The slower migrating protein band corresponds to the heavy chain of the FLAG IgG_1 antibody (Fig. 4.8B, α-FLAG IgG_1 heavy chain) and was detected because both the anti-52 kDa Ro/SSA and anti-FLAG antibodies were raised in mice. The faster migrating band in the immunoprecipitated samples was the 52 kDa Ro/SSA protein band as it co-migrated with the protein detected in the HeLa lysate (Fig. 4.8B, 52kDa Ro/SSA). The presence of the 52 kDa Ro/SSA protein in both the pCMV-Tag2 and pCMV_Tag2-ExoU_S142A immunoprecipitated samples suggests that the 52 kDa Ro/SSA protein did not bind specifically to ExoU but interacted directly with the anti-FLAG agarose.

It is apparent that the identification of CD98 and the 52 kDa Ro/SSA proteins in the anti-FLAG ExoU immunoprecipitated sample was spurious as we have demonstrated that these proteins do not interact with ExoU. Further analysis of bands 1-7 from our immunoprecipitation reaction revealed that bands 1-3 appear to be variously ubiquitinated ExoU and bands 4-7 are mainly ExoU fragments. Bands 1-3 may represent ExoU modified with 4, 3, and 1 ubiquitin moieties respectively. In addition to the lysine residue 178 of ExoU being ubiquitinated, there is also some ubiquitination of lysine residue 428 observed in bands 1 and 2. In band 1, lysine 428 is 30-40% occupied and in band 2 there is a low level of lysine 428 ubiquitination. In contrast, lysine 178 of ExoU is almost fully occupied in both these bands.

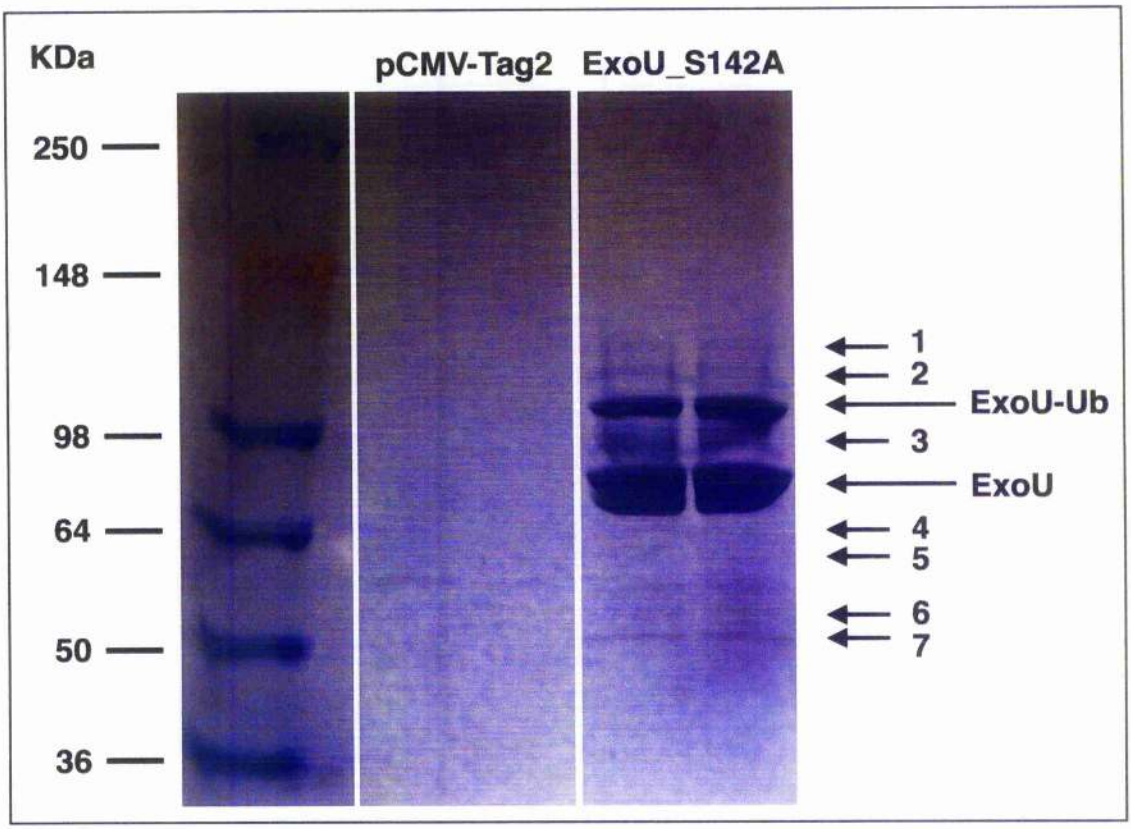


Fig. 4.7. Attempted identification of the eukaryotic cofactor of ExoU by co-immunoprecipitation.

HeLa cells were transfected with the empty pCMV-Tag2 vector or pCMV-Tag2-ExoU_S142A and immunoprecipitated with anti-FLAG affinity gel. Eluted proteins were subjected to SDS-PAGE and the resulting gel was coomassie stained. ExoU and ubiquitinated ExoU protein bands are indicated (ExoU and ExoU-Ub) in addition to 7 minor protein bands (1-7) that were unique to the ExoU_S142A sample.

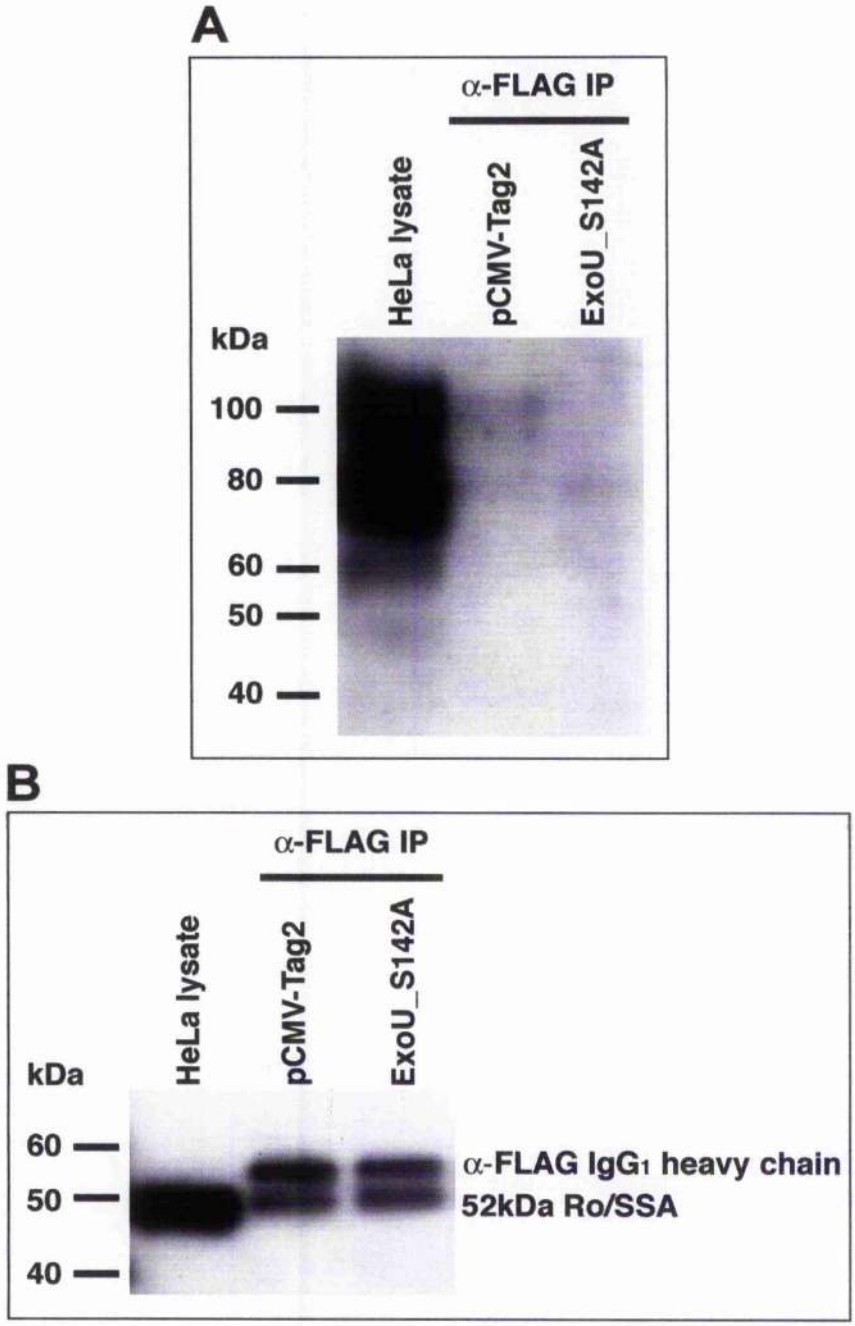


Fig. 4.8. Testing the co-immunoprecipitation of CD98 and 52kDa Ro/SSA with ExoU.

HeLa cells transfected with the empty pCMV-Tag2 vector or pCMV-Tag2-ExoU_S142A were immunoprecipitated with anti-FLAG affinity gel. Eluted proteins were immunoblotted with an anti-CD98 antibody (**A**) or an anti-52kDa Ro/SSA antibody (**B**). As a positive control, immunoblotting of HeLa lysate with anti-CD98 and anti-52kDa Ro/SSA was also carried out. The anti-FLAG IgG_1 heavy chain and 52kDa Ro/SSA protein bands are indicated in the anti-52kDa Ro/SSA immunoblot.

4.3.5. Effect of ubiquitination on turnover of ExoU

The most studied consequence of ubiquitination of a protein is to target that protein for destruction by the proteosome. For a protein to be targeted for proteolysis it requires a polyubiquitin chain of at least four ubiquitin molecules. We have demonstrated that ExoU is mainly diubiquitinated, therefore rendering it unlikely to be targeted for proteasomal degradation. Protein ubiquitination, however, is involved in a number of other processes, for example protein localisation, transcriptional activation, chromatin structure, kinase activation, DNA repair and ribosome function. Ubiquitination can also result in protein degradation by the lysosome. To determine whether diubiquitination of ExoU leads to its degradation, we compared the kinetics of ubiqutinated ExoU degradation to that of unmodified ExoU.

HeLa cells transfected with pCMV-Tag2-ExoU_S142A were treated with cyclohexamide to prevent further protein synthesis. At a number of time points after cycloheaxamide treatment, lysates were prepared and immunoblotted with anti-FLAG (Fig. 4.9A). The amount of protein in each band was measured as a percentage of that observed at 0 hours (Fig. 4.9B). It is evident that the amount of ExoU exceeded that of ubiquitinated ExoU at all time points (Fig. 4.9A) and that both forms of the protein decreased over time after treatment with cyclohexamide (Fig. 4.9B). Although the initial decrease in the amount of ExoU and modified ExoU was similar, after 12 hours there was significantly more degradation of ubiquitinated ExoU compared to non-ubiquitinated ExoU. Unmodified ExoU decayed with a half-life of between 7-8 hours in contrast to ubiquitinylated ExoU which consistently turned over rather faster, with a half-life between 5-6 hours (Fig. 4.9B). By 24 hours, there was no detectable ubiquitinated ExoU but still about 13% of unmodified ExoU compared to that observed in the zero hour sample. Thus, diubiquitination of ExoU appeared to lead to a small but significant increase in degradation compared to the non-ubiquitinated form.

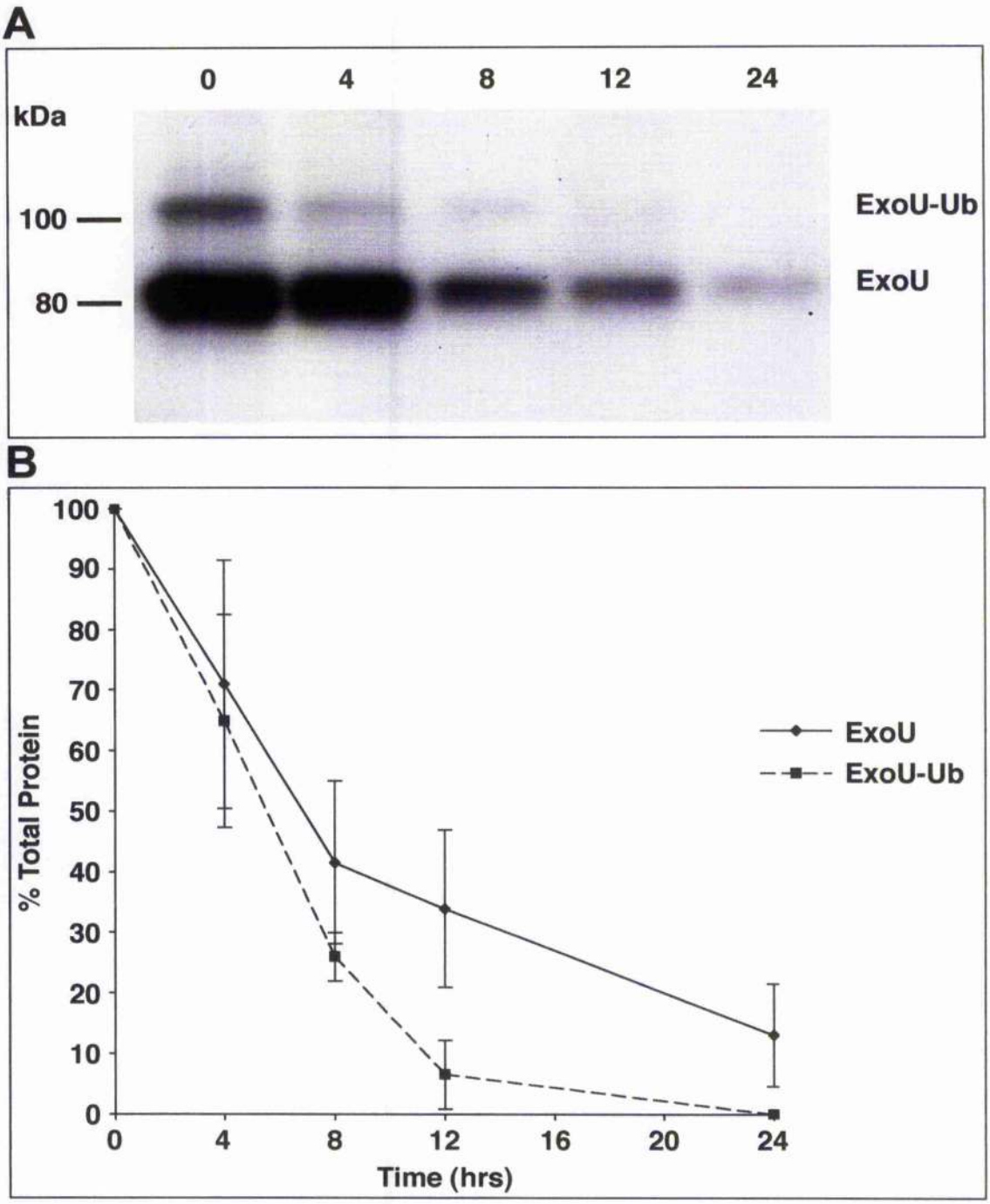


Fig. 4.9. Half-life of ExoU and ubiquitinated-ExoU in HeLa cells.

A) HeLa cells were transfected with pCMV-Tag2-ExoU_S142A and treated with cyclohexamide 12 hours after transfection. Lysates prepared 0, 4, 8, 12 and 24 hours after cyclohexamide treatment were immunoblotted with an anti-FLAG antibody. **B)** The intensity of each ExoU (ExoU) and ubiquitinated ExoU (ExoU-Ub) band was measured and expressed as a percentage of the total protein in the ExoU and ubiquitinated ExoU bands at the 0 time point. The experiment was carried out in triplicate and the average values were plotted with standard deviation bars.

4.3.6. ExoU is localised to the plasma membrane after transfection

In agreement with our immunoblot analysis, ExoU was not detected by immunoflourescence when HeLa cells were transfected with a construct encoding FLAG-tagged ExoU (Fig. 4.10A, ExoU). However, when pCMV-Tag2-ExoU_S142A was transfected into HeLa cells, the phospholipase inactive FLAG-tagged ExoU was visible by immunofluorescent staining (Fig. 4.10A, S142A). Immunofluorescence of HeLa cells transfected with ExoU S142A revealed that ExoU localised specifically to the plasma membrane (Fig. 4.10A, S142A). The distribution of ExoU at the plasma membrane appeared punctate in nature, with ExoU seeming to cluster in small dot-like regions around the cell. Some ExoU staining was also observed in the cytoplasm of the HeLa cells.

To confirm the sub-cellular localisation of ExoU S142A, the lysate of HeLa cells transfected with pCMV-Tag2-ExoU_S142A was fractionated into a particulate membrane-enriched fraction and a cytosolic fraction by ultracentrifugation. Immunoblotting these two fractions with an anti-FLAG antibody showed that the majority of ExoU separated with the particulate membrane–enriched fraction while a smaller amount of ExoU was found in the cytosolic fraction (Fig. 4.10B, top panel). It was also evident that diubiquitinated ExoU was localised exclusively in the particulate fraction of HeLa cells (Fig. 4.10B, ExoU*). To assess the success of the fractionation technique the same membrane was stripped and reprobed with an anti-calnexin antibody and an anti-GAPDH antibody. The integral membrane protein calnexin was detected exclusively in the particulate membrane-enriched fraction (Fig. 4.10B, middle panel) while the cytosolic protein GAPDH separated predominantly with the cytosolic fraction (Fig. 4.10B, bottom panel). There was a small amount of GAPDH detected in the particulate fraction (Fig. 4.10B, bottom panel) that was probably the consequence of the incomplete removal of the supernatant from the pellet during the fractionation protocol.

When HeLa cells were transfected with FLAG-tagged ExoU S142A and stained using an anti-FLAG antibody without prior permeabilisation of the cells, no ExoU was detected (results not shown). This suggests that ExoU was not localised to the outside of the cell, but remained localised at the inner plasma membrane.

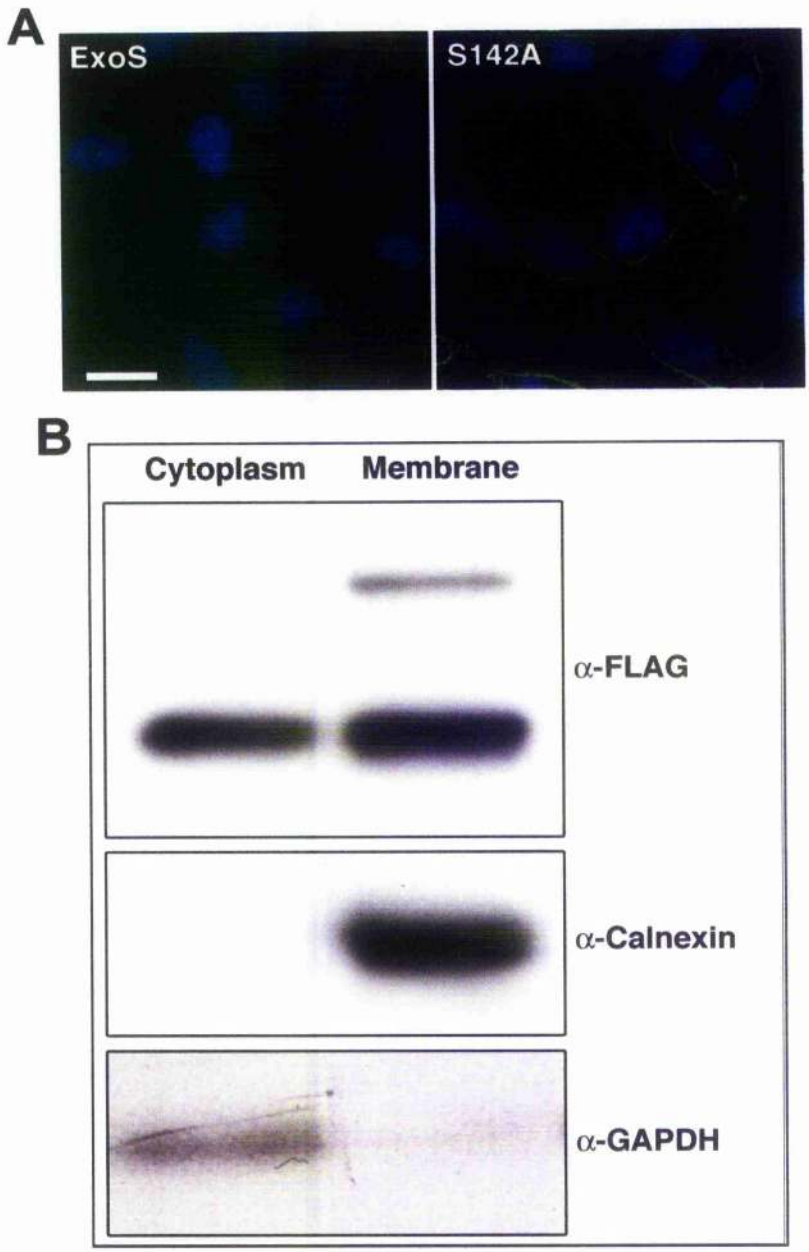


Fig. 4.10. Localisation of ExoU in HeLa cells after transfection.

A) HeLa cells were transfected with pCMV-Tag2-ExoS (ExoS) or pCMV-Tag2-ExoS_S142A (S142A) and stained for ExoU (green) after 16 h. Deconvolved images are shown; nuclei are counterstained with DAPI. Scale bar = 10 μm. **B)** HeLa cells transfected with pCMV-Tag2-ExoU_S142A were fractionated into cytoplasm and particulate membrane-enriched fragments and analysed for ExoU by immunoblotting with an anti-FLAG antibody. The blot was stripped and reprobed with antibodies for calnexin (membrane protein) and GAPDH (cytoplasmic protein) to guage purity of fractions.

4.3.7. The C-terminus is essential but not sufficient for ExoU localisation

To determine which regions of ExoU were required for localisation to the plasma membrane, we assessed the localisation of the N-terminal deletion mutants N7 and N8 (Fig. 4.2A) and the C-terminal deletion mutants C1 – C7 (Fig. 4.3A) of ExoU S142A in transfected HeLa cells. Immunofluorescent analysis showed that the N-terminal 122 amino acids were not required for localisation of ExoU S142A to the plasma membrane as both N7 and N8 retained the wild type plasma membrane localisation phenotype (Fig. 4.11, N7 and N8). The C-terminal deletion mutants C1 - C6 did not localise to the plasma membrane but were situated uniformly throughout the cytoplasm (Fig. 4.11, C1 – C6). In contrast, the C7 C-terminal deletion mutant of ExoU S142A, which is truncated four amino acids before the end of the protein, did localise to the plasma membrane (Fig. 4.11, C7). These results indicate that the region between amino acids 679 and 683 that is required for diubiquitination of ExoU is also required for localisation to the plasma membrane.

Plasma membrane localisation of the C-terminal alanine substitution mutants (Fig. 4.4A) also correlated with diubiquitination of ExoU. Mutation of only the tryptophan residue at position 681 altered localisation of ExoU S142A. When any of the other residues in the C-terminal 9 amino acids of ExoU were substituted with alanine, they all retained their plasma membrane localisation (Fig. 4.12). In contrast, the W681A mutant was mainly found in the cytosol of HeLa cells with only a small amount being associated with the plasma membrane (Fig. 4.12, W681A).

These results indicate that the C-terminal region between amino acids 679 and 683, and particularly the tryptophan at position 681, are required for plasma membrane localisation. To determine whether the C-terminal region is sufficient for plasma membrane localisation, a construct containing the C-terminal 33 amino acids of ExoU fused to EGFP was transfected into HeLa cells and the localisation of EGFP assessed. Both EGFP and EGFP fused to the C-terminal 33 amino acids of ExoU showed uniform staining throughout the cytoplasm (Fig. 4.13). This demonstrated that the C-terminal 33 amino acids of ExoU were not sufficient for plasma membrane localisation.

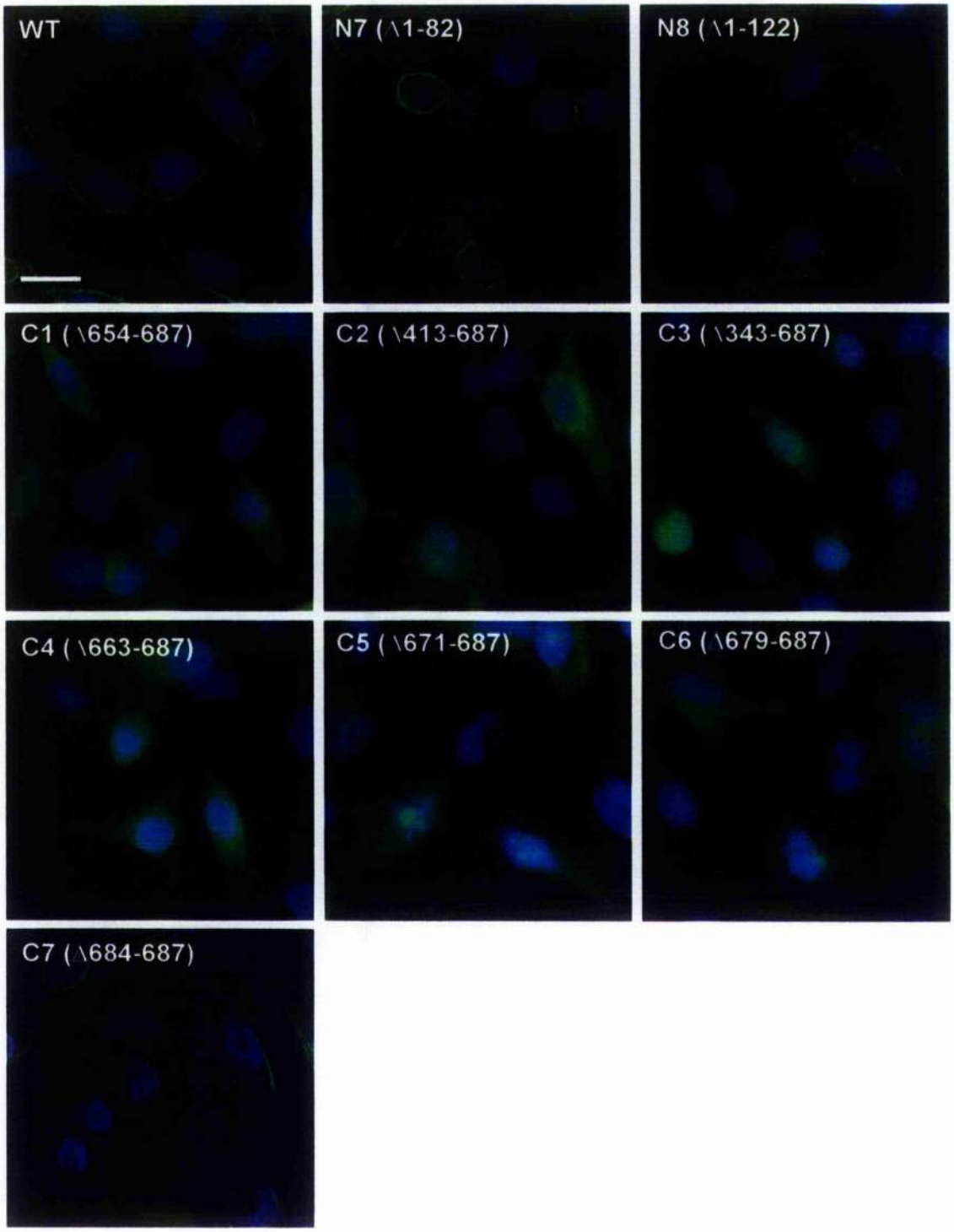


Fig. 4.11. Localisation of N- and C-terminal deletion mutants of ExoU.

HeLa cells were transfected with pCMV-Tag2-ExoS_S142A (WT) or various N- or C-terminal deletion mutants of pCMV-Tag2-ExoS_S142A and stained for ExoU (green) after 16 h. Deconvolved images are shown; nuclei are counterstained with DAPI. Scale bar = 10 μm.

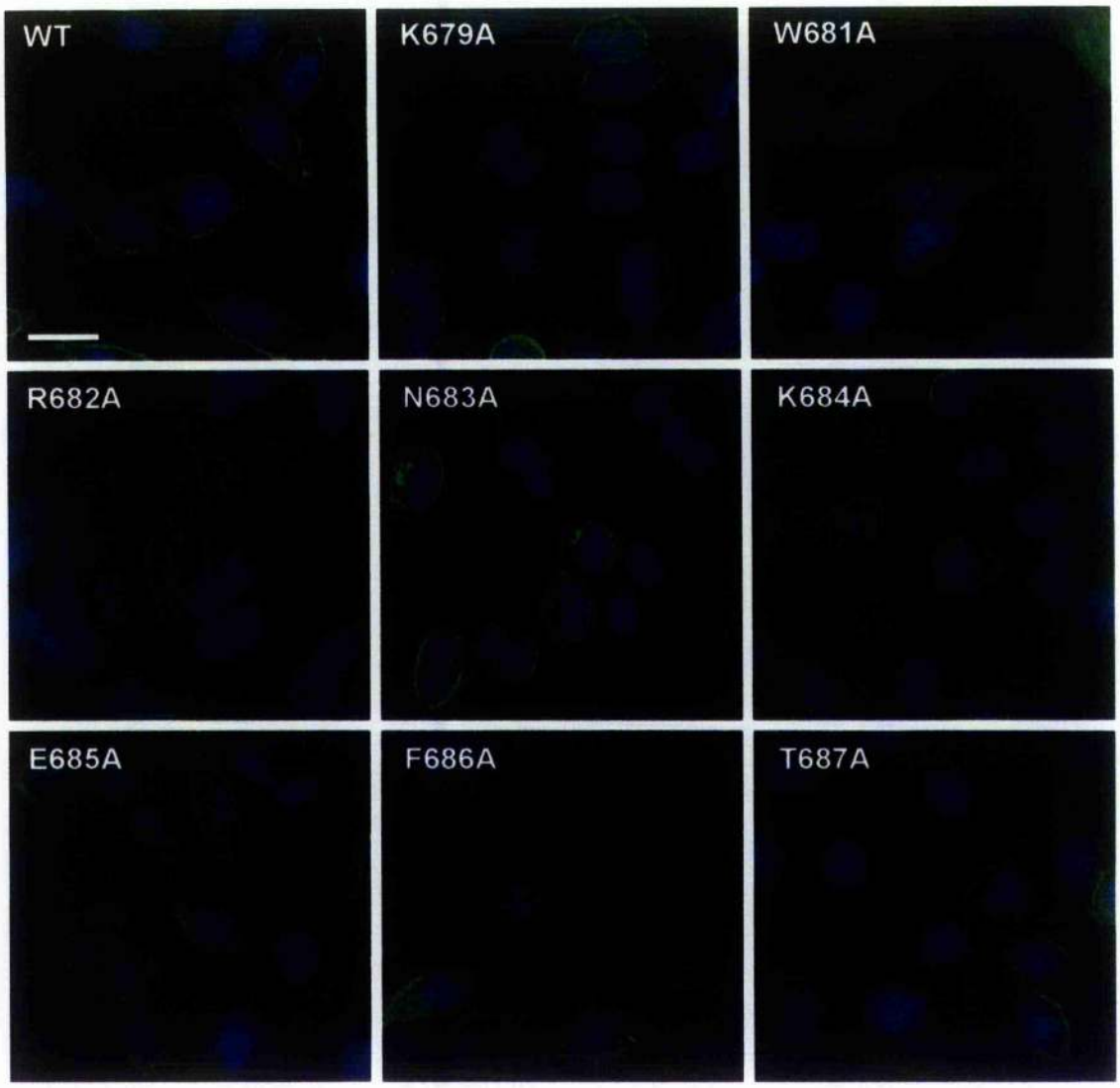


Fig. 4.12. Localisation of the C-terminal amino acid mutants of ExoS.

HeLa cells were transfected with pCMV-Tag2-ExoS_S142A (WT) or various C-terminal amino acid substitution mutants of pCMV-Tag2-ExoS_S142A and stained for ExoU (green) after 16 h. Deconvolved images are shown; nuclei are counterstained with DAPI. Scale bar = 10 μm.

Fig. 4.13. Localisation of an EGFP-ExoU C-terminal fusion protein.

Representative fields showing fluorescence of cells transfected with constructs encoding EGFP or a fusion protein of EGFP with the C-terminal 33 amino acids of ExoU (EGFP-ExoU653-687). Scale bar = 10 μm.

4.3.8. Ubiquitination is not required for plasma membrane localisation

The experiments above demonstrated that the C-terminal deletion mutants of ExoU S142A that were not diubiquitinated were also not localised to the plasma membrane and the W681A mutant that showed only a very small amount of modification also showed a greatly reduced plasma membrane localisation. This correlation between the residues required for the two phenotypes and the observation that the diubiquitinated form of ExoU is found exclusively in the particulate membrane-enriched fraction of HeLa cell lysates, suggests that diubiquitination is either the cause or consequence of plasma membrane localisation.

To distinguish between the possibilities that diubiquitination results in plasma membrane localisation or plasma membrane localisation results in diubiquitination, we examined the localisation of the K178R mutant of ExoU S142A. When HeLa cells were transfected with a construct encoding ExoU S142A K178R, immunofluorescent staining showed that this mutant retained the wild type membrane localisation phenotype (Fig. 4.14, K178R). Thus, abolishing the ability of ExoU to be diubiquitinated did not prevent plasma membrane localisation. Diubiquitination must therefore only occur after prior localisation of ExoU to the plasma membrane and the requirement for the C-terminus in diubiquitination is probably a consequence of its role in plasma membrane localisation.

The mass spectroscopy results indicated that there was also a small amount of ubiquitination of lysine residue 428 in ExoU (section 4.3.4). To establish whether ubiquitination at this site effected ExoU localisation, we assessed the localisation of the K428R mutant of ExoU S142A. The K428R mutant localised to the plasma membrane of HeLa cells (Fig. 4.14, K428R) showing that modification of this residue had no role in plasma membrane localisation.

Fig. 4.14. Localisation of ubiquitination mutants of ExoU.

HeLa cells were transfected with pCMV-Tag2-ExoS_S142A (WT) or the lysine K178R or K428R mutants of pCMV-Tag2-ExoS_S142A and stained for ExoU (green) after 16 h. Deconvolved images are shown; nuclei are counterstained with DAPI. Scale bar = 10 μm.

4.3.9. Localisation after infection mirrors that observed after transfection

When HeLa cells were transfected with constructs encoding ExoU, the toxin was expressed using the eukaryotic transcription and translation machinery located at the rough endoplasmic reticulum. Thus, trafficking of the toxin to the plasma membrane must occur from this site. During infection of mammalian cells with *P. aeruginosa* the ExoU toxin would be secreted fully formed into the cytoplasm of the cell. As a consequence, any trafficking and subsequent localisation must start from the cytosol at the point of ExoU injection. We therefore decided to establish whether ExoU was localised to the plasma membrane after infection of HeLa cells and if the regions of ExoU essential for localisation after transfection were also required for localisation after infection.

HeLa cells that were infected with the pseudomonal strain PA103 lacking all knonw TTS toxins showed only background staining when immunostained with ExoU anti-serum (Fig. 4.15, PA103ΔTΔU). When HeLa cells were infected with PA103ΔTΔU complemented with the pseudomonal expression plasmid pUCP19 containing *exoU* S142A and *spcU*, staining revealed that ExoU was injected into the cytoplasm of eukaryotic cells by the TTS system localised to the plasma membrane of these cells (Fig. 4.15, S142A). Examining the localisation of various ExoU mutants in HeLa cells after secretion by *P. aeruginosa* also generally mirrored the results obtained from our transfection studies. The mutation of the diubiquitinated lysine residue 178 did not prevent plasma membrane localisation (Fig. 4.15, K178R) confirming that diubiquitination is not required for plasma membrane localisation. The importance of the C-terminus in plasma membrane localisation was strengthened by the observation that deletion of the last 9 amino acids of ExoU abolished plasma membrane localisation after infection (Fig. 4.15, C6) but deletion of the last 4 amino acids did not (Fig. 4.15, C7). However, when the W681A mutant of ExoU S142A was injected into HeLa cells by the TTS system, localisation of this mutant to the plasma membrane was observed (Fig. 4.15, W681A).

In order to examine how ExoU was trafficked from the cytoplasm of eukaryotic cells after injection by the TTS system, we generated a construct that encoded EGFP fused to the C-terminus of ExoU in the pUCP19-exoU_S142A-spcU vector. Although fusion of

EGFP to the C-terminus of ExoU, which I have previously shown to be important in localisation, may alter the distribution of ExoU, fusion to the N-terminus would have interfered with secretion of the toxin into the eukaryotic cell. When HeLa cells where infected with PA103ΔTΔU either lacking or containing the plasmid encoding the ExoU S142A EGFP fusion protein no EGFP fluorescence was observed (data not shown), which is in agreement with other studies that show that the TTS system cannot translocate GFP. No conclusions could therefore be drawn as to how ExoU gets from the cytoplasm of eukaryotic cells to their plasma membranes.

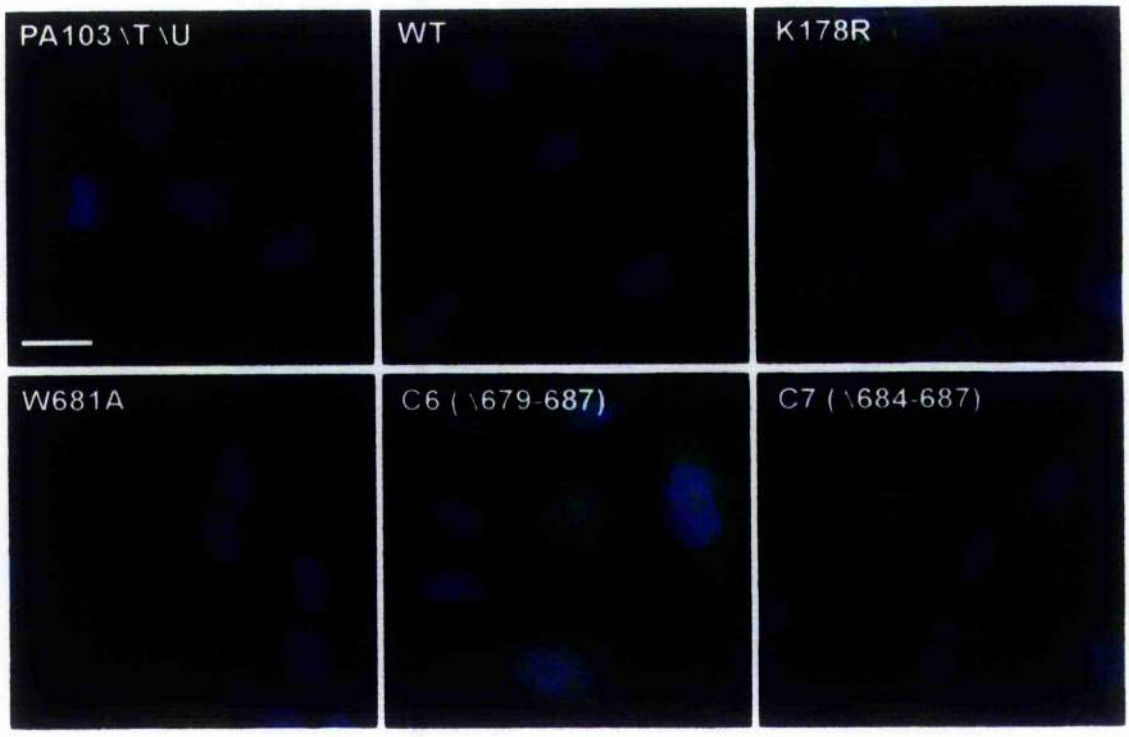


Fig. 4.15. Localisation of ExoU and ExoU mutants after infection.

Representative fields showing anti-ExoU immunoflourescent staining of HeLa cells infected with PA103 with the known TTS toxins deleted (PA103ΔTΔU) and this strain complemented with a number of ExoU mutants in the pUCP19-exoU-spcU expression vector. HeLa cells were injected with ExoU_S142A (WT), ExoU_S142A_K178R, ExoU_S142A_W681A, ExoU_S142A_C6 (Δ679-687) and ExoU_S142A_C7 (Δ684-687) via the TTSS. Scale bar = 10 μm.

4.3.10. The C-terminus of ExoU is required for toxicity after transfection

Previous studies have shown that the C-terminus of ExoU is required for toxicity in CHO cells and in *S. cerevisiae*. In order to define further the C-terminal region required for ExoU cytotoxicity and to determine whether the region we identified as being essential for plasma membrane localisation and diubiquitination of ExoU correlates with the ability of ExoU to kill cells, we carried out a luciferase assay to assess the cytoxicity of a number of ExoU mutants after transfection.

HeLa cells were co-transfected with the constitutive luciferase expressing plasmid pGL2-control and a number of pCMV-Tag2 constructs expressing ExoU or ExoU mutants. As only metabolically active cells are able to express luciferase from the pGL2-control plasmid, luciferase production was used as a measure of cell viability. When HeLa cells were transfected with pCMV-Tag2-ExoU there was over a 100-fold reduction in luciferase production compared to cells transfected with the empty pCMV-Tag2 vector (Fig. 4.16, pCMV and ExoU). When the active serine residue 142 was mutated to an alanine, luciferase production was as high as that observed with the empty pCMV-Tag2 vector (Fig. 4.16, S142A) confirming that the S142A mutant renders ExoU non-toxic. When we transfected HeLa cells with a construct expressing ExoU with the C-terminal residues 663 – 687 deleted (Fig. 4.16, C4) luciferase production was as high as that observed by HeLa cells transfected with either the empty pCMV-Tag2 vector or the inactive ExoU S142A mutant. Therefore a region within the C-terminal 25 amino acids is essential for ExoU toxicity in HeLa cells. HeLa cells transfected with ExoU truncated at either residue 671 or 679 (Fig. 4.16, C5 and C6) produced much more luciferase than cells transfected with wild type ExoU but significantly less luciferase than HeLa cells transfected with the inactive ExoU S142A mutant. This result suggested that a region between amino acids 663 and 670 was required for full toxicity of ExoU but that deletion of the final 9 residues was sufficient to substantially reduce the activity of ExoU. When the last 4 amino acids of ExoU were deleted (Fig. 4.16, C7), ExoU retained its wild type toxicity phenotype. Therefore the same C-terminal region, amino acids 679 - 683, required for diubiquitination and plasma membrane localisation of ExoU was also required for full toxicity of ExoU in HeLa cells.

The contribution of the single amino acids in the C-terminal 679 – 684 region to ExoU cytoxicity was investigated by measuring the luciferase production from HeLa cells co-transfected with pGL2-control and one of the single alanine substitution mutants (Fig. 4.16, K679A, W681A, R682A, N683A). Mutation of no single amino acid to alanine in this region altered the toxicity of ExoU including mutating the tryptophan residue that we have shown to be important to ExoU localisation and modification.

In order to assess the contribution of ubiquitination to ExoU toxicity, we tested the action of the lysine mutants K178R and K428R in HeLa cells. Mutation of either lysine residue to arginine, did not affect the toxicity of ExoU (Fig. 4.16, K178R and K428R) as HeLa cells transfected with either construct produced approximately the same amount of luciferase as cells transfected with wild type ExoU.

Further indication of the importance of the ExoU C-terminus in cytotoxicity and the differential toxicity of the C-terminal deletion mutants was provided by immunoblotting lysates of HeLa cells transfected with various C-terminal deletion mutants. As noted before, wild type ExoU cannot be observed by Western blot analysis of transfected cells (Fig. 4.17, ExoU) and the toxin had to be rendered non-toxic, for example by mutation of the active site serine 142, in order for it to be visualised (Fig. 4.17, S142A). When HeLa cells were transfected with a construct encoding ExoU truncated 25 amino acids before the end (Fig. 4.17, C4), a similar amount of protein was observed on the immunoblot as that seen for the ExoU S142A mutant. When the C-terminal 17 or 9 residues of ExoU were deleted and transfected into HeLa cells, ExoU was detectable by immunoblotting (Fig. 4.17, C5 and C6) although there was substantially less protein present compared to the S142A or C4 ExoU mutants. When the final 4 amino acids of ExoU were deleted, no protein was visualised in the lysate of transfected HeLa cells on a Western blot (Fig. 4.17, C7). Although this was an uncontrolled experiment, with no measure of the transfection efficiency and no loading control, the ability to observe the C5 and C6 ExoU mutants by Western blot analysis indicates that they are not as toxic as wild type ExoU. However the apparent decrease in signal compared to HeLa cells transfected with the non-toxic ExoU S142A mutant after the same amount of DNA has been transfected, suggests that the C5 and C6 ExoU mutants still retain some toxicity as shown in the luciferase toxicity assay.

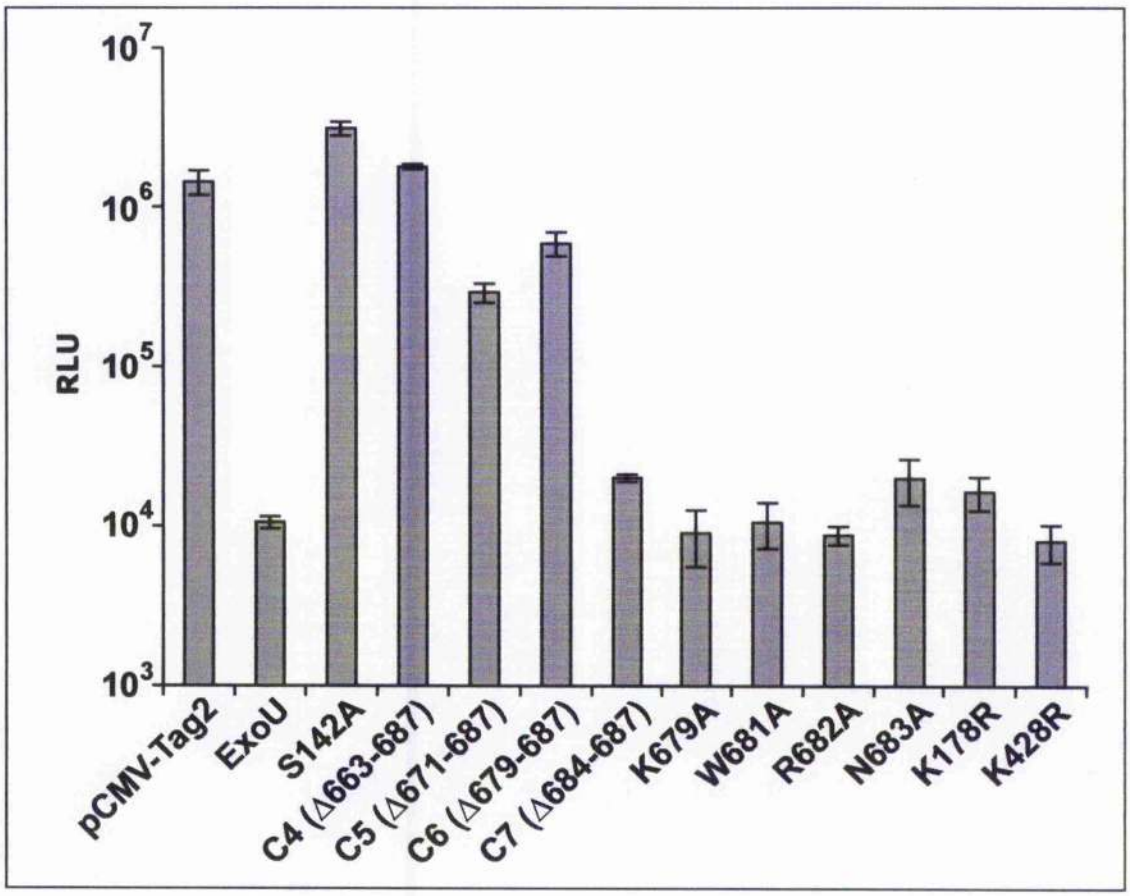


Fig. 4.16. Toxicity of ExoU and ExoU mutants after transfection.

HeLa cells were co-transfected with the luciferase expressing plasmid pGL2-control and either empty pCMV-Tag2 vector or the pCMV-Tag2 vector containing *exoU* or *exoU* mutants. 48 hours after transfection, luciferase production was measured as the relative light units (RLU) produced from the cell lysates over a 10 second period. The average results of three separate transfections are shown and error bars represent standard deviation of the mean.

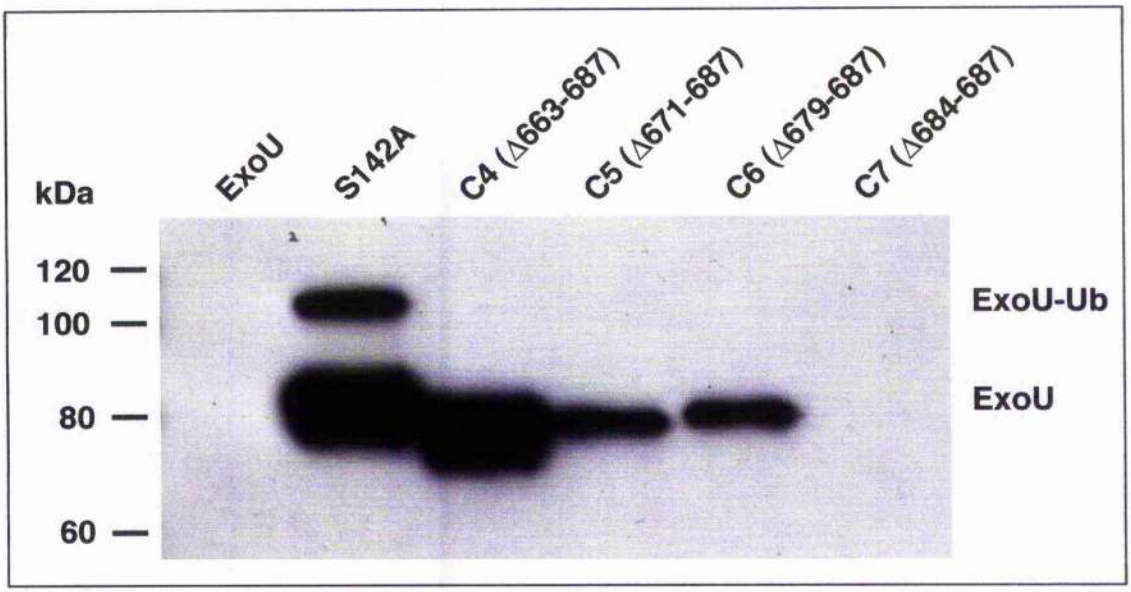


Fig. 4.17. Expression of C-terminal deletion mutants of ExoU after transfection. HeLa cells were transfected with pCMV-Tag2 encoding active ExoU, lipase-inactive ExoU_S142A or a number of C-terminal deletion mutants of WT ExoU. 16 hours after transfection the HeLa cell lysates were analysed by immunoblotting with an anti-FLAG antibody. ExoU and ubiquitinated ExoU (ExoU-Ub) protein bands are indicated.

4.3.11. The C-terminus of ExoU is required for toxicity after infection

To confirm the cytoxicity results gained from the transfection studies in an infection setting, we used an LDH assay to assess cell death resulting from infection of HeLa cells with *P. aeuriginosa* expressing ExoU and various ExoU mutants. The stable cytosolic enzyme LDH is released upon cell lysis and provides a good measure of cell death, with 100% cell death corresponding to the amount of LDH released when all the cells are lysed.

When HeLa cells were infected with PA103ΔTΔU very few cells were lysed 3 hours after infection (Fig. 4.18, PA103ΔTΔU). When this pseudomonal strain was complemented with wild type *exoU* and *spcU* in the pUCP19 expression vector, about 65% of cells were lysed after 3 hours (Fig. 4.18, pUCP19-exoU-spcU). Mutating the active site serine 142 to an alanine in pUCP19-exoU-spcU rendered ExoU non-toxic as shown by the lack of cell death after infection with PA103ΔTΔU secreting ExoU S142A (Fig. 4.18, S142A). Mutating either the diubiquitinated lysine residue 178 to an arginine or the tryptophan residue 681 to an alanine did not alter ExoU toxicity, with at least 65% of cells being lysed after infection (Fig. 4.18, K178R and W681A). When ExoU lacking the final 9 amino acids was secreted into HeLa cells by the TTSS, very few cells were killed (Fig. 4.18, C6). However, when the last 4 amino acids of ExoU were deleted and this mutant was injected into HeLa cells, it resulted in ExoU wild type levels of cell death (Fig. 4.18, C7). Therefore amino acids 679 – 683 are required for ExoU toxicity both after transfection and infection but the diubiquitinated lysine residue 178 and the tryptophan residue 681 are not essential for the observed cytoxicity.

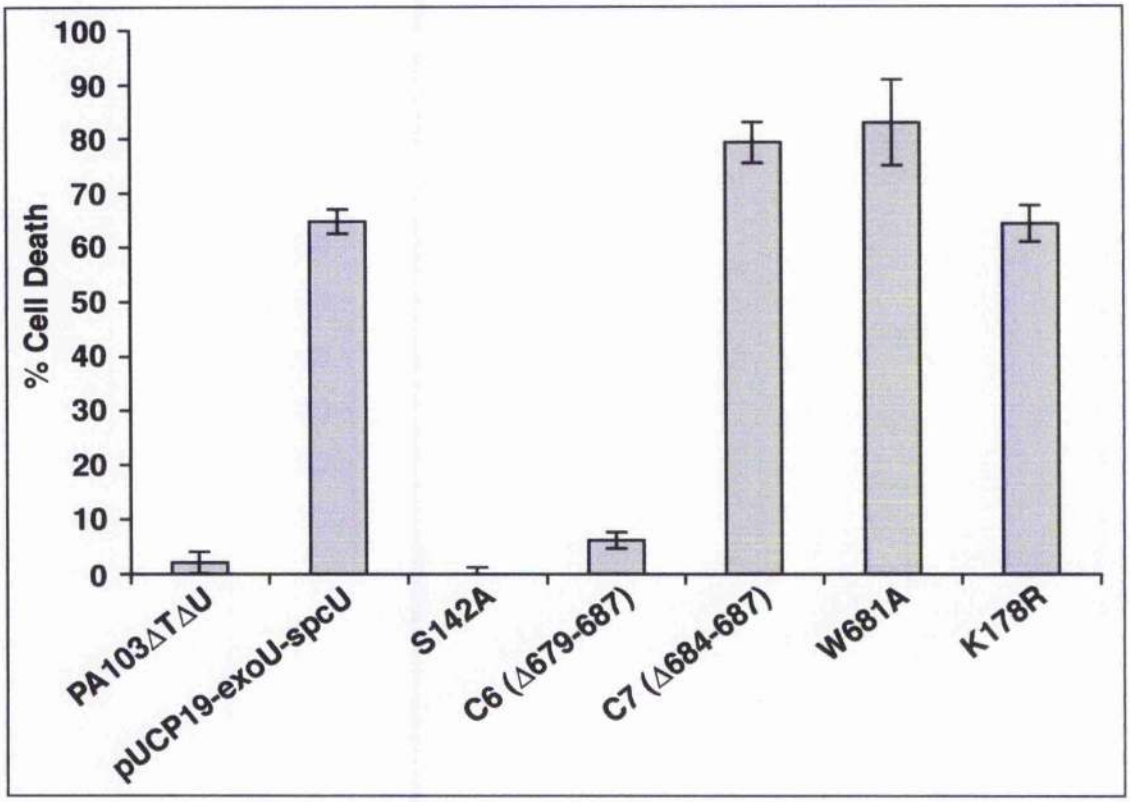


Fig. 4.18. Toxicity of ExoU and ExoU mutants after infection.

HeLa cells were infected with a pseudomonal strain lacking all known TTS toxins (PA103ΔTΔU) or this strain complemented with a construct encoding ExoU and its cognate chaperone, SpcU (pUCP19-exoU-spcU) or this strain complemented with ExoU mutants and SpcU. Cell death was calculated by measuring LDH release from infected cells 4 hours after infection and is expressed as a percentage of LDH release from complete cell lysis. The average results of three separate infections are shown and error bars represent standard deviation of the mean.

4.3.12. The C-terminus of ExoU is required for phospholipase activity

We have demonstrated that the C-terminus of ExoU is essential for localisation of the toxin to the plasma membrane, diubiquitination of lysine residue 178 and toxicity. In order to examine the requirement of the C-terminus of ExoU on the catalytic activity of this enzyme we measured the phospholipase activity of recombinant ExoU and a number of recombinant ExoU mutants.

It has been previously demonstrated that ExoU needs an, as yet unknown, eukaryotic cofactor for activation. Therefore, when the phospholipase activity of recombinant ExoU alone was measured, no activity was detected (Fig. 4.19, rExoU). When we added HeLa cell lysate to recombinant ExoU, it had an activity of about 57 nmoles/min/g (Fig. 4.19, rExoU + lysate). Although HeLa cell lysate exhibits a small amount of phospholipase activity (Fig. 4.19, Lysate) the majority of that observed was the result of the recombinant ExoU action. As previously demonstrated, mutation of the active site serine abolished phospholipase activity, with activity reduced to background lysate levels (Fig. 4.19, S142A + Lysate). Truncation of ExoU at residue 679 also resulted in a loss of phospholipase activity (Fig. 4.19, C6 + Lysate). Deletion of the last 4 amino acids of ExoU did not affect the phospholipase activity of the protein (Fig. 4.19, C7 + Lysate). Also mutation of the diubiquitinated lysine residue 178 did not alter activity of ExoU (Fig. 4.19, K178R + Lysate). Mutation of the tryptophan residue 681 did not alter the cytotoxicity of ExoU in either the transfection or infection toxicity assays described above but in the phospholipase assay, the W681A mutant showed a much reduced phospholipase activity compared to wild type recombinant ExoU although it exhibited significantly more phospholipase activity than the lysate background control (Fig. 4.19, W681A + Lysate).

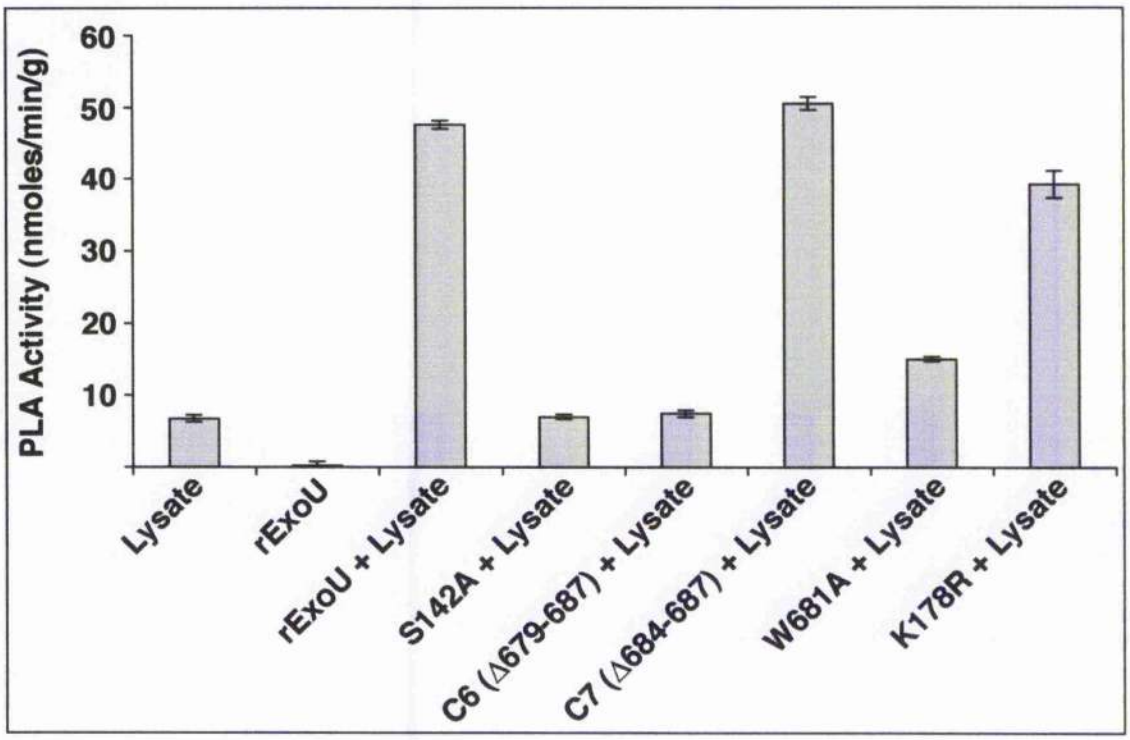


Fig. 4.19. Phospholipase activity of recombinant ExoU and ExoU mutants.

The PLA_2 activity of recombinant ExoU and a number of recombinant ExoU mutants was measured as the nmoles of free thiol released from hydrolysis of the arachidonoyl thioester bond at the *sn-2* position in aracidonyl thio-PC in 1 minute by 1 gram of recombinant protein. The intrinsic PLA_2 activity of HeLa cell lysate and recombinant ExoU in the absence of HeLa cell lysate is indicated. Each assay was performed in triplicate. Error bars represent standard deviation of the mean.

4.3.13. The eukaryotic cofactor for ExoU is localised in the particulate, membrane-enriched fraction of HeLa cells

The requirement of the same ExoU C-terminal residues for toxicity and plasma membrane localisation may be accounted for by the presence of the ExoU eukaryotic cofactor at the plasma membrane. In order to assess whether the eukaryotic cofactor for ExoU phopsholipase activity was membrane localised, we fractionated HeLa cell extracts into cytoplasmic and particulate membrane-enriched fractions and determined the ability of each fraction to activate recombinant ExoU in the *in vitro* phospholipase assay.

Our results demonstrate that as expected, neither the recombinant ExoU, the total HeLa cell extract, the cytoplasmic HeLa cell fraction or the particulate membrane-enriched HeLa cell fraction were able to hydrolyse phospholipids effectively on their own (Fig. 5.20, rExoU, Lysate, Lysate C, Lysate M). As illustrated previously (section 5.3.12), when ExoU was incubated with total HeLa cell extract it was shown to be active (Fig. 5.20, rExoU + lysate). Recombinant ExoU incubated with the cytoplasmic HeLa cell fraction exhibited very little phopsholipase activity (Fig. 5.20, rExoU + lysate C), whereas recombinant ExoU incubated with the particulate membrane-enriched HeLa cell fraction was very active (Fig. 5.20, rExoU + lysate M). Therefore, the eukaryotic cofactor required for the phopsholipase activity of recombinant ExoU fractionates with the particulate membrane-enriched HeLa cell fraction.

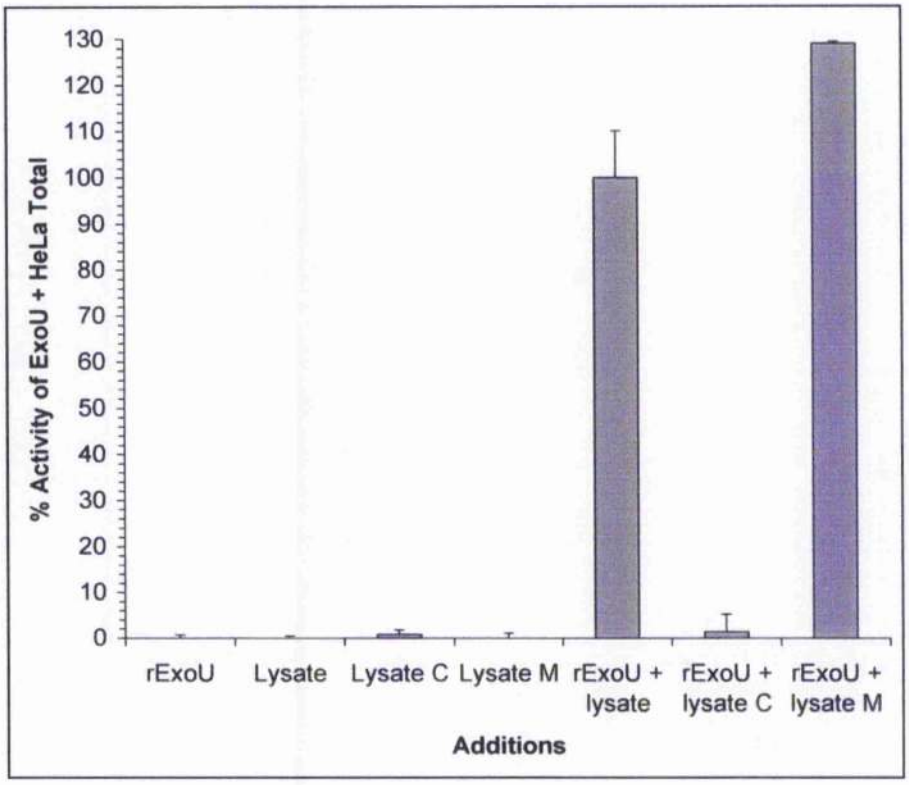


Fig. 4.20. Co-factor for ExoU resides in a particulate HeLa cell fraction

Phospholipase activity of recombinant ExoU was determined alone or in the presence of unfractionated HeLa cell extract (lysate) or the HeLa cell lysate fractionated into a particulate (lysate M) or cytoplasmic (lysate C) fraction.

4.3.14. ExoU binds specifically to certain phospholipids

The C-terminus of ExoU is required for directing the toxin to the plasma membrane of eukaryotic cells. The C-terminus may be essential for binding to a protein that anchors ExoU to the plasma membrane or it may interact directly with the plasma membrane. Although there are no obvious plasma membrane localisation signals within the C-terminus of ExoU it may anchor the rest of the protein in place by interacting with specific phospholipids in the membrane. In order to test this hypothesis, phosphoinositide coated beads were used to pull down ExoU from the lysate of HeLa cells transfected with pCMV-Tag2-ExoU_S142A. Proteins eluted off the phosphoinositide coated beads were then analysed by Western blotting with the anti-FLAG antibody (Fig. 4.21A). ExoU appears to interact specifically with phosphatidylinositol-3-phosphate (PI(3)P), phosphatidylinositol-4-phosphate (PI(4)P) and phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$) and ubiquitinated ExoU seems to interact with PI(3)P and PI(4)P. There did not appear to be any binding to phosphatidylinositol (PtdIns, PI) phosphatidylinositol-5-phosphate (PI(5)P). phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$), phosphatidylinositol-3,5-bisphosphate ($PI(3,5)P_2$) or phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$. Unfortunately, this method produced highly variable results when repeated.

In an attempt to use a more consistent method to assess the binding of ExoU to phospholipids we measured the binding of recombinant ExoU to phospholipids immobilised on a nitrocellulose membrane. The membrane was incubated with recombinant ExoU S142A, washed and then immunoblotted with an anti-His antibody. Recombinant ExoU S142A reproducibly interacted strongly with the phosphatidylinositol monophosphates, with intermediate strength to the phosphatidylinositol bisphosphates and phosphatidic acid and more weakly to phosphatidylinositol triphosphate, phosphatidylserine and lysophosphatidic acid (Fig. 4.21B, S142A).

To determine if the region we identified as being important for localisation of ExoU to the plasma membrane was also required for the observed binding to the phospholipids, we assessed the binding of a number of recombinant ExoU mutants. Mutation of the lysine residue 178 or the tryptophan residue at 681 did not alter the binding affinity of

ExoU for the phospholipids (Fig. 4.21B, K178R and W681A). Truncation of ExoU at amino acid 679 or 684 also did not affect the binding of ExoU to phospholipids (Fig. 4.21B, C6 and C7). These results indicate that the plasma membrane localisation of ExoU cannot be explained by the binding of the toxin to particular phospholipids.

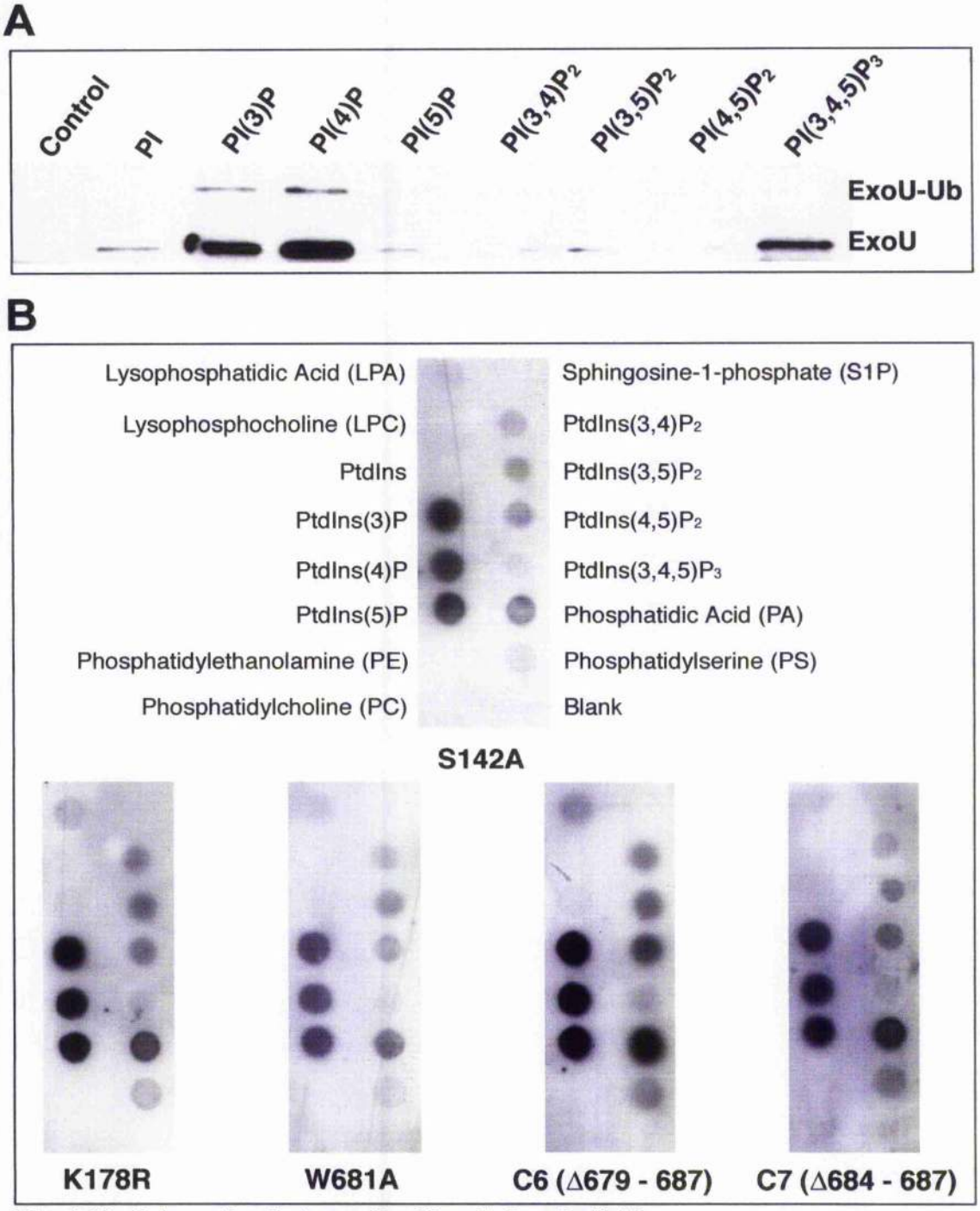


Fig. 4.21. Interaction between ExoU and phospholipids.

A) Phosphoinositide coated agarose beads were used to pull down proteins from the lysate of HeLa cells transfected with pCMV-Tag2-ExoU_S142A. The presence of ExoU in the proteins pulled down by the phosphoinositide coated beads was assessed by immunoblotting with an anti-FLAG antibody. **B)** The binding of recombinant ExoU mutants to various phospholipids was assessed by incubating membranes with the phospholipids spotted onto them with the recombinant ExoU mutants and immunoblotting with an anti-His antibody.

4.3.15. Phospholipids do not activate ExoU phospholipase activity

The requirement of a eukaryotic cofactor to activate ExoU that we have been unable to identify by co-immunoprecipitation combined with the specific binding of ExoU to particular phospholipids, led us to speculate that a phospholipid may be the activating factor. To test this theory, we investigated whether any of the phosphatidylinositol mono- or bisphosphates or phosphatidylserine were able to activate the PLA_2 activity of ExoU. The phospholipase assay showed that none of the tested phospholipids were able to substitute for HeLa lysate in providing the activating cofactor for ExoU activity (Fig. 4.22).

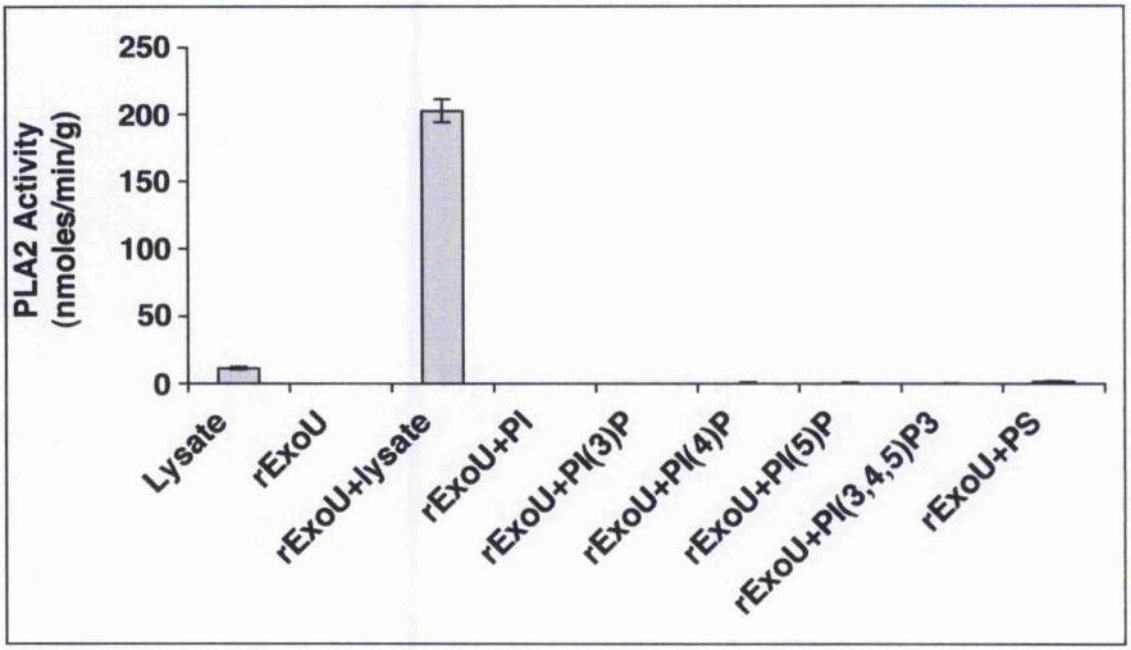


Fig. 4.22. Contribution of phospholipids to the phospholipase activity of recombinant ExoU.

The PLA_2 activity of recombinant ExoU in the presence of HeLa cell lysate or a number of different phospholipids was measured as the nmoles of free thiol released from hydrolysis of the arachidonoyl thioester bond at the *sn-2* position in aracidonyl thio-PC in 1 minute by 1 gram of recombinant protein. The intrinsic PLA_2 activity of HeLa cell lysate and recombinant ExoU in the absence of HeLa cell lysate is indicated. Each assay was performed in triplicate. Error bars represent standard deviation of the mean.

4.4. DISCUSSION

In this chapter we studied what happened to ExoU after it had been transfected or injected into cells by the TTS system in order to provide an insight into how this toxin is activated. Initially we observed that ExoU was modified to a higher molecular weight form in eukaryotic cells and mass spectrometry analysis revealed that this was the result of diubiquitination of lysine residue 178. The majority of the ubiquitin-ubiquitin linkages were through lysine residue 63, but lysine 48 ubiquitin linkages were also observed. Mass spectrometry analysis also revealed that some ExoU was modified by 1, 3, or 4 ubiquitin moieties and that, in addition to ubiquitination of lysine residue 178 in ExoU, there was also a small amount of ubiquitination of lysine residue 428. Other modifications of ExoU indicated by mass spectrometry analysis included N-terminal acetylation and the probable phosphorylation of serine residue 30 in a small proportion of the molecules analysed. Analysis of the regions required for diubiquitination of lysine residue 178 revealed that the C-terminus of ExoU was essential for this modification. Deletion of just the last nine amino acids abolished diubiquitination of ExoU, whereas deletion of the last four amino acids did not, indicating that the region between residues 679 and 683 was important for this modification. Analysis of the contribution of the individual amino acids within this C-terminal region demonstrated that the tryptophan residue at position 681 was important, with just a very small amount of diubiquitination occurring when this tryptophan was mutated to an alanine. We demonstrated that although diubiquitination of ExoU did not have a dramatic effect on the turnover of the toxin in eukaryotic cells, it did lead to a small but significant increase in degradation.

In addition to studying the modification of ExoU, we also determined the localisation of this toxin and studied how the two phenomena were related. Immunofluorescent staining of HeLa cells transfected or injected with ExoU via the TTS needle demonstrated that ExoU was localised to the inner plasma membrane of eukaryotic cells in a punctate fashion. Immunoblotting of cellular fractions of HeLa cells expressing ExoU also showed that ExoU was mainly found in the particulate membrane-enriched fraction with the diubiquitinated toxin exclusively separating with this fraction. Analysis of the localisation of the lysine 178 mutant (which is unable to be diubiquitinated) revealed that this ExoU mutant retained its plasma membrane

distribution, indicating that ubiquitination was a consequence not a cause of plasma membrane localisation. The same C-terminal region that was shown to be important for diubiquitination of ExoU was also shown to be important for localisation. Deletion of the last 9 amino acids of ExoU abolished plasma membrane localisation whereas deletion of the last 4 amino acids did not. The tryptophan residue 681 required for wild type levels of diubiquitination was also implicated in localisation, with this mutant showing a diminished level of plasma membrane localisation at least in transfected cells. Although essential, we demonstrated that the C-terminal 33 amino acids of ExoU were not sufficient for localisation, indicating that other regions of ExoU are also required for plasma membrane targeting. Unfortunately, our attempts to study the trafficking of an ExoU-EGFP fusion protein from the bacterium to the eukaryotic plasma membrane failed. This was probably due to the inability of the stable GFP protein to unfold and thus pass through the TTS needle [196]. With regards to membrane localisation, we also assessed the binding specificity of ExoU to various phospholipids immobilised on either beads or nitrocellulose membranes. The phosphoinositide coated bead pull-down assay produced highly variable results probably due to the inconsistent loss of beads during the washing steps. The overlay assay of recombinant ExoU on nitrocellulose membranes spotted with immobilised phospholipids produced highly reproducible results and indicated that ExoU binds strongly to phosphatidlyinositol monophosphates, with intermediate strength to phosphatidlyinositol bisphosphates and phosphatidic acid and more weakly to phosphatidlyinositol triphosphates, phospatidylserine and lysophatidic acid. These binding properties, however, did not require the C-terminus of ExoU and thus cannot explain the observed plasma membrane localisation of this toxin.

In an attempt to determine the functional implications of ExoU diubiquitination and plasma membrane localisation we analysed the toxicity and phospholipase activity of a number of ExoU mutants. We showed that the same C-terminal region between amino acids 679 and 683 that was required for diubiquitination and plasma membrane localisation was also required for wild type levels of ExoU toxicity and phospholipase activity. Studying the toxicity of a number of ExoU C-terminal mutants also implicated regions N-terminal to this small 679-683 region in ExoU toxicity. For example, deletion of the C-terminal 25 amino acids led to a complete abolition of ExoU toxicity, whereas deletion of the C-terminal 17 or 9 amino acids lead to a reduction but not a

complete elimination of toxicity. We showed that although the same C-terminal region was responsible for toxicity and ubiquitination, diubiquitination of lysine residue 178 was not required for toxicity or phospholipase activity. We also demonstrated that the tryptophan 681 residue was not required for ExoU toxicity although it was required for wild type levels of phospholipase activity. This discrepancy is probably due to the differences in sensitivity of the assay systems. Whereas the phospholipase assay was able to demonstrate that the ExoU tryptophan 681 mutant had only about 20% of the activity of wild type ExoU, this activity was probably sufficient to cause death in eukaryotic cells. Therefore, any cell that was successfully transfected or injected with the 20% active ExoU tryptophan 681 mutant would die and thus the cell death rate would be the same as that for cells transfected or injected with fully toxic ExoU.

In this study we also tried to isolate the eukaryotic cofactor required for ExoU activity by co-immunoprecipitation of ExoU interacting proteins. This method generated a few possible ExoU binding proteins but further testing of the most promising candidates revealed that they were false positives. Although we were unable to isolate the cofactor, we did localise it to the particulate membrane-enriched fraction of HeLa cells as demonstrated by the capacity of this fraction to activate the phospholipase activity of recombinant ExoU and the inability of the cytosolic fraction to do so. None of the phospholipids we assayed were able to activate recombinant ExoU, providing further support for a proteinaceous eukaryotic cofactor.

In an attempt to explain our results, we have developed two alternative models for ExoU activation (Fig. 4.23). We have demonstrated that the C-terminus of ExoU is required for plasma membrane localisation, diubiquitination and phospholipase activity that leads to cell death. We have also shown that the eukaryotic cofactor required for phospholipase activity fractionates with the particulate membrane-enriched fraction of HeLa cells. Therefore, the cofactor may be localised at the site of ExoU action, the plasma membrane. In the first model, the C-terminus of ExoU is required for plasma membrane localisation by virtue of its ability to bind to the membrane-bound eukaryotic cofactor. Once at its site of action in the presence of its cofactor, ExoU is able to act as a phospholipase and destroy the plasma membrane resulting in cell death. ExoU is also diubiquitinated at the plasma membrane, possibly as a result of its interaction with the membrane-bound co-factor, although the consequences of this modification are

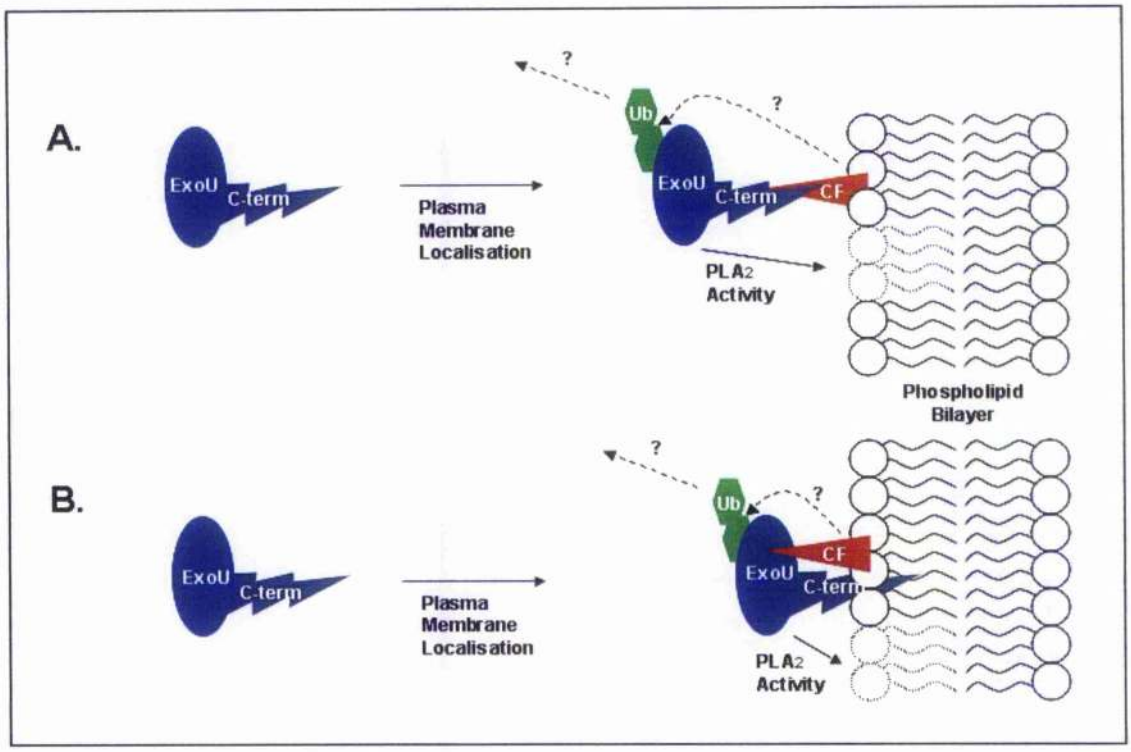


Fig. 4.23. Model of ExoU interaction with the plasma membrane.

The C-terminus (C-term) of ExoU is involved in localising the toxin to the plasma membrane either by **(A)** binding to a membrane-bound eukaryotic cofactor (CF) of by **(B)** binding directly to the phospholipid bilayer where it is then able to bind the cofactor. Membrane localisation of ExoU allows this phopsholipase to exert its catalytic activity (PLA_2 Activity). Membrane localisation also leads to the addition of two ubiquitin molecules (Ub) to lysine 178 of ExoU possibly through the action of the cofactor. The consequence of ExoU diubiquitination is unknown.

unclear. The second model is similar in most respects to the first but in this case the C-terminus is responsible for plasma membrane localisation due to its ability to directly interact with the phospholipid bilayer. Once ExoU is membrane bound, the eukaryotic cofactor is able to bind and ExoU can act as a phospholipase and is diubiquitinated. Both these models suggest that the C-terminus is primarily responsible for plasma membrane localisation and its importance in diubiquitination and toxicity is a consequence of this localisation. The importance of membrane localisation for ExoU toxicity is similar to that observed for $cPLA_2$. Human $cPLA_2$ is targeted to membranes via its Ca^{2+}-dependent lipid-binding C2 domain, where it is then able to hydrolyse phospholipids [520]. When the C2 domain is deleted, $cPLA_2$ is no longer able to hydrolyse liposomal or micellar substrates although it can still hydrolyse a monomeric lysophospholipid [520]. Thus the lipid-binding domain and catalytic domain of $cPLA_2$ are functionally distinct but are both required for hydrolysis when the substrate is an aggregated phospholipid. In the same way, although abolition of the C-terminus of ExoU renders the protein non-toxic to eukaryotic cells and inactive against an aggregated phospholipid substrate (our phospholipase assay utilised a micelle comprised of the synthetic substrate aracidonoyl thio-PC), C-terminally truncated ExoU may retain its activity against monomeric phospholipids.

It is intuitive that ExoU requires targeting to its phospholipid substrates in order to act as a phospholipase and cause cell death. We have implicated the C-terminus in this targeting but are unclear as to whether this targeting results from interaction with a membrane-bound protein or directly with the phospholipids bilayer. Human $cPLA_2$ is targeted to the membrane by its calcium dependent C2 domain and patatin contains a hydrophobic region of 50 amino acids at the N-terminus that may be involved in membrane binding [449, 520]. ExoU lacks homology to either of these domains and also does not exhibit homology to any known lipid interacting domains, thus if the C-terminus binds directly to the lipid bilayer it is via a novel domain. We have demonstrated that the tryptophan residue 681 is important for membrane localisation after transfection, diubiquitination and phospholipase activity. It is unclear why there is a discrepancy between localisation of the ExoU tryptophan mutant after transfection and injection by the TTS needle but it may be the result of different trafficking mechanisms. It is probable that the diminished membrane localisation of the tryptophan mutant results in the low levels of diubiquitination and the decreased phospholipase activity.

Thus the tryptophan residue 681 may be important in the binding of ExoU to either the membrane-bound cofactor or the lipid bilayer. Support for the latter model, in which tryptophan is involved in direct membrane binding, is supplied by the observation that tryptophan is a key residue in supporting lipid-protein interactions [528]. For example, tryptophan residues are often found at the membrane-water interface of membrane proteins and tryptophan residues have been implicated in the interfacial binding of human secreted phospholipases to zwitterionic phospholipid bilayers [529, 530]. The side chain of tryptophan is an indole ring joined to a methylene group and it has been shown that this polar-aromatic amino acid has a special affinity for a region near the lipid carbonyls at the membrane-water interface [528].

As discussed in the introduction to this chapter, a number of lipases require modification or cofactor binding for the formation of an active conformation. Human $cPLA_2$ requires phosphorylation and interfacial activation and pancreatic triglyceride lipase requires colipase to open a flexible lid domain that allows the lipid substrates access to the catalytic site of the lipase [446, 522, 525, 526]. ExoU exhibits no obvious homology to the flexible lid domains of either of these lipases, but it is possible that the C-terminus is required for lipid binding or cofactor binding that induces a conformation change which allows ExoU to be active. Thus, in addition to targeting ExoU to its substrates, the C-terminus may also be required for inducing a phospholipase active conformation by virtue of its potential binding properties.

Mass spectrometry analysis revealed that ExoU was modified in a number of ways in eukaryotic cells, which theoretically might be involved in the activation of this toxin. ExoU is diubiquitinated at lysine residue 178, N-terminally acetylated and possibly phosphorylated at serine residue 30. We clearly demonstrated that diubiquitination of lysine residue 178, or the lesser ubiquitination of lysine residue 428, was not required for the phospholipase activity of ExoU. Also, phosphorylation of serine 30 does not appear important in ExoU activation as it is clear from previous studies that deletion of the first 52 amino acids of ExoU does not diminish the toxicity of this protein [436]. In the same way, N-terminal acetylation can be disregarded as an activating modification because deletion of the N-terminal methionine of ExoU does not abolish toxicity [436]. The absence of a modification that is solely responsible for ExoU activation is consistent with the recent findings of Sato et. al. whose results suggest that a eukaryotic

cofactor is required at the time of phospholipid hydrolysis [447]. Obviously, neither their results nor ours rule out the possibility of other modifications, in addition to the presence of the eukaryotic cofactor, being required for ExoU activation. Also, although phosphorylation of serine 30 is not required for ExoU activation, it remains a possibility that phosphorylation of another residue that was not identified in the mass spectrometry analysis is required.

Our discovery that ExoU is diubiquitinated at lysine residue 178 was unexpected and we remain uncertain as to the functional significance of this modification. To date two other TTS toxins have been demonstrated to be ubiquitinated in eukaryotic cells, SopE and SopB in Salmonella [259, 531]. SopE is a GEF for the Rho-family GTPases Cdc42 and Rac1 [256]. It is injected into eukaryotic cells by the TTS system of Salmonella along with a number of other toxins including SptP, a GAP for Cdc42 and Rac1 [258]. SopE induces profuse membrane ruffling, actin cytoskeleton rearrangements and subsequent bacterial uptake [256]. SopE is then rapidly degraded by ubiquitin-mediated proteasomal degradation and the longer-lived SptP is able to reverse the cellular changes induced by SopE by inactivating Cdc42 and Rac1 [259]. SopB is also translocated into eukaryotic cells by the TTS apparatus of Salmonella and acts as an inositol phosphatase that specifically dephosphorylates inositol (1,3,4,5,6) – pentakisphosphate producing Ins (1,4,5,6)P_4, which is an indirect activator of Cdc42 [286]. SopB has also been shown to be ubiquitinated in eukaryotic cells but in contrast to SopE, this ubiquitination does not lead to rapid degradation by the proteasome [531]. It is unclear what the consequences of SopB ubiquitination are but it has been suggested that ubiquitination of this membrane bound toxin may lead to its targeting to and degradation by the lysosome [531].

Ubiquitination is a multi-step process that results in the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and a lysine residue in the target protein. This covalent attachment of the 76-residue ubiquitin protein to the target protein usually requires the concerted action of three separate enzymes [532]. The ubiquitin-activating enzyme, E1, forms a high-energy thiol ester with the carboxyl group of the C-terminal glycine residue of ubiquitin, thus activating this region for nuclephilic attack. A ubiquitin-conjugating enzyme, E2, then transiently carries the activated ubiquitin molecule as a thiol ester. A ubiquitin ligase, E3, then transfers the

activated ubiquitin from E2 to the substrate lysine residue. In most organisms including humans, there is a single E1 enzyme, a significant number of E2 enzymes and many more E3 enzymes. For example, the human genome project revealed more than 40 E2s and over 500 E3s [533]. Ubiquitination is a reversible process and ubiquitin removal is carried out by group of enzyme called deubiquitins (dubs) of which there are over 80 in humans [533].

Ubiquitin itself contains 7 lysine residues that have the potential to be targeted by ubiquitination, thereby leading to the formation of a polyubiquitin chain. A yeast screen revealed that all 7 lysine residues, lysine 6, 11, 27, 33, 48 and 63, are ubiquitinated *in vivo* although some linkages are more common than others [534]. The most common ubiquitin linkage is through lysine residue 48 followed by linkages through lysine residues 63 or 11 and then through lysine residues 33, 27 or 6 [534]. Most polyubiquitin chains contain the same ubiquitin linkage throughout the chain, although there are some examples of branched chains, for example a yeast protein was identified that was simultaneously modified at lysine residue 29 and 33 [534]. The ability of ubiquitin to modify a diverse range of proteins on either one or more lysine residue within the target protein, and to itself be ubiquitinated at a number lysine residues provides a vast array of potential modifications. A protein may be monoubiquitinated, multi-monoubiquitinated, polyubiquitinated, multi-polyubiquitinated and the polyubiquitination may proceed through any one of 7 lysine linkages. This diversity in modification also results in a diversity of function for ubiquitination. The most common consequence of ubiquitination is to target proteins for degradation, but this modification can also functionally modulate a protein and alter its membrane trafficking [535].

The most well studied consequence of ubiquitination is to target the ubiquitinated protein for proteolysis by the 26s proteasome [536]. Ubiquitin-mediated proteasomal degradation is used both to rid cells of misfolded or truncated polypeptides and to degrade fully functional proteins as a means of regulation. For example, the turnover of mitotic cyclins is ubiquitin dependent and enables regulated cell cycle progression [537]. Degradation by the proteasome requires the target protein to be polyubiquitinated with at least four ubiquitin moieties linked through lysine 48 [538]. The ubiquitinated protein is recruited to the 26s proteasome complex were it is unfolded

and then translocated into an interior chamber where the substrate is hydrolysed by a nucleophilic mechanism to produce small peptides. Ubiquitin is not degraded by the proteasome as it is released from the targeted protein by dubs.

Recently a number of additional functions have been assigned to ubiquitination that result either from monoubiquitination or polyubiquitination through lysine linkages other than lysine 48 [535, 539, 540]. For example, monoubiquitination has been implicated in protein trafficking, endocytosis and gene expression and silencing. Lysine 63-linked polyubiquitin chains have also been shown to signal in four pathways: DNA damage tolerance, the inflammatory response, protein trafficking, and ribosomal protein synthesis. The ability of ubiquitin to alter protein stability, protein function, protein trafficking and protein-protein interactions enable it to play a role in these diverse systems.

Our results show that ExoU was mainly diubiquitinated through lysine residue 178 with the two ubiquitins being linked through lysine 63. A small amount of lysine 48-linked ubiquitin was also present in the diubiquitinated ExoU protein band and this result, combined with the small amount of tri- and tetraubiquitinated ExoU observed, suggests that a small proportion of the toxin may be polyubiquitinated through lysine 48 and targeted for proteasomal degradation. However, as the main modification of ExoU was lysine 63-linked diubiquitination of lysine residue 178, I shall focus my discussion on the possible consequences of this modification.

In a number of cases, lysine 63-linked polyubiquitin chains are capable of altering the activity of a protein. One way the lysine 63-linked polyubiquitin chain can achieve this is by providing a platform to which downstream proteins with ubiquitin-interacting domains can bind. For example, when TRAF6 is polyubiquitinated with a lysine 63-linked chain it is able to interact with a novel zinc binding domain in TAB2 [540]. The TAB2-associated TAK1 kinase is then activated and phosphorylates and activates IKK. IKK mediates phosphorylation of IκBα that leads to the lysine 48-linked polyubiquitination and proteolysis of IκBα. NF-κB, which is normally bound by IκB, is then free to enter the nucleus and activate expression of many target genes. Thus, in this inflammatory signalling pathway ubiquitination is involved in both the activation of IKK and the degradation of IκBα, and lysine 63-linked polyubiquitination of TRAF6

mediates the activation step [540]. For ExoU we have shown that 63-linked diubiquitination of lysine 178 does not alter the toxicity or phospholipase activity of this protein, proving that this modification is not required for either ExoU activation or inactivation.

Another role that lysine 63-linked polyubiquitination plays is in endocytosis of membrane-bound proteins. Ubiquitin acts as an internalisation signal at the plasma membrane and is involved in the regulated endocytosis of many signal-transducing receptors, transporters and channels [541]. Although in most cases monoubiquitination is sufficient for the rapid internalisation of membrane-bound proteins, a number of yeast proteins require conjugation with lysine 63-linked ubiquitin chains for maximal internalisation. For example, the yeast nutrient permeases Fur4p and Gap1 can be internalised when monoubiquitinated but are only efficiently endocytosed when polyubiquitinated with a lysine 63-linked chain [542-544]. In addition to the initial internalisation of plasma membrane bound proteins, ubiquitin also functions as a signal at a later stage in the endocytic pathway, in the sorting of proteins into the internal vesicles of multivesicular body (MVB) [541]. The endosomal system comprises of three main components, the early endosomes, the late endosomes or MVBs and the lysosomes. When proteins are initially internalised they are located in the early endosomes, these compartments then mature into late endosomes that accumulate many intracellular vesicles by internal budding of the outer limiting membrane, hence the alternative name MVBs. Fusion of the MVBs to lysosomes results in the degradation of the contents of the MVBs. Thus the proteins that are sorted into the vesicles of the MVBs are targeted for degradation, whereas those that remain on the limiting membrane of the MVB are recycled back to the plasma membrane. The accurate sorting of proteins into the invaginated vesicles is therefore an important step in determining which proteins are degraded and which are recycled, and ubiquitin plays a central role in this sorting. Proteins that are ubiquitinated are concentrated to the site on the limiting membrane of the MVB where vesicle invagination is to occur, these proteins are then internalised into the vesicles [541]. During the invagination step, ubiquitin is removed from the protein by a Dub, therefore only the targeted protein is degraded.

The role that ubiquitination plays in both the initial internalisation of plasma membrane proteins and the subsequent sorting into internal vesicles in the MVB suggests a number of potential consequences of diubiquitination of ExoU. We have shown that ExoU is membrane localised and is diubiquitinated at this location so it is possible that this toxin is internalised into early endosomes. Although polyubiquitination through lysine 63 has not been implicated in sorting of proteins into the internal vesicles of MVBs it is conceivable that, like endocytosis, a lysine 63-linked polyubiquitin chain may enhance this process. Thus, diubiquitinated ExoU may be targeted to the internal vesicles of MVBs. If this targeting occurred, it could explain the slight decrease in the half-life of diubiquitinated ExoU that we observed, as the modified toxin may be targeted to and degraded by the lysosome. Lysosomal degradation of ExoU would be a useful eukaryotic defence mechanism to try to overcome the cytotoxic effects of this potent phospholipase. In contrast, diubiquitination of ExoU may be advantageous for the potency of this toxin for example by increasing its membrane targets. When the enzymatic activity of ExoU was first elucidated, it was observed that expression of ExoU is *S. cerevisiae* led to the destruction of yeast vacuoles [439]. Thus in addition to hydrolysing plasma membrane lipids, ExoU must be able to target vacuolar membranes in yeast. Vacuoles are the yeast functional equivalent of lysosomes. Therefore, if diubiquitination of ExoU targets the toxin to intracellular organelles such as endosomes or lysosomes this may increase its potency by increasing its targets.

Another possible consequence of ExoU diubiquitination may be to facilitate intercellular spread of the toxin. In addition to being targeted for lysosomal degradation, the internal vesicles of MVBs can also be released extracellularly [545]. It has been observed in many cell types, including epithelial cells, that the limiting membrane of MVBs can fuse to the plasma membrane and release their contents into the extracellular milieu [546]. When the MVB internal vesicles are secreted in this fashion they are called exosomes. Exosomes have been implicated in a number of processes including intercellular communication during the immune response by virtue of carrying peptide-loaded major histocompatibility complex (MHC) molecules [545]. Exosomes are also excellent candidates to transfer membrane proteins from cell to cell without the need for direct cell contact as it has been suggested that exosomes can bind to target cells and be endocytosed. A recent report has implicated exosomes in the transmission of infectious glycosylphosphatidylinositol-linked prion particles,

bypassing the need for cell to cell contact in the dissemination of prions [547]. In a similar way, if diubiquitnation targeted ExoU to the intracellular vesicles of MVBs, the toxin may be able to spread between cells via exosomes and thus increase its cytotoxic effect.

In addition to our lack of knowledge as to the consequence of ExoU diubiquitination, it is also unclear what targets ExoU for ubiquitination. As previously stated, an E3 ubiquitin ligase is required to transfer the activated ubiquitin from E2 to the target protein, therefore ExoU must bind to an E3 ligase. It is possible that the eukaryotic cofactor required for ExoU activation and the E3 ligase responsible for ExoU ubiquitination reside within the same protein or protein complex. This may provide a clue as to what the eukaryotic cofactor for ExoU is, although the identification of over 500 E3 ligases in humans means the field of potential candidates is wide. All E3 ligases contain either a HECT or RING finger domain [532]. Thus, it is possible that the ExoU eukaryotic cofactor also contains one of these domains. In addition for the requirement of an interacting E3 ligase, ExoU also must contain a specific signal that targets it for ubiquitination. There are a number of different signals that target proteins for ubiquitination including phosphorylation, hydroxylation, glycosylation, deacetylation, aminoacylation, oxidation and inappropriately exposed hydrophobic regions [536]. It is unclear what targets ExoU for ubiquitination but it is not the phosphorylation of the serine 30 residue, as the N-terminal mutant with 88 amino acids deleted is still diubiquitinated.

To summarise, in this chapter we have demonstrated that ExoU is localised to the plasma membrane where it is then able to act as a phospholipase and destroy the cell. Membrane localisation of ExoU also results in diubiquitination of lysine residue 178 through a lysine 63-linked chain. It is unclear what the consequence of ExoU diubiquitination is, as it does not alter the activity of ExoU and only leads to a slight increase in degradation. We have suggested that diubiquitination may be a cellular defence mechanism to target this potent pseudomonal toxin for lysosomal degradation. Alternatively diubiquitination may be a bacterial strategy to increase the targets of ExoU or to aid its intercellular spread. The plasma membrane localisation and consequent phospholipase activity and diubiquitination of ExoU is dependent on a small domain at the C-terminus of this toxin with the tryptophan residue 681 playing an

important role. We do not know how this C-terminal domain targets ExoU to the plasma membrane but suggest that it either binds to a membrane-bound cofactor or directly to the phopsholipid bilayer. As in chapter 3, our attempts to identify the eukaryotic cofactor failed, but we successfully localised it to the particulate membrane-enriched cellular fraction, which should help in further purification of this elusive factor. In conclusion, our results suggest a reason for the importance of the C-terminus in ExoU toxicity reported previously by many groups. We also provide further insight into the targeting and processing of this pseudomonal toxin once it has been injected into the eukaryotic cell by the TTS system.

CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1. SUMMARY OF RESULTS

The aim of this study was to further define the mechanisms of action of the *Pseudomonas aeruginosa* TTS toxins in eukaryotic cells. To achieve this we utilised a *Saccharomyces cerevisiae* model to study the effects of ExoS expression and to screen for host mutants resistant to the cytotoxic effects of ExoS, ExoU and ExoY. We also used a human epithelial cell line to examine the modification, localisation and toxicity of ExoU and determine which regions of the toxin were required for these activities. Our studies resulted in a number of novel findings that I will summarise below.

Using the *S. cerevisiae* model, we have shown for the first time that ExoY and the GAP and ADPRT domains of ExoS are cytotoxic to yeast. We have also demonstrated the utility of the tetracycline-inducible activator-repressor system for examining the toxicity of bacterial toxins in *S. cerevisiae*. Using the tetracycline-inducible system we showed that the ADPRT domain of ExoS disrupted the actin cytoskeleton of yeast resulting in a phenotype similar to that observed in a stabilised actin mutant. We also revealed that the ADPRT domain of ExoS inhibits DNA synthesis in yeast. In addition, analysing the effect of ExoS expression on α-factor synchronised yeast showed that ExoS results in an increase in mating projection formation and aberrant bud development. We used the *S. cerevisiae* haploid deletion library to screen for mutants resistant to the cytotoxic effects of ExoS, ExoY or ExoU. The only yeast mutants identified in our screen were unable to transcribe the TTS toxin genes and thus resistant to galactose-induced expression. Our failure to identify any yeast mutants capable of transcribing ExoS, ExoY or ExoU but resistant to their effects suggests that no non-redundant, non-essential protein is required for their activity.

By analysing the trafficking of ExoU in HeLa cells we discovered that ExoU is localised to the plasma membrane when expressed within the host cell or injected fully formed by the TTS system of *P. aeruginosa*. We demonstrated that this sub-cellular localisation requires the C-terminus of ExoU and the small region between amino acids 679 and 683 is essential for this localisation. Our results showed that targeting of ExoU

to the plasma membrane resulted in diubiquitination of lysine residue 178 through a lysine 63-linked ubiquitin chain. This localisation was also required for toxicity as deletion of the C-terminal region of ExoU that abrogated plasma membrane localisation also resulted in a phospholipase inactive, non-toxic protein. Diubiquitination of ExoU did not alter the enzymatic activity of this toxin but resulted in a small increase in its degradation. We also demonstrated that the eukaryotic cofactor required for ExoU toxicity separated with the membrane-enriched particulate cellular fraction.

5.2. BIOLOGICAL IMPLICATIONS AND DIRECTIONS FOR FUTURE WORK

Bacterial virulence factors enable pathogenic bacteria to infect and cause disease in susceptible hosts. These virulence factors must act in concert to facilitate the colonisation, survival and replication of the bacteria in the hostile host environment. *P. aeruginosa* produces many virulence factors that enable it to infect a wide range of hosts and sites within these hosts. Although the enzymatic action of many of the pseudomonal virulence factors is known, in most cases their precise mechanisms of action remains to be elucidated. For example, the catalytic activities of the four TTS toxins of *P. aeruginosa* are known, many of their targets have been identified and the consequences of their expression have been detailed. However, in many cases the link between the TTS toxins catalytic targets and their cellular effects remains unclear. The activation of the pseudomonal TTS toxins is another area that requires further research, particularly regarding the identity of the eukaryotic cofactors for ExoU and ExoY and also the localisation of these toxins. Our work sought to investigate these questions and I will consider the implications of our results to the understanding of the activation and action of the pseudomonal TTS toxins and also discuss the future work suggested by our results.

Our results regarding the effects of ExoS on *S. cerevisiae* parallel many of those observed within mammalian cells. It has been previously demonstrated in mammalian cell culture systems that ExoS is cytotoxic, disrupts the actin cytoskeleton and inhibits DNA synthesis [350, 351]. We have also demonstrated these phenotypes in yeast, which suggests that ExoS targets highly conserved eukaryotic pathways and justifies the use of this simple eukaryotic model in studying ExoS action. The literature detailing the contribution of GAP and ADPRT domain to the ExoS induced phenotypes is

unclear, with some reports suggesting a role for the GAP domain in cell morphology changes and other experiments indicating that the ADPRT domain is responsible for all the observed phenotypes [378, 395]. Our results clearly demonstrate that in *S. cerevisiae* the ADPRT domain is responsible for the extreme cytotoxicity, actin disruption and inhibition of DNA synthesis observed. However, we also show that the GAP domain is active in yeast as it causes growth inhibition. The actin phenotype observed when the ADPRT domain of ExoS was expressed in yeast provides further insight into how this domain leads to the cell morphology changes observed in mammalian cells. It has been proposed that the changes in cell morphology result from the ability of ExoS to ADP-ribosylate the Rho family of small GTPases or the actin-binding proteins ezrin, radixin and moesin [372, 376]. It is, however, unclear which of these potential targets is modified in natural infection and how this modification leads to the observed cell morphology phenotype. Our results in yeast suggest that whatever the target, its ADP-ribosylation results in an actin phenotype similar to that of a yeast mutant expressing stable, depolymerisation resistant actin [498]. In order to confirm that the ADPRT domain of ExoS stabilises actin, one could determine whether the ADPRT domain made actin resistant to the drug, Latrunculin A. Lactrunculin A binds to and sequesters actin monomers, thus preventing the formation of new actin polymers. The highly dynamic nature of the actin cytoskeleton means that after 5-10 minutes of exposure to Lactrunculin A, the entire yeast actin cytoskeleton is completely disrupted. If the ADPRT domain of ExoU did stabilise actin, it would protect the actin structures we observed from depolymerisation after treatment with Lactrunculin A in the same way that the V159A depolymerisation resistant actin mutant is protected from the effects of this drug [498].

Our *S. cerevisiae* deletion library screen did not identify any strains that were able to express ExoS, ExoU or ExoY but were resistant to their cytotoxic effects. This indicates that there are no non-essential, non-redundant proteins required for the action of these toxins. In the case of ExoS, this may not be surprising as the ADPRT domain generally acts to inhibit its targets, thus knocking out a target would have similar consequences. Also, the targets of the ADPRT domain of ExoS are likely to be essential proteins as their inhibition results in cell death. For ExoU, its ability to hydrolyse phospholipids may directly result in cell death due to membrane destruction. Therefore, knocking out a protein would not inhibit this method of cell destruction.

ExoY increases the concentration of cAMP inside the target cell and this is likely to have multiple consequences. Thus, unless ExoY inhibition of cell growth results from the cAMP activation of a single non-essential pathway, no yeast deletion mutant would be identified. Although our *S. cerevisiae* haploid deletion screen failed to identify any interesting toxin targets, there are a number of other yeast library screen strategies that could be used. The same haploid yeast deletion library could be screened for mutants that were more sensitive to the toxins by identifying deletion strains that grew slower than wild type yeast expressing the toxins. This method was used by one group to identify yeast mutants with altered sensitivities to the *P. aeruginosa* pyocyanin toxin [511]. They assessed the growth of each mutant individually when treated with the toxin and compared their growth to wild type yeast treated with pyocyanin. This method would be unsuitable for studying ExoS due to the extreme toxicity of this protein and also would not identify any essential proteins. Another screen that would enable essential proteins to be assessed relies on the principle of haploinsufficiency. Haploinsufficiency occurs when the loss of function of one gene copy results in an abnormal phenotype, in this case increased sensitivity to a TTS toxin. This screen would use the heterozygous deletion library that consists of mutants with a deletion in one copy of each gene. If the toxin acted on a specific protein target, it follows that decreasing the dosage of this protein may sensitise the heterozygous yeast strain to the toxin. This approach has been used to examine possible targets of a number of drugs [510]. To enable high throughput screening, the yeast heterozygous strains were pooled, treated with the drug and the relative growth of each strain was assessed at set time points [510]. This strategy would also be unsuitable for studying ExoS as the heterozygous library strain cannot be transformed with *exoS* under the control of the *GAL1* promoter so it would be impossible to identify strains more sensitive to the effects of this toxin. A third possible method to examine host proteins involved in TTS toxin action would be to screen a yeast overexpression library in which every yeast mutant overexpresses a different protein. If the TTS toxin targeted and inactivated a protein, overexpression of this protein might render the yeast resistant to the toxin. Such a screen could be done in a yeast strain that enables the use of the tetracycline-inducible expression system and would therefore be suitable for ExoS.

All the TTS toxins secreted by *P. aeruginosa* require a eukaryotic cofactor for activity to restrict their action to the target cell and the identities of the cofactors for ExoU and

ExoY remain unknown. Our attempts to identify the cofactors of ExoU and ExoY using the *S. cerevisiae* deletion library screen failed, which suggests that no non-essential, non-redundant protein fulfils this role. We were also unable to purify the ExoU cofactor by coimmunoprecipitation indicating that the interaction may be weak, transient or require additional conditions that are lost during coimmunoprecipitation, for example lipid binding. We were however able to localise the ExoU cofactor to the membrane-enriched particulate fragment demonstrating for the first time that the cofactor is localised at the site of action of ExoU. This localisation of the ExoU cofactor may assist in its identification as it narrows down the possible candidates. One could fractionate the particulate fragment proteins with regards to size and charge, and use the simple phospholipase assay used in this study to determine which fraction activated rExoU. Once the number of proteins in an activating fraction had been reduced sufficiently, they could be identified by mass spectrometry and individually tested in the phospholipase assay. Our observation that ExoU is ubiquitinated may help in the identification of the cofactor. The ExoU cofactor may also function as an E3 ubiquitin ligase and as such would contain a HECT or RING finger domain [532]. The haploinsufficiency library screen may also be useful in identifying the cofactors for ExoU and ExoY. If the dosage of the cofactor was reduced by half this may render the heterozygous deletion strain more resistant to the toxic effects of ExoU or ExoY. This approach would enable essential genes to be screened but would not identify the cofactors if they were encoded by non-redundant genes as is the case for the 14-3-3 cofactor of ExoS.

For some bacterial toxins to act they require localisation to their site of action. For example, the membrane localisation domain of ExoS is required for Ras ADP-ribosylation, although deletion of this domain does not abolish ExoS induced toxicity [368]. In the case of ExoU we have demonstrated that toxicity is dependent on localisation, with the C-terminus directing ExoU to the plasma membrane where it can then act in concert with its membrane-localised cofactor as a phospholipase and destroy the cell. This involvement of the C-terminus in targeting ExoU to the plasma membrane explains the requirement of this domain for toxicity observed by a number of groups. It remains unclear how the C-terminus anchors ExoU at the plasma membrane, whether it is through direct lipid binding or binding to a membrane bound cofactor, although we have illustrated that the tryptophan at position 681 is important in the

interaction. It is also unclear how ExoU is trafficked from its site of injection by the TTS needle in the cytosol of the eukaryotic cell to the plasma membrane. Our initial attempts to follow the trafficking events by using GFP-tagged ExoU failed, as GFP is too stable to unfold and pass through the TTS needle [196]. In order to overcome this problem one could use a newly developed approach that involves labelling the toxin with a tetracysteine tag and treating the live bacteria with FlAsH. FlAsH is a membrane-permeant fluorescein-based biarsenical dye that is non-fluorescent when unbound but emits a bright green fluorescent light when it forms a high affinity complex with the tetracysteine tag [548]. The tetracysteine motif consists of a 6 amino acid core (Cys-Cys-X-X-Cys-Cys) often flanked by optimised linker stretches of 6 amino acids. Therefore, only about 18 amino acids need to be inserted into the toxin of interest and one must aim to insert this motif into a region that will not destroy the secretion, localisation or activity of the toxin. The FlAsH bound tetracysteine-tagged toxin can be followed from the bacteria into the eukaryotic cell, as these complexes remain intact during secretion through the TTS needle [549]. This technique has recently been used to assess the secretion of IpaB and IpaC from *Shigella flexneri* through the TTS needle into eukaryotic cells [549]. A modification of this method where the target eukaryotic cell is treated with FlAsH so that the translocated tetracysteine-tagged toxin becomes fluorescent upon entry into the target cell has also been used to study the localisation of the *Yersinia entercolitica* TTS toxins YscM1 and YscM2 [550]. This technique enables the trafficking of proteins in living cells to be conveniently monitored by fluorescence and also allows for detailed ultrastructural information to be gained from electron microscopical snapshots of the infected cells [551].

One of the most unexpected results from this study into the activation and activity of pseudomonal TTS toxins was the diubiquitination of ExoU. Although this modification is not required for ExoU phospholipase activity, it is likely to confer some benefit to the bacteria or possibly to the host cell. Diubiquitination through lysine residue 63 of ubiquitin suggests that modified ExoU will be targeted to the endosomal pathway. This might lead to an increase in membrane targets, a means of intercellular spread by exosomes or degradation by the lysosome. Degradation by the lysosome may be a eukaryotic defence mechanism or advantageous to the bacteria to allow the controlled removal of the toxin as is the case for the Salmonella TTS toxin SopE [258]. In order to

further study the consequences of ExoU diubiquitination, it would be interesting to study the trafficking of this modified toxin in isolation from the unmodified form. One way in which this could be achieved is through ubiquitin-mediated fluorescence complementation, which is a method recently developed to show that ubiquitinated Jun is targeted to lysosomal vesicles for degradation [552]. This method relies on the ability of fluorescent proteins, such as enhanced yellow fluorescent protein and enhanced cyan fluorescent protein, to be separated into two complementary fragments that only fluoresce when brought together. Thus, one fragment is fused to ubiquitin and the complementary fragment is fused to the target protein, in our case ExoU. When ExoU is ubiquitinated, the two fluorescent fragments would be brought together and the resulting fluorescence would allow diubiquitinated ExoU trafficking to be followed. In order to test the hypothesis that ExoU diubiquitination results in intracellular spread by exosomes, one could measure the presence of extracellular ExoU after infection of eukaryotic cells with *P. aeruginosa* expressing ExoU.

Our work has answered a number of questions regarding the activation and activity of the TTS toxins of *P. aeruginosa* and in the process has posed many more questions. A great deal more work needs to be carried out in order to unravel the complex interactions between the pseudomonal pathogen and human host as the greater our understanding of this interaction, the greater our ability to influence things in favour of the host.

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