Activation and functional studies of the Type VI secretion systems in *Pseudomonas aeruginosa*

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Declaration of Originality

The work presented in this thesis is my own, and the contributions of others are duly noted.

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Abstract

Pseudomonas aeruginosa is a versatile and prevalent opportunistic pathogen. It encodes a large arsenal of pathogenicity factors, and secrets a plethora of proteins using specialised protein secretion systems. The type VI secretion system (T6SS) delivers proteins directly into neighbouring bacteria or eukaryotic cells using a mechanism homologous to the T4 bacteriophage tail spike.

Three T6SS are encoded on the *P. aeruginosa* genome. The study of the H1-T6SS has been facilitated by the fact it can be activated by the manipulation of the RetS/Gac/Rsm regulatory cascade by deletion of *retS*. However, the precise signals required for activation of this cascade, resulting in H1-T6SS activation, are unknown. This work investigates the role of subinhibitory concentrations of antibiotics in activating the system, and shows that kanamycin is able to induce production of core H1-T6SS components. This activation requires a functional Gac/Rsm cascade, but it is not known if this is due to direct signalling via the cascade, or due to a dominant effect of RsmA repression.

The H2-T6SS is characterized in this work. We highlight key differences between the H2-T6SS cluster in PAO1 and PA14, including the presence of additional core T6SS components and putative secreted effectors. A strain is generated in which expression of the PA14 H2-T6SS cluster can be activated and tightly controlled by arabinose inducible promoters. The activity of the promoters is confirmed by the H2-T6SS dependent secretion of Hcp2 specifically upon arabinose induction.

We further consider two putative H2-T6SS secreted substrates, VgrG14 and Rhs14. Production of these proteins is observed following arabinose induction, but their secretion is not detected. The Rhs14 protein is characterised, and its possible role as a H2-T6SS dependent effector is discussed. Finally, the H2-T6SS system in PA14 is shown to inhibit the internalisation of *P. aeruginosa* PA14, in contrast to the previously published observations of the H2-T6SS promoting internalisation of PAO1.

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Appendices

Work included in this thesis has subsequently been published and is available online:

Chapter 3:

Jones C, Allsopp L, Horlick J, Kulasekara H, Filloux A (2013) Subinhibitory Concentration of Kanamycin Induces the *Pseudomonas aeruginosa* type VI Secretion System. PLoS ONE 8(11): e81132. doi:10.1371/journal.pone.0081132

Chapter 4:

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Abbreviations

ACD	Actin cross-linking domain
AHL	Acyl homoserine lactone
AI	Autoinducer
AT	Autotransporter
bEBPs	Bacterial enhancer binding proteins
CDI	Contact dependent growth inhibition
CF	Cystic fibrosis
Csr	Carbon storage regulator
Cup	Chaperone usher pathway
DGC	Diguanylate cyclase
dot	Defective in organelle trafficking
EPS	Extracellular polymeric substances
Exo	Exoenzyme
Exs	Exoenzyme S synthesis regulatory protein
Fur	Ferric uptake regulator
GacS	Global activator of antibiotic and cyanide synthesis.
gfp	Green fluorescent protein
Gsp	General secretory pathway
H1-T6SS	Hcp secretion island 1 Type Six Secretion System
Hcp	Heamolysin co-regulated protein
His	Hcp secretion island
Hpt	Histidine phosphotransfer domains
icm	Intracellular multiplication
kDa	Kilo Daltons
LadS	Loss of adherence sensor
LPS	Lipopolysaccharide
Mbp / bp	Mega base pair / base pair
Omp	Outer membrane porin
PAGI	*P. aeruginosa* genomic island
PcrV	*Pseudomonas* low calcium response antigen V
PDE	Phosphodiesterase
Pel	Pelicle forming polysaccharide
Pop	*Pseudomonas* outer protein
Psc	*Pseudomonas* secretion component
Psl	Polysaccharide synthesis locus
QS	Quorum Sensing
RetS	Regulator of exopolysaccharide and T3SS Sensor
RetSp	RetS periplasmic domain
Rhs	Recombination hot spot
Rsm	Regulator of secondary metabolism
Sec	Secretion
Ssp	Small secreted proteins
Stk	Serine threonine kinase
Stp	Serine threonine phosphatase
TxSS	Type (1-5) Secretion System
Tae	Type VI amidase effector
Tag	Type VI associated gene
Tat	Twin arginine translocation
Tle	Type VI lipase effectors
Tps	Two partner secretion
Tse	Type six effector
Tsi	Type VI immunity
Tss	Type Six Secretion
VgrG	Valine glycine repeat
Xcp	Extracellular protein
YD-repeat	Tyrosine-aspartate repeat

Chapter 1 Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative, rod-shaped bacterium, with a cell diameter of 0.5-1.0 µm and length of 1.5-3.0 µm. It has an optimum growth temperature of 37 °C, but it is able to grow between 4 °C and 42 °C. *P. aeruginosa* has relatively simple nutritional requirements, often reported to grow in distilled water (Favero et al., 1971). *P. aeruginosa* is a facultative anaerobe, able to use nitrate as a terminal electron acceptor, in the absence of oxygen. A single polar flagellum allows *P. aeruginosa* to swim, and type IV pili allow cells to twitch and swarm on solid and semi-solid surfaces (Garrity, 1989; Kohler et al., 2000).

The genome sequence of *P. aeruginosa* PAO1 was published in 2000, allowing the genetic make-up of this organism to be interrogated and exploited (Stover et al., 2000; Winsor et al., 2011). The 6.3 Mbp genome is among the largest of bacterial genomes, encoding 5570 open reading frames, similar in number to the eukaryotic yeast *Saccharomyces cerevisiae*, equipping *P. aeruginosa* with an enormous potential for diversity.

Pseudomonas spp. are remarkably metabolically diverse, and are able to utilise 146 different organic compounds for growth (Stanier et al., 1966). The ability of *Pseudomonas* spp. to degrade a wide range of substrates, including aromatic compounds and halogen derivatives has made them a useful tool for bioremediation. For example, *Pseudomonas putida* is able to degrade phenolic compounds polluting soils and industrial effluents (Krastanov et al., 2013).

P. aeruginosa also shows enormous environmental versatility, and can be isolated from terrestrial, freshwater and marine environments (Khan et al., 2007), as well as being ubiquitous in man-made environments (Bonten et al., 1999; Pirnay et al., 2003). *P. aeruginosa* can either exist in these environments as planktonic, unicellular organisms, or as sessile cells attached to surfaces in multicellular aggregates known as biofilms (Coggan and Wolfgang, 2012). The formation of biofilms is clinically relevant as discussed further in Section 1.4.

P. aeruginosa has a large host range, capable of infecting plants, animals, and humans. It causes soft-rot disease in plants, leading to economic losses in tobacco, lettuce and sugar cane (Elrod and Braun, 1942), and *Arabidopsis thaliana* is a useful plant model for the study of *P. aeruginosa* pathogenicity (Rahme et al., 1997). The observation that *P. aeruginosa* uses many of the same virulence factors to infect both plants and animals makes plant infection models highly relevant (Rahme et al., 2000). *P. aeruginosa* infects a large range of insects and animals which are widely used as models of infection, including the *Galleria mellonella* (Miyata et al., 2003), *Drosophila melanogaster* (Apidianakis and Rahme, 2009), *Caenorhabditis elegans* (Tan et al., 1999) and mice (Hoffmann et al., 2005).

P. aeruginosa can be a commensal of healthy humans, but has become a prevalent opportunistic human pathogen. The various types of infections caused by *P. aeruginosa* are described in Section 1.2. The ability of *P. aeruginosa* to cause infections is facilitated by its large arsenal of cell-associated and extracellular virulence factors, which allow it to communicate with host cells, to attack and defend, and to acquire nutrients during infection. *P. aeruginosa* encodes for a large number of protein secretion systems, facilitating the secretion of a number of diverse proteins (Bleves et al., 2010). The mechanisms *P. aeruginosa* employs to secrete proteins are reviewed in Section 1.6.

A large proportion, around 8.4% of the genes encoded on the *P. aeruginosa* PAO1 genome are predicted to be devoted to regulatory mechanisms (Stover et al., 2000), many of which are involved in the regulation of pathogenicity. *P. aeruginosa* encodes many complex regulatory networks, which allow appropriate and timely deployment of pathogenicity factors. These mechanisms contribute to the success of this organism as an opportunistic pathogen, and are described in detail in Sections 1.8 and 1.9.

1.2 *P. aeruginosa* the opportunistic pathogen

1.2.1 Opportunistic infections

In addition to being an environmental organism, *P. aeruginosa* is a common opportunistic pathogen of humans, and infections can be acquired in the community or in hospital. Community-acquired infections include outer ear, skin and soft tissue infections in those with diabetes mellitus, ulcerative keratitis in contact lens users, and soft tissue infections associated with burn wounds (Driscoll et al., 2007).

Hospital-acquired *P. aeruginosa* infections can be life threatening with high levels of morbidity and mortality. *P. aeruginosa* infects a range of sites, including the lungs, surgical wounds, urinary tract, and can cause septicaemia (Driscoll et al., 2007). *P. aeruginosa* is responsible for 11-13.8% of nosocomial infections where a microbiological cause is identified in three studies from Korea, Italy and Switzerland (Kim et al., 2000; Lizioli et al., 2003; Pittet et al., 1999), and is the second most common cause of healthcare associated pneumonias in the United States (Kollef et al., 2005). *P. aeruginosa* is therefore clearly responsible for a large proportion of the infectious disease burden in hospitals.

Infections caused by *P. aeruginosa* are a particular problem for those with immune deficiencies including cancer, HIV, and recipients of organ transplantation. *P. aeruginosa* is responsible for 14-21% of bacteraemia in patients with acute leukaemia, a higher rate compared to non-cancerous patients (Chatzinikolaou et al., 2000). In individuals infected with HIV the incidence of *P. aeruginosa* bacteraemia is ten times higher compared to those not infected by the virus (Vidal et al., 1999).

1.2.2 *P. aeruginosa* infections and Cystic Fibrosis

The class of *P. aeruginosa* infections with the most striking morbidity and mortality occur in Cystic Fibrosis (CF) patients, where *P. aeruginosa* airway infection is the cause of death in around 80% of cases (Anon, 2007). *P. aeruginosa* is able to colonise the CF lung, leading to chronic, long term infection, severe pneumonia and death from pulmonary failure (O'Sullivan and Freedman, 2009). A study of young children with CF used a combination of culture techniques and serological screening to show that 97.5% of CF patients were colonised with *P. aeruginosa* by the age of three (Burns et al., 2001).

CF is a genetic disease caused by an inherited mutation in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a chloride channel and regulatory protein (Rosenfeld et al., 2003). Patients with CF have an altered pulmonary environment in which *P. aeruginosa* is able to colonise and survive (Mehta, 2005). The 'low volume' hypothesis describes how the loss of regulation of chloride channels and the resulting compensatory excess sodium and water reabsorption into cells lead to dehydration of the airway surface (Worlitzsch et al., 2002). In healthy individuals the normal mucocilliary clearance mechanism of the lungs is effective in clearing invading microorganisms, preventing infection. In the CF lung, the lubricating layer between epithelial cells and mucus is reduced, cilia become compressed, and the normal mucocilliary

clearance mechanism is inhibited, resulting in an environment conducive to persistent *P. aeruginosa* infection (Worlitzsch et al., 2002).

1.2.3 Antibiotic resistance

P. aeruginosa infections are resistant to commonly used antibiotics, and this resistance can be intrinsic or acquired by *P. aeruginosa* during infection. *P. aeruginosa* is naturally resistant to many antibiotics due to the low permeability of the bacterial outer membrane (Hancock, 1997). Water filled channels in this membrane, known as porins, allow small molecules, including antibiotics, to pass through. In *E. coli* general porins OmpF and OmpC allow non-specific uptake of molecules (Cai and Inouye, 2002). In contrast, *P. aeruginosa* produces highly specialised porins for specific molecules, which do not readily allow non-specific uptake of antibiotics, resulting in a slower rate of uptake (Hancock and Brinkman, 2002). Two *P. aeruginosa* porins are believed to allow limited non-specific transport of substrates. OprF is a major *P. aeruginosa* porin with a copy number of approximately 200,000 per cell (Nestorovich et al., 2006), responsible for the outer membrane transit of molecules between 200 and 3,000 Da (Bellido et al., 1992). OprD, despite being a specific porin for amino acid transport, may allow passage of small molecules below 200 Da (Huang and Hancock, 1993). This slower rate of antibiotic uptake results in resistance since antibiotics are not able to accumulate to active concentrations inside the cell. Mutations inactivating the OprD porin lead to carbapenem resistance by blocking uptake of this molecule (Wolter et al., 2004).

Efflux pumps export multiple antimicrobials, preventing molecules from reaching their target sites, and thus enhancing the antibiotic resistance of *P. aeruginosa*. Characterised efflux pumps from *P. aeruginosa* include MexA/B, MexC/D, MexF/E and MexY/X, which have overlapping specificities for antibiotics from different classes (Masuda et al., 2000). The slower rate of uptake through *P. aeruginosa* porins allows multi drug efflux pumps to work efficiently, since they are not

overwhelmed by large antibiotic concentrations (Lambert, 2002). Mutations leading to the over expression of efflux pumps have been reported in clinical isolates, which increase their intrinsic resistance due to hyper-export of antimicrobials (Saito et al., 1999). The combination of slow diffusion of antibiotics across the cell membranes and active and efficient efflux via these pumps result in the high levels of intrinsic resistance of *P. aeruginosa* to antibiotics.

Resistance to beta-lactam antibiotics can be achieved through mutations allowing the constitutive expression of a chromosomal beta-lactamase AmpC (Bagge et al., 2002). Plasmid borne Beta-lactamases and carbapenemases can be acquired by *P. aeruginosa* through horizontal gene transfer (Woodford et al., 1998). Bacterial enzymes, which transfer acetyl, phosphate, or adenylyl groups onto the aminoglycoside, inactivating it. These modifying enzymes are present on plasmids, which may be acquired during treatment (Poole, 2005). Mutations in key residues of antibiotic targets can change the active site conformation and inhibit the antimicrobial activity, while maintaining the function of the protein. An example in *P. aeruginosa* is the mutation of the *gyrA* gene encoding a subunit of DNA gyrase, a target of the quinolones (Akasaka et al., 2001).

Data published by the Health Protection Agency show that the number of reported cases of *P. aeruginosa* bacteraemia has remained relatively stable at around 3000 cases per year between 2007 and 2011. However, the percentage of *P. aeruginosa* isolates reported as not being sensitive to a combination therapy of piperacillin/tazobactam has risen from 5% in 2007 to 7% in 2011 (Figure 1.1) (Anon, 2012). In the same period, the percentage of *P. aeruginosa* bacteraemia isolates not sensitive to imipenem (14%), meropenem (9%), ceftazidime (8%) ciprofloxacin (11%) and gentamicin (6%) have remained constant, indicating that around 10% of *P. aeruginosa* isolates are resistant to at least one commonly used antibiotic.

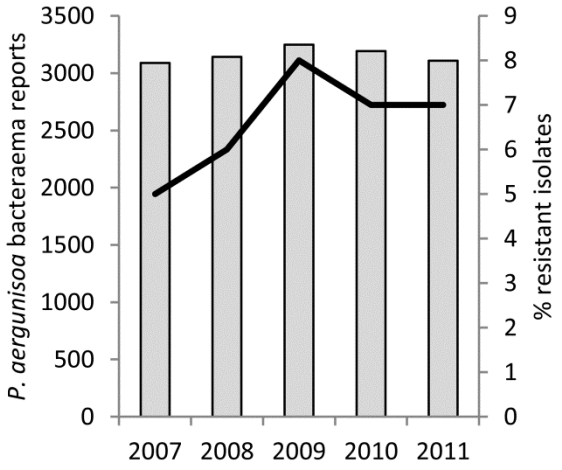


Figure 1.1 Annual reports of *P. aeruginosa* bacteraemia 2007-2011.
Number of *P. aeruginosa* bacteraemia reports to the Health Protection Agency (UK) between 2007 and 2011 (grey bars, primary axis). The percentage of those isolates resistant to a combination therapy of piperacillin and taazobactam is indicated by the black line (secondary axis).

There are many mechanisms by which *P. aeruginosa* is able to develop antibiotic resistance, and the emergence of multi drug resistant strains is a concern. Long-term antibiotic treatment leads to the emergence of multi-drug resistant strains, which contributes to the difficulty in eradicating infections. The spread of antibiotic resistance could ultimately render all current antimicrobials ineffective against the most minor *P. aeruginosa* infections. Currently, *P. aeruginosa* infections are treated using combination therapy of the aminoglycoside tobramycin and the polymyxin antibiotic colistin, which has been shown to be more efficient than mono-therapy, and leads to a significant reduction in cell counts (Herrmann et al., 2010).

1.3 *P. aeruginosa* pathogenicity factors: cell associated

The ability of *P. aeruginosa* to cause the large range of diseases described previously is due to its arsenal of pathogenicity factors, which are both cell-associated and extracellular. The cell-associated pathogenicity factors described here are summarised in Figure 1.2. Cell associated

virulence factors are important in infection since they mediate the initial contact and recognition with host cells, leading to adherence and colonisation, essential early stages of infection.

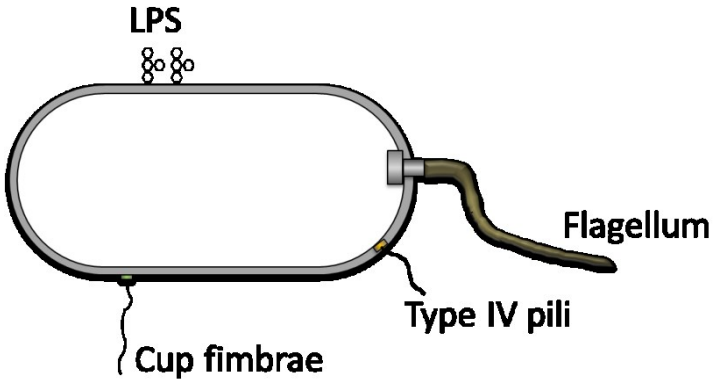


Figure 1.2 *P. aeruginosa* cell associated pathogenicity factors.
Cellular localisation of lipopolysaccharide (LPS), Cup fimbriae, Type IV pili and flagellum of *P. aeruginosa.* Not to scale. LPS is present on the entire cell surface, and the exact number and location of Cup fimbriae are not known. Type IV pili are located at the cell pole, as is the single flagellum.

Type IV pili

P. aeruginosa encodes type IV pili, which are a major extracellular appendage involved in attachment and motility. Type IV pili are 5 to 7 nm thick rod-like fibres with variable length (Mattick, 2002). The type IV pili account for 90% of the adherence capacity of *P. aeruginosa* (Hahn, 1997). The pilus is located at the cell pole, and is involved in the movement of *P. aeruginosa* cells across a semi-solid surface by a process known as twitching motility. Pilus mediated twitching motility allows *P. aeruginosa* to spread across a surface, aiding colonisation. This is achieved by the extension of a pilus from the cell, which is tethered to the surface, and then retracted by the bacterium, leading to movement of the cell (Bradley, 1980). Type IV pili are also involved in attachment of *P. aeruginosa* to host cells through specific binding to carbohydrate moiety of glycosphingolipids asialo-G(M1) and asialo-G(M2) (Hahn, 1997). Type IV pili are assembled by a machine similar to the T2SS, as described further in Section 1.6, and play a role in biofilm formation, described in Section 1.4.

Flagella

P. aeruginosa has a single polar flagellum, which facilitates swimming motility. The importance of the flagella is clear from the observation that non flagellated strains of *P. aeruginosa* are less virulent (Montie et al., 1982). Subsequently, the flagellum has been implicated in adhesion, invasion and inflammation. Flagellin, the major component of the flagellar filament, mediates adhesion to corneal cells, and to host mucins (Hazlett and Rudner, 1994; Scharfman et al., 2001), and flagellin is potent in causing inflammation in the lungs of mice (Liaudet et al., 2003). Additionally, flagellin is recognised by Toll-like receptor (TLR) 5 on human epithelial cells (Zhang et al., 2005).

Fimbriae

P. aeruginosa possesses a range of fimbriae which are important in the attachment of cells to surfaces. These are known as Cup fimbriae, and are assembled by the chaperone-usher pathway (Vallet et al., 2001). The minimum requirements of a Cup pathway consists of a major fimbrial subunit, a chaperone and an usher, important in the assembly of the pilus fibre. Five such Cup fimbriae (*cup*A-E) are encoded by *P. aeruginosa* genomes (Filloux, 2011). Cup pathways may contain additional components, including minor pilins and modified pilins with additional receptor binding domains that act as adhesins (Vallet et al., 2001).

Endotoxin

P. aeruginosa lipopolysaccharide (LPS) is a major component of the outer surface of the outer membrane. LPS is an endotoxin composed of lipid A region, a core LPS region, and the O-antigen, which is cell surface located. Variation in the O-antigen yields two forms of LPS, either smooth

(expressing long O-side chains) or rough (expressing few, short O-side chains). The O-antigen is highly immunogenic, and smooth-LPS is an essential virulence factor since these isolates are significantly more virulent (Cryz et al., 1984). LPS can be recognised by the TLR4 receptor on eukaryotic cells. The A-antigen in *P. aeruginosa* strains isolated from CF patients shows modifications leading to increased recognition by TLR4 (Ernst et al., 2003).

1.4 *P. aeruginosa* biofilm formation

As well as living planktonically as isolated single cells, bacteria can also form multicellular communities known as biofilms. Biofilm formation involves adhesion to a surface, and thus the previously described cell-associated factors, many of which are important in adhesion, also play a role in biofilm formation. Biofilm formation is important in environmental and infection settings. This section describes the characteristics of biofilms, components important in biofilm formation, and the complex developmental cycle involved. Additionally, the influence of biofilms on antibiotic resistance is discussed.

Biofilms are a particular problem in the healthcare setting. Indwelling medical devices are often colonised by *P. aeruginosa* biofilms, presenting a common route of infection (Morris et al., 1999). Biofilms can also occur during infections within the body, and it is estimated that 65% of human bacterial infections involve biofilms (Potera, 1999)

1.4.1 Biofilm characteristics

Biofilms are multicellular, matrix- enclosed, surface associated aggregates of cells. The mature biofilm structure can form tower or mushroom shapes protruding from the colonised surface into the surrounding liquid (Lawrence et al., 1991). These structures are interspersed with open water

channels and pores (Sauer, 2003). The matrix within which cells are enclosed is a slime-like substance made from extracellular polymeric substances (EPS). EPS is produced by the bacteria and provides structural support to the biofilm (Flemming and Wingender, 2010). Water channels and pores allow the exchange of nutrients and oxygen by cells deep within the biofilm community (Hall-Stoodley et al., 2004). Biofilm bacteria are protected from predatory cells, adverse environmental conditions, and starvation. Bacteria within biofilm communities have a competitive advantage over their planktonic counterparts, allowing *P. aeruginosa* cells to persist under conditions in which planktonic cells would normally be eradicated. Although the majority of studies mentioned here discuss mono-species *P. aeruginosa* biofilms, it is likely that mixed species biofilms occur in natural settings.

1.4.2 Components of the biofilm matrix

The EPS which makes up the matrix of *P. aeruginosa* biofilms is composed of polysaccharides, proteins and nucleic acids and is essential for the colonisation of surfaces (Sutherland, 2001). EPS is highly varied, and the matrix changes in composition during the various stages of biofilm development.

Alginate

P. aeruginosa produces a capsular polysaccharide called alginate, a high molecular weight polymer (Linker and Jones, 1964). *P. aeruginosa* alginate is a linear polymer of β-1,4 linked D-manuronic acid and L-guluronic acid residues. The alternative sigma factor encoded by *algT* is essential for alginate production (Flynn and Ohman, 1988). The synthesis of alginate depends on an operon of 12 genes responsible for synthesis, polymerisation and export which are silent in non-mucoid *P. aeruginosa* but is active in mucoid strains. (Deretic et al., 1987)

P. aeruginosa cells overproducing alginate form a characteristic 'mucoid' colony morphology, where colonies are slimy and encased in a viscous material, as shown in Figure 1.3 (Rao et al., 2011). Specific genetic events lead to emergence of alginate overproducers, and a selective process occurs in the cystic fibrosis lung where these alginate overproducers successfully compete and predominate. The majority of these strains carry mutations in the *mucA* gene, resulting in defective MucA, unable to carry out its role as an anti-sigma factor, leading to alginate overproduction (Xie et al., 1996). The selection of these mutants suggests that the overproduction of alginate is advantageous during CF lung infections. Alginate is able to hold a large amount of water, and thus maintain an aqueous environment within the matrix, allowing bacterial cells to survive longer during periods of desiccation (Chang et al., 2007).

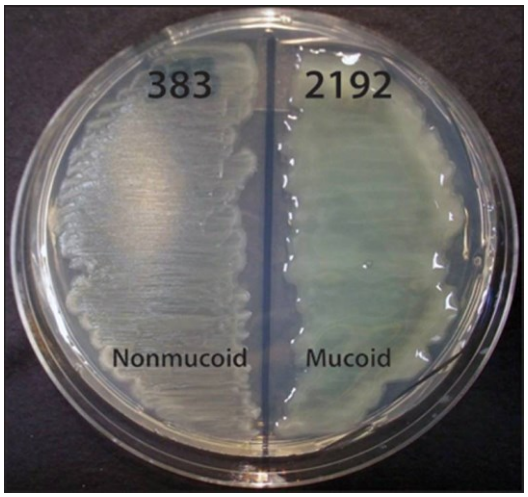


Figure 1.3 *P. aeruginosa* mucoid colony morphology
CF *P. aeruginosa* isolates streaked on L agar. Strain 383 (left), shows a non-mucoid morphology, while strain 2192 (right) shows a characteristic mucoid morphology. Reproduced from (Rao et al., 2011)

The role of alginate in biofilm production is not clear, as it is not essential for in vitro biofilm formation (Stapper et al., 2004). However, it has an important role in shaping biofilms and increases

antibiotic resistance when over produced (Hentzer et al., 2001). This information has led to the search for alternative polysaccharides responsible for biofilm production in the absence of alginate.

Psl and Pel polysaccharide

The search for alternative polysaccharides has identified several biosynthetic operons, one of which, a polysaccharide synthesis locus, *psl*, was important for biofilm integrity (Friedman and Kolter, 2004b; Jackson et al., 2004). *psl* is a repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose, the product of an 11 gene operon, which show homology to carbohydrate synthesis genes (Byrd et al., 2009; Matsukawa and Greenberg, 2004). Psl has been shown to have a role in initial adherence of bacterial to surfaces (Ma et al., 2006), and is a component of mature biofilms, affecting their structure and stability (Ma et al., 2009b).

Psl polysaccharide is not required for biofilm formation in PA14 since this strain lacks part of the *psl* operon. A second aggregative polysaccharide component of the biofilm matrix was discovered in a transposon screen for poor biofilm formation in *P. aeruginosa* PA14. The disruption of a gene cluster, later named *pel*, resulted in an obvious defect in biofilm formation at the air liquid interface, the pellicle, important in the early and late stages of biofilm formation (Friedman and Kolter, 2004a; Vasseur et al., 2005). The Pel polysaccharide locus contains seven genes, homologous to polysaccharide biosynthesis genes. The cluster lacks some essential genes, and may interact with other systems for complete polysaccharide synthesis (Franklin et al., 2011). The Psl polysaccharide seems to be dominant over the Pel polysaccharide, since in the presence of both loci, Psl predominates, and disruption of Pel has only a small impact on biofilm formation (Colvin et al., 2012).

eDNA

Nucleic acids are found in abundance in the biofilm matrix, principally DNA, which is a critical component. The extracellular DNA (eDNA) isolated from *P. aeruginosa* biofilms is derived from genomic DNA, and its presence can be explained by autolysis of cells and release of cytosolic DNA (Allesen-Holm et al., 2006). The structure of early biofilms can be disrupted by exposure to DNase1, showing that DNA is a major component, however more developed biofilms are more tolerant to DNase treatment (Whitchurch et al., 2002). Large amounts of DNA can be observed throughout biofilms at 48h of growth, by confocal laser scanning microscopy. However, at 144h the biofilms contain less DNA, and it is concentrated in the interior of stalks or mushroom shaped caps, not evenly distributed as at 48h (Allesen-Holm et al., 2006).

Accessory biofilm components

Several components have been shown to have accessory roles in the biofilm matrix. LPS is important in biofilm formation, and affects adherence of cells, and cell-free LPS is a major component of the matrix (Lau et al., 2009). A number of carbohydrate binding proteins, including CdrA and cytotoxic lectins, may be important in the interaction of molecules within the biofilm matrix, and formation of a stable matrix (Borlee et al., 2010; Tielker et al., 2005). CdrA is secreted via a two-partner secretion mechanism, and is described in more detail in Section 1.6. Two lectins, LecA and LecB, are important in biofilm formation, but their mode of transport is not understood.

1.4.3 The biofilm development cycle

Biofilm formation is characterised by distinct steps, which result in the formation of mature, complex, biofilm structures. As outlined in Figure 1.4, five distinct stages are involved: reversible

attachment (1), irreversible attachment (2), maturation 1 (3), maturation 2 (4), and dispersal (5) (Sauer, 2003; Sauer et al., 2002).

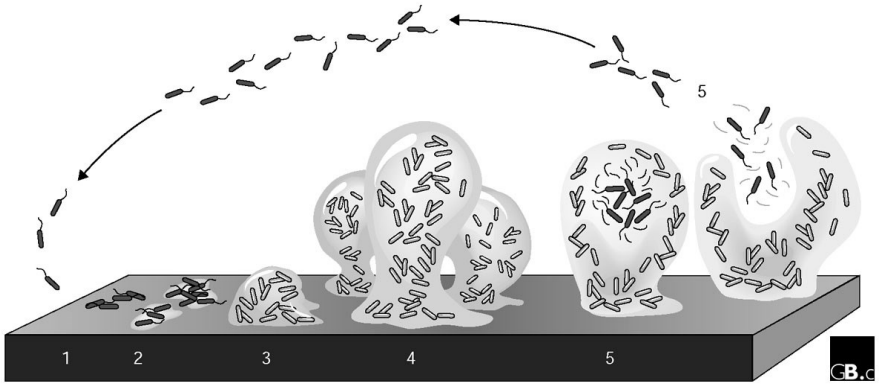


Figure 1.4 Stages of *P. aeruginosa* biofilm formation
Five distinct stages of biofilm formation are shown. Stage 1: Reversible attachment. Stage 2: Irreversible attachment. Stage 3: Early maturation. Stage 4: Late maturation. Stage 5: Biofilm dispersal. Following stage 5, cells are released from the biofilm and return to a planktonic lifestyle, and are able to reinitiate the biofilm formation process. Upper diagram originally published by Biomed central and reproduced with permission from (Sauer, 2003).

Analysis of DNA microarrays and proteomics approaches have shown that the different stages of biofilm formation represent distinct episodes in the lifestyle of *P. aeruginosa*, and bacteria in each stage are physiologically distinct from cells in other stages of the process. The formation of a mature biofilm is a complex developmental process (O'Toole et al., 2000).

Reversible attachment

The reversible attachment phase is the initial event in biofilm formation, where bacteria become associated with, but not attached to, the surface. The ability of *P. aeruginosa* cells to swim increases their ability to locate a surface. Under certain conditions (glucose minimal media) non-motile strains were deficient in biofilm formation, but in other conditions (citrate minimal medium) they were proficient, therefore the dependency on flagella is conditional (Klausen et al., 2003b; O'Toole and Kolter, 1998). Once a cell is located at a surface, it is not yet committed to biofilm formation. Cells

may be enclosed in only small amounts of matrix, and are able to leave the surface and resume a planktonic lifestyle (O'Toole and Kolter, 1998).

sadB has been shown to be involved in the regulation of the frequency by which *P. aeruginosa* cells attach to and detach from surfaces (Caiazza and O'Toole, 2004). A *sadB* mutant is able to attach to a surface, but unable to form microcolonies despite being proficient in twitching and swarming motility. The production of SadB is regulated by intracellular levels of the second messenger c-di-GMP as discussed in Section 1.8 (Merritt et al., 2007).

Irreversible attachment

The transition from reversible to irreversible attachment of the bacterial cell to the surface is essential for biofilm formation, as cells must maintain a strong contact with the surface. Attachment is dependent on type IV pili, with hyperpiliated strains rapidly forming strongly adherent biofilms (Deziel et al., 2001). Flagella may enhance attachment to the surface (Sauer et al., 2002). The *cup* fimbrial genes are also necessary for initial biofilm development, as was discovered in a transposon screen in non-piliated *P. aeruginosa* used to overcome the dominant impact of type 4 pili (Vallet et al., 2001).

Following attachment to a surface bacteria need to increase in number in order to form a nascent microcolony. This increase can come from division of cells attached to the surface, or by recruiting new cells into the developing biofilm from the aqueous environment (Tolker-Nielsen et al., 2000). The process of biofilm maturation is thus a combination of an increase in cell number and the rearrangement of cells, and continued production of matrix components until the complex architecture, and associated channels, are formed.

The production by bacteria of extracellular polymeric substances (EPS) facilitates irreversible attachment, and the production of EPS is a hallmark of the early stages of biofilm formation. Using an *algC*::*lacZ* fusion it was shown that cells attached to Teflon mesh or a glass surface for 15 minutes showed increased expression of the alginate biosynthesis gene *algC* (Davies et al., 1993; Davies and Geesey, 1995). These extracellular polymers act as a sticky slime-like substance, attaching cells to the surface and each other (Sutherland, 2001).

Analysis of global gene expression during initial biofilm formation has been performed in *P. putida*. After 6 hours of attachment to silicone, 2-D protein gel electrophoresis and cDNA subtractive hybridisation was performed (Sauer and Camper, 2001). This experiment found 45 differences in the protein profile of planktonic and sessile cells, and 40 differences in mRNA production. Proteins involved in amino acid metabolism, and flagellar synthesis were downregulated following 6 hours of attachment. Conversely, proteins involved in the synthesis of type IV pili and lipopolysaccharides were upregulated (Sauer and Camper, 2001).

Biofilm maturation

Time lapse confocal laser scanning microscopy has proven a powerful tool for visualisation of the biofilm maturation process. The structure of the mature biofilm is dependent on the growth medium used. When citrate was the sole carbon source, initial microcolonies formed from clonal growth of sessile cells, which then spread evenly across the surface by means of type IV pili, forming flat biofilms. Biofilms grown with glucose as the sole carbon source formed differentiated motile and non-motile subpopulations. The non-motile population form microcolonies at distinct foci, which act as the stalks of mushroom shaped structures. The motile sub population climb on these stalks by means of type IV pili mediated motility and aggregate to form mushroom caps, as illustrated by Figure 1.5 (Klausen et al., 2003a; Klausen et al., 2003b). This is likely due to differences

in the iron concentrations of the media since citrate chelates iron (Harmsen et al., 2010). Twitching motility is induced in *P. aeruginosa* biofilms by iron limitation, and so iron limited biofilms are likely to be flat as they lack the non-motile sub-population required for stalk formation (Patriquin et al., 2008) .

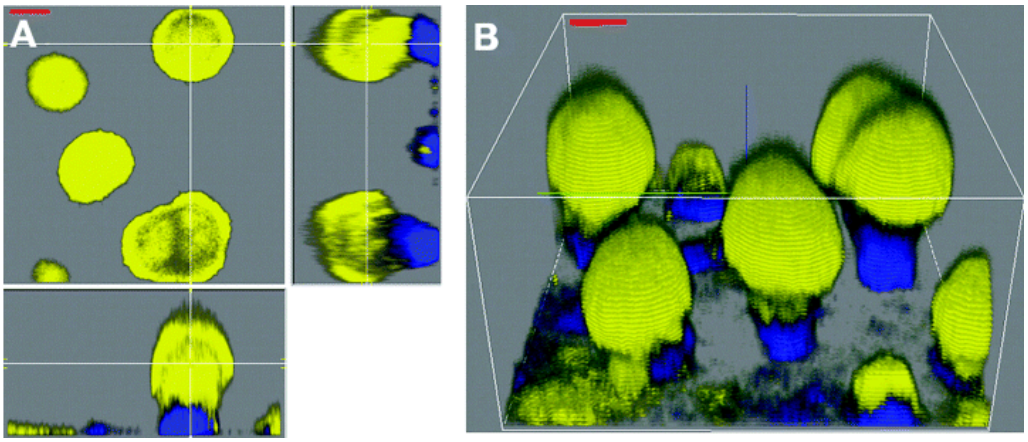


Figure 1.5 Formation of mushroom-shaped structures in *P. aeruginosa* biofilms
Confocal laser scanning microscopy images of mushroom shaped structures consisting of fluorescent yellow *P. aeruginosa* wild type cells forming the mushroom caps, and cyan florescent *P. aeruginosa pilA* mutants forming stalks. Images show 4-day old biofilms initiated with 1:1 mixtures of each strain grown on citrate minimal media. Reproduced with permission from (Klausen et al., 2003a). Panel A shows vertical CLSM sections, while panel B shows the corresponding 3-D CLSM images. Red scale bar indicates 20 µm.

Microarray analysis has been performed to investigate differences in gene expression between *P. aeruginosa* planktonic cells and mature biofilm cells. 73 genes were differentially expressed in these two conditions, many of which were of unknown function. 34 genes were up-regulated in biofilm conditions, which related to the increased expression of bacteriophage genes with unknown relevance. 39 genes were down-regulated in biofilm conditions, including type IV pili and flagella genes (Whiteley et al., 2001).

Analysis of the proteome by 2D gel electrophoresis shows over 50% of detectable proteins had a six fold or greater change in expression level when planktonic cells were compared to mature biofilm cells (Sauer et al., 2002).

Biofilm dispersal

The final stage, dispersal, is characterised by individual cells, or cell clumps, detaching from the surface, leaving void spaces behind (Sauer et al., 2002). Dispersing *P. aeruginosa* cells revert to their planktonic lifestyle, re-initiating biofilm formation on uncolonised surfaces, completing the life cycle (Stoodley et al., 2001). The proteome of cells in the late stages of biofilm formation was more similar to that of planktonic cells than mature biofilm cells, with cells reverting to the planktonic lifestyle (Sauer et al., 2002).

Bacteria possess mechanisms to allow dispersal from biofilms, including reducing in adhesiveness and breakdown of the biofilm matrix. The enzyme alginate lyase is produced by *P. aeruginosa* and is involved in detachment of *P. aeruginosa* due to alginate degradation (Boyd and Chakrabarty, 1994). Dispersal has been observed from microcolonies once they have reached a certain size (Purevdorj-Gage et al., 2005). Biofilm dispersal can be triggered by environmental factors such as shifts in carbon availability (Sauer et al., 2004) iron availability (Banin et al., 2006) and in response to nitric oxide (Barraud et al., 2006). Biofilm dispersal has been linked to the second messenger cyclic di-GMP. Decreased c-di-GMP levels are related to motile lifestyle and to cell dispersal from biofilms (Simm et al., 2004). Regulation by c-di-GMP is discussed in more detail in Section 1.8.

1.4.4 Antibiotic resistance in *P. aeruginosa* biofilms

P. aeruginosa is intrinsically resistant to many antimicrobials, and can acquire resistance to others, as described previously. However, biofilm cells can be up to 1000 fold more resistant to antibiotics than planktonic cells, making biofilms very hard to eradicate (Hoyle and Costerton, 1991). Several hypotheses exist as to why biofilms are highly resistant to antibiotics. Firstly, the ability of

antibiotics to penetrate the biofilm matrix may be reduced, especially in deeper areas of the biofilm (Stewart, 2002). Oxygen limitation and low metabolic activity in the interior of the biofilm may also affect antibiotic activity (Walters et al., 2003). The nature of biofilms also results in decreased susceptibility to eradication by the immune system, contributing to the persistence of biofilm infections (Jensen et al., 2010).

Periplasmic glucans synthesised by the *ndvB* locus have been shown to be a genetic cause of antibiotic resistance in *P. aeruginosa* biofilms. A *ndvB* mutant produces biofilms with comparable architecture to wild type biofilms, but do not show high levels resistance to antibiotics (Mah et al., 2003). These periplasmic glucans interact physically with antibiotics, and are proposed to prevent antibiotics reaching their active site by sequestration in the periplasm. Periplasmic glucans are capable of binding to and sequestering tobramycin or kanamycin within biofilms, and preventing them from reaching their target (Mah et al., 2003; Sadovskaya et al., 2010).

1.5 Extracellular pathogenicity factors

In addition to cell-associated virulence factors, and their contribution to biofilm formation as already described, *P. aeruginosa* also produces a range of extracellular pathogenicity factors. These factors are essential for the progression of infection, and allow *P. aeruginosa* to cause substantial damage to, or invade host cells, sequester nutrients from the environment, and interact with the immune system. This section briefly describes some of the non-protein extracellular pathogenicity factors produced by *P. aeruginosa*; siderophores, pyocyanin, hydrogen cyanide and rhamnolipids.

Siderophores

Pyoverdin and pyochelin are siderophores produced by *P. aeruginosa*, which allow it to acquire iron under iron limiting conditions by sequestering it from host proteins and delivering it into the bacterial cytosol for incorporation into bacteria proteins. Specific cell receptors such as FpvA allow re-uptake of the siderophore-iron complex, dependant on the TonB energy transducer (Beare et al., 2003). Additionally, pyoverdin is a yellow/green pigment that is fluorescent under UV light, characteristic of *P. aeruginosa*.

Pyoverdin is required for full virulence in animal models and it regulates the production of virulence factors. Pyoverdin-deficient mutants demonstrate no virulence in a burned mouse model, and virulence was restored when purified pyoverdin was supplemented during infection (Meyer et al., 1996). Secreted pyoverdin regulates the production of three virulence factors, exotoxin A, and endoprotease, and pyoverdin itself (Lamont et al., 2002). This regulation is dependent on a transmembrane signalling system including an outer membrane receptor for iron bound pyoverdin, a signal-transducing protein and a sigma factor (Lamont et al., 2002).

Pyocyanin

Pyocyanin is a blue/green redox active secondary metabolite that generates reactive oxygen species, essential for full virulence in a mouse model (Lau et al., 2004) Pyocyanin is zwitterionic, and can penetrate host cell membranes and interrupt eukaryotic cellular pathways (Lau et al., 2004). Additionally, pyocyanin has a potent antibacterial effect, with Gram positive organisms being more susceptible (Baron and Rowe, 1981). Pyocyanin has been shown to interfere with ciliary beating potentially reducing muco-cilliary clearance of *P. aeruginosa* (Kanthakumar et al., 1993)

Hydrogen cyanide

Hydrogen cyanide is a potent toxin, inhibiting mitochondrial respiration by binding to the ferric iron of cytochrome oxidase enzyme (Lenney and Gilchrist, 2011). Hydrogen cyanide is responsible for killing in the nematode *C. elegans* model, and mutants not producing cyanide are avirulent in this host (Gallagher and Manoil, 2001). Biologically significant amounts of cyanide are present in sputum from the airways of *P. aeruginosa* infected CF patients (Ryall et al., 2008; Sanderson et al., 2008). Due to inhibition of mitochondrial respiration, cyanide induces anaerobic respiration, causing a toxic build-up of lactic acid (Baud et al., 1996). Cyanide levels reported in CF sputa are within the toxin range (Ryall et al., 2008).

Rhamnolipids

P. aeruginosa produces rhamnolipids, which are ramose containing glycolipid surfactants with a detergent-like structure (Jarvis and Johnson, 1949). Their role remains highly speculative, and they are involved in a plethora of phenotypes. Rhamnolipids emulsify hydrophobic substrates and allow them to be taken up by *P. aeruginosa* (Noordman and Janssen, 2002), and have antimicrobial properties against a range of bacteria (Ito et al., 1971).

Rhamnolipids have several roles in the virulence of *P. aeruginosa*. They integrate into the epithelial cell membrane, disrupting tight junctions and disrupting epithelia (Zulianello et al., 2006), as well as killing polymorphonuclear leukocytes and macrophages, and inhibiting phagocytosis (Jensen et al., 2007; McClure and Schiller, 1992, 1996). Rhamnolipids have been detected in the sputum of CF patients (Kownatzki et al., 1987), where they may promote persistent infections by reducing muco-ciliary clearance of *P. aeruginosa* (Read et al., 1992).

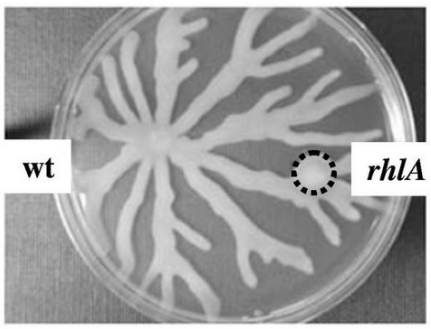


Figure 1.6 *P. aeruginosa* PA14 swarming motility requires rhamnolipids
Wild type *P. aeruginosa* PA14 and PA14 Δ *rhlA* mutant, defective in rhamnolipid production, spotted onto 0.5% swarm agar and incubated for 16h. The dotted line indicates the colony formed by the non-swarming *rhlA* mutant. Reproduced with permission from (Caiazza et al., 2005)

Rhamnolipids are also important for biofilm formation by *P. aeruginosa* by maintaining the fluid channels in mature biofilms allowing water and oxygen flow (Davey et al., 2003), and in the formation of biofilm structures since disruption of rhamnolipids synthesis results in flat biofilms (Pamp and Tolker-Nielsen, 2007). Rhamnolipids have been implicated in swarming motility where they are postulated to reduce the surface tension allowing *P. aeruginosa* to swarm on semi-solid surfaces, since disruption of rhamnolipids synthesis results in loss of the characteristic swarming patterns, as shown in Figure 1.6 (Caiazza et al., 2005).

1.6 *P. aeruginosa* pathogenicity factors: Secreted proteins

A large number of secreted proteins contribute to the pathogenicity of *P. aeruginosa*, and are delivered from the bacterial cytoplasm by dedicated protein secretion systems. This section describes these secretion systems, explaining how proteins are exported across the bacterial membranes, and in some cases, the host cell membrane. The nature and effects of the many *P. aeruginosa* secreted proteins and their role in virulence will be discussed. The T6SS is considered separately in section 1.7.

Protein secretion systems in Gram negative bacteria have been classified, and there are currently six types. These are named Type I Secretion System (T1SS) through to the Type VI Secretion System (T6SS). Secretion systems export proteins in one- or two-steps, as shown in Figure 1.7. One-step mechanisms involve secretion of proteins across both bacterial membranes in a single step by dedicated machinery, and periplasmic intermediates do not accumulate in a secretion mutant. The T1SS, T3SS, T4SS and T6SS work in this way. Two-step mechanisms involve initial secretion across the membrane using universal transporters, and separate dedicated machinery is used for transport across the outer membrane. The T2SS and T5SS secrete proteins in two steps. The recently described T7SS from my *Mycobacterium tuberculosis* is not included here due to the fact is not found in Gram negative bacteria, but is involved in the secretion of proteins across the inner membrane and the highly impermeable cell wall of the Gram positive mycobacteria (Abdallah et al., 2007).

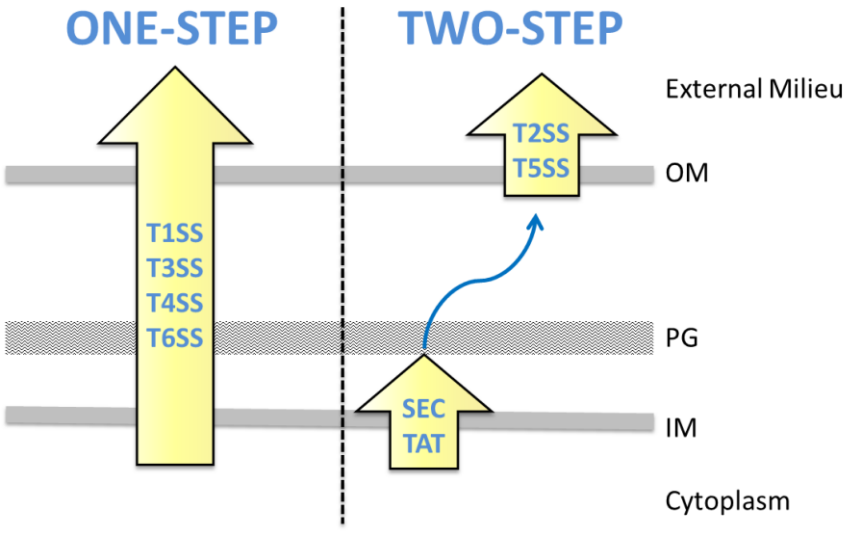


Figure 1.7 One and Two-step protein secretion mechanisms
Gram negative protein secretion systems are either a one- or two-step process. One-step secretion (left) involves transport across the bacterial inner membrane (IM) and outer membrane (OM) in a single step, while two-step secretion (right) involves initial transport into the periplasm via Sec or Tat, and dedicated machinery for outer membrane transport.

1.6.1 Inner Membrane Translocation

Protein export by two-step mechanisms (T2SS and T5SS) first requires translocation across the bacterial inner membrane. The Sec (secretion) and Tat (twin-arginine-translocation) systems provide a route across the inner membrane for substrates of both the T2SS and T5SS, as well as for periplasmic-localised or outer membrane proteins. Once in the periplasm, T2SS and T5SS substrates are available for secretion across the outer membrane by dedicated machinery.

Despite their common function, the mechanisms used by the Sec and Tat systems to transport proteins are different. The Sec system transports unfolded single linear polypeptides and is essential for viability, while the substrates of the Tat system are folded in the cytoplasm prior to transport including folded proteins carrying co-factors (Weiner et al., 1998) and multi-protein complexes (Theuer et al., 1993).

Proteins are targeted to the Sec or Tat systems by a cleavable N terminal signal sequence. The signal sequences for targeting to the Sec and Tat systems are structurally similar, but not identical, as illustrated by Figure 1.8. Targeting to the Sec translocon is mediated by a 20 amino acid N terminal sequence, made up of three parts. The amino terminal n-region of the signal sequence is positively charged, and followed by a hydrophobic core h-region, terminating with a polar carboxyl-terminal c-region (von Heijne, 1990). The c-region is required for recognition by signal peptidase which cleaves the signal peptide to yield the mature protein, typically at an 'A-x-A' motif (Paetzel et al., 2002). Proteins targeted to the Tat translocon possess an N terminal signal sequence which also possess a positively charged n-region which is much longer compared to the sec sequence. Tat substrates also have a hydrophobic h-region, and a short polar c-region for recognition by the signal peptidase. Proteins targeted to the Tat machinery have two conserved arginine residues within their signal

sequence, located between the n and h regions, and important for substrate recognition (Berks, 1996).

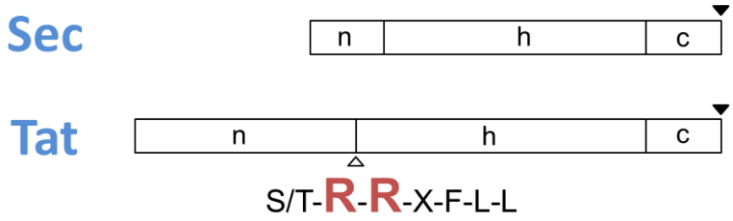


Figure 1.8 Sec and Tat signal sequences
Amino terminal n-regions, hydrophobic h-region and carboxy terminal c-regions shown for Sec and Tat signal sequences. Black arrow indicates the site of cleavage by signal peptidase, white arrow indicates the location of the Tat specific twin arginine sequences as shown.

The Sec Translocon

Proteins can be targeted to the Sec translocon co-translationally or post-translationally, as indicated by Figure 1.9. The co-translational route involves a protein known as signal recognition particle, SRP, which binds to the signal sequence of a Sec targeted protein as it emerges from the ribosome. The entire Ribosome-SRP-nascent polypeptide complex is targeted to the Sec machinery (Luirink et al., 2005). A signal recognition particle receptor transfers the nascent polypeptide to the Sec machinery. Proteins transported in this way generally end up inserted into the inner membrane (Luirink et al., 2005). The post-translational route is used by the majority of Sec-targeted secreted, periplasmic and outer membrane proteins, and involves recognition of substrates by the secretion specific chaperone SecB. SecB binds to proteins post-translationally, following release from the ribosome complex, and maintains them in a secretion competent unfolded state as well as targeting them to the Sec machinery at the inner membrane (Driessen, 2001).

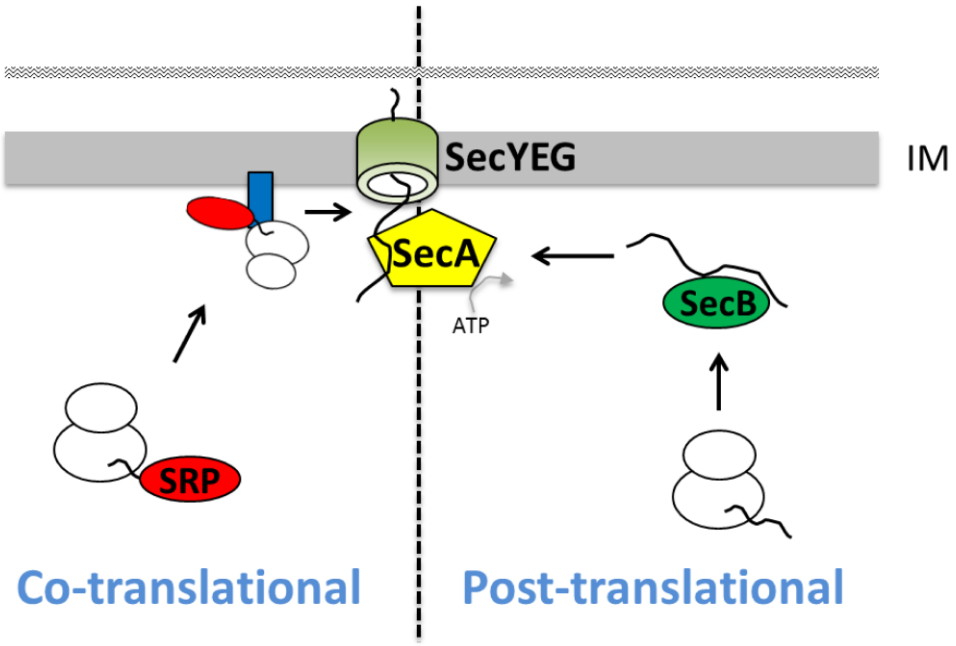


Figure 1.9 Targeting of proteins to the Sec translocon
Proteins can be targeted to the Sec machinery co-translationally (left) or post-translationally (right). IM denotes the inner membrane, where the SecYEG channel is located (green ring). The details of each mechanism are included in the text. Red oval: signal recognition particle (SRP), green oval: SecB. Blue rectangle represents SRP receptor, and the yellow pentagon represents SecA. The white stacked ovals represent the ribosome, with an emerging Sec targeted polypeptide.

The Sec translocon consists of three integral membrane proteins, SecY, SecE and SecG, and a cytoplasmic ATPase, SecA (de Keyzer et al., 2003). SecA associates with SecYEG and receives the Sec-targeted polypeptide from the ribosome or the SecB chaperone. SecA threads the unfolded protein through the SecYEG membrane channel, using energy from ATP hydrolysis and the proton motive force (Hartl et al., 1990). The SecYEG channel is only opened once a Sec-targeted polypeptide is bound, and unwanted leakage through the pore is avoided by providing a tight seal around the translocating protein (Van den Berg et al., 2004). Sec targeted polypeptides are exported in a 'step-wise' manner into the periplasm, with multiple cycles of SecA binding and ATP hydrolysis contributing to translocation (van der Wolk et al., 1997).

The Tat Translocon

Since the Sec translocon is limited to unfolded protein transport, the Tat system provides a vital route across the periplasm for folded proteins. These include proteins containing co-factors or multi-protein complexes which are incompatible with the Sec translocon (De Buck et al., 2007; Weiner et al., 1998). During the study of periplasmic hydrogenases from *Desulfovibrio vulgaris* it became clear that a Sec-independent system was present, since only one subunit of this hydrogenase contained a signal sequence, but both subunits were required for their translocation. This indicated that these proteins were secreted as a complex in a folded state (Vandongen et al., 1988).

The mechanism of translocation by the Tat system is summarised in Figure 1.10. The minimal Tat translocon is composed of two subunits, TatA and TatC, while a third component, TatB, is also found in the more complex Proteobacteria (Lee et al., 2002; Sargent et al., 1999). TatB is very similar to TatA, and may be specialised for more stable TatC interactions (Yen et al., 2002). TatB is not essential, since TatA can complement for the lack of TatB (De Keersmaeker et al., 2005). TatC is an integral membrane protein, with six transmembrane domains, able to bind twin arginine signal sequence domains (Alami et al., 2003; Holzapfel et al., 2007). Following substrate binding a conformational change occurs in TatC that pulls the substrate through the membrane, facilitated by weakening of the membrane by TatA and the proton motive force. TatA proteins, when purified following overexpression, form ring-like structures with a variable diameter, which may allow transient weakening and disruption of the membrane for substrate transport (Bruser and Sanders, 2003; Gohlke et al., 2005). Transport via the Tat translocons is proposed to be in a single step, after which the membrane quickly re-seals, consistent with a large proton flux accompanying Tat transport (Alder and Theg, 2003).

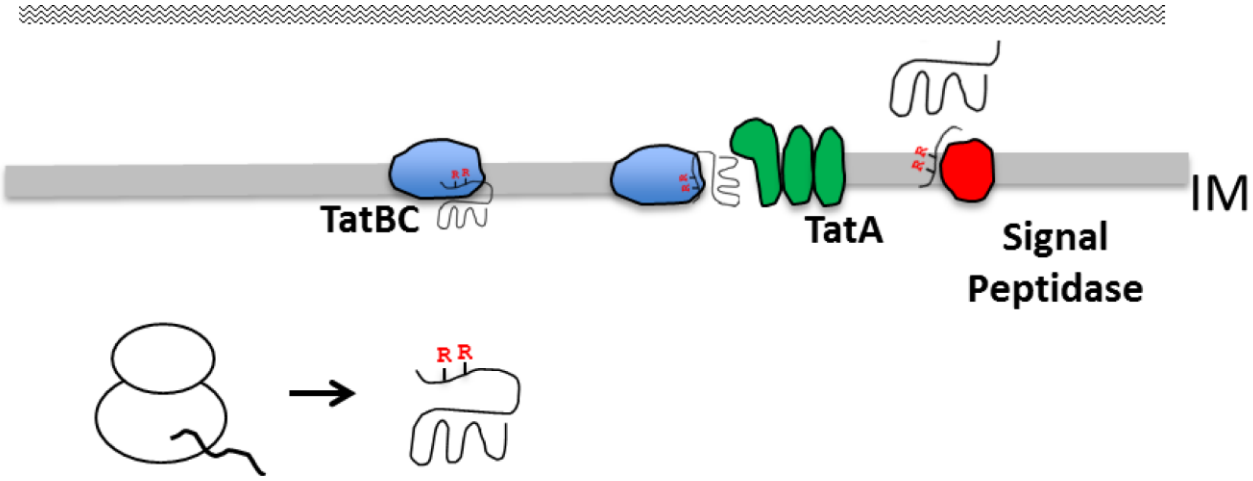


Figure 1.10 Targeting of proteins to the tat translocon
Tat targeted proteins, containing a twin-arginine motif (RR) are released form the ribosome (white ovals) and are folded in the cytoplasm. The signal sequence is then recognised by TatBC (blue) and exported across the inner membrane (IM) in a Tat A dependent manner (green). Once in the periplasmic space, the signal sequence is cleaved by signal peptidase (red) to release the mature protein.

1.6.2 The Type I Secretion System (T1SS)

T1SS is among the simplest protein secretion systems, delivering unfolded proteins through the bacterial envelope in a single step, thus independent of the Sec and Tat translocons. The Type I machinery is a three component translocator, illustrated by Figure 1.11 The T1SS spans the bacterial inner and outer membranes, allowing transport of proteins from the cytoplasm directly into the extracellular medium. Two components, located at the inner membrane, are the ABC (ATP binding cassette) protein and an adaptor protein which extends into the periplasm. The third component, located at the outer membrane, forms a beta barrel pore with α-helical projections into the periplasm (Koronakis et al., 2000). The periplasmic sections of the outer membrane pore and the inner membrane adaptor protein interact and form a continuous channel across the cell envelope (Holland et al., 2005).

Proteins targeted to the T1SS contain a non-cleavable C terminal secretion signal, although the amino acid sequence of this signal is not highly conserved (Mackman et al., 1986). The signal is believed to partly rely on the helical secondary structure of the C terminal 60 residues of the protein (Zhang et al., 1995). This C terminal signal sequence interacts with the ABC protein and the adapter protein (Benabdelhak et al., 2003) which is thought to induce a conformational change in the ABC transporter, promoting ATP binding and hydrolysis, and providing the energy required for the translocation of the substrate through the pore (Letoffe et al., 1996).

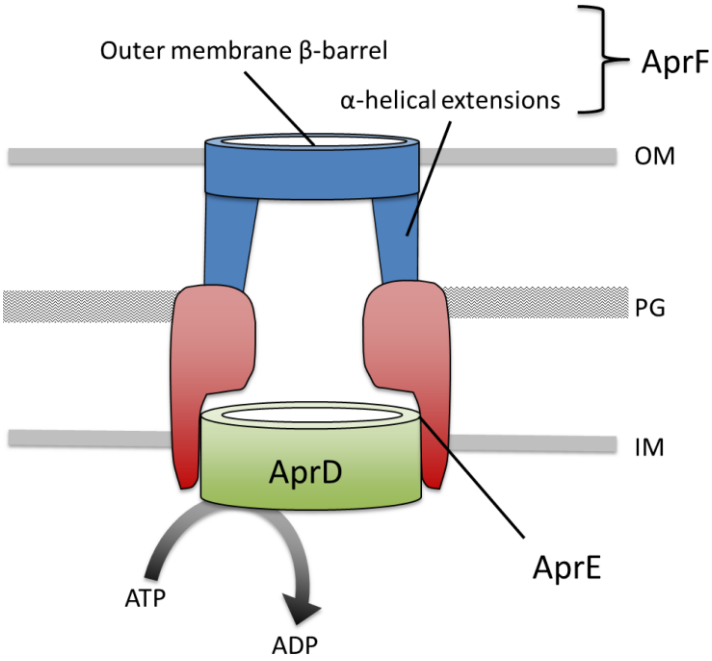


Figure 1.11 Model of the T1SS
The three components of the P. aeruginosa Apr T1SS. The AprD ATPase (green) is located in the inner membrane (IM), while AprF (blue) is located in the outer membrane (OM) and forms a β-barrel with alpha helical projections. ArpF and ArpE are linked by the AprE adaptor protein (red).

P. aeruginosa encodes two T1SSs. The first is encoded by the *apr* genes, responsible for the secretion of an alkaline protease AprA, and a protein of unknown function AprX, (Duong et al., 2001; Guzzo et al., 1991). AprA alkaline protease is responsible for the degradation of host tissues, allowing bacterial invasion and subsequent systemic infection (Heck et al., 1986; Matsumoto, 2004).

The *apr* T1SS is composed of the AprD ABC transporter, the AprE adapter protein and the AprF outer membrane component.

The second characterised *P. aeruginosa* T1SS is the HasAp system, which is involved in the secretion of a haemophore (Letoffe et al., 1998). HasAp is important for the scavenging of iron from haemoglobin, allowing iron acquisition by *P. aeruginosa* in the iron limited environment of host tissues (Letoffe et al., 1998). In this system the ABC transporter is HasD, the adaptor is HasE, and the outer membrane protein is HasF. A fourth protein, HasR, is an outer membrane receptor which allows for the internalisation of the HasAp-heam complex (Letoffe et al., 1998).

1.6.3 The Type II Secretion System (T2SS)

Components and Assembly of the T2SS

The Type Two Secretion System (T2SS) is able to secrete proteins to the external environment in a two-step process dependant on Sec or Tat for initial inner membrane translocation. T2SS substrates carry the required N terminus secretion signal for targeting to Sec or Tat, which is cleaved by signal peptidase after translocation across the inner membrane. Once in the periplasm, substrates are targeted to the dedicated T2SS machinery for transport across the outer membrane. A prerequisite for T2SS dependent secretion is for substrates to be properly folded before leaving the periplasm, which suggests that the as yet poorly defined signal for targeting to the T2SS is dependent on the final 3D conformation of the protein (Francetic and Pugsley, 2005; Voulhoux et al., 2000). The T2SS system is composed of between 11 and 16 proteins, and several T2SS proteins have homologous counterparts in the type IV pilus apparatus, suggesting an evolutionary link between these two systems (Peabody et al., 2003). Comparison of T2SS and type IV pilus assembly are shown in Figure 1.12.

Type IV pili are long, extracellular appendages, typically 50-80 Å in diameter and up to 4 µm in length, exposed on the surface of *P. aeruginosa* cells. These appendages allow *P. aeruginosa* to move by twitching motility mediated by extension, tethering and retraction of the Type IV pili (Bradley, 1980). The pilus structure is composed of multiple copies of a single subunit, known as the pilin, which oligomerise in a helical conformation (Forest and Tainer, 1997). The pilus extends from the cell via an outer membrane pore, the secretin, driven by the energy for an assembly ATPase, and is retracted by the action of a retraction ATPase (Chiang et al., 2008). This pilus extension and retraction mechanism is responsible for the twitching motility of *P. aeruginosa*. Our understanding of pilus biogenesis has been instrumental in the characterisation of the T2SS.

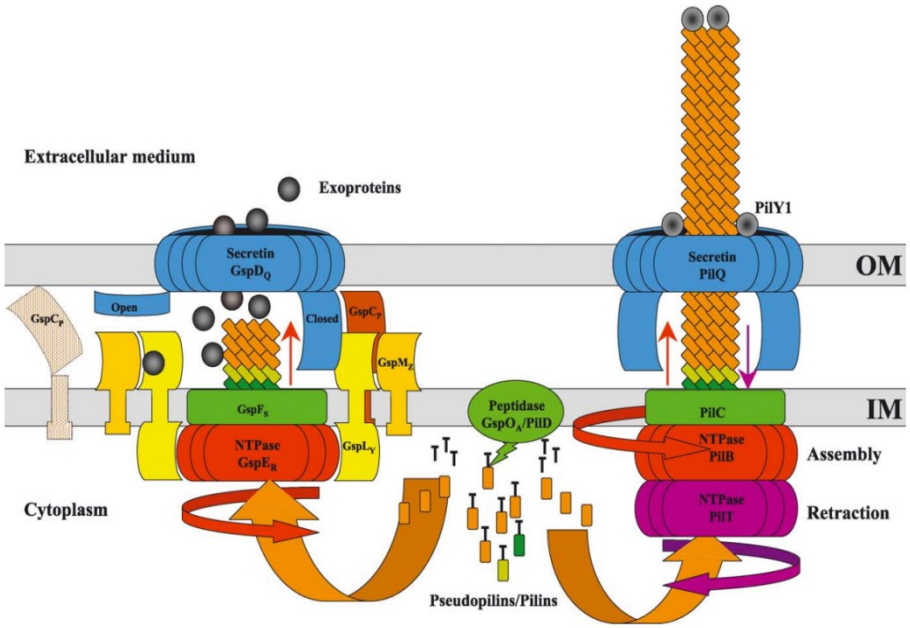


Figure 1.12 The *P. aeruginosa* T2SS
Comparison of the T2SS (left) with the type IV pilus assembly apparatus (right). Corresponding components in the two systems share the same colour. Both systems contain an outer membrane (OM) secretin (blue), and an inner membrane (IM) ATPase (Red). While the type Iv pilus is made up of many rectangular orange pilin subunits and extends from the surface of the cell, the T2SS pseudopilus is shorter, and contained within the periplasm. Additional components are referred to in the text. Reproduced with permission from Elsevier (Filloux, 2004).

The T2SS was originally described as the terminal branch of the general secretory pathway, while the initial branch is provided by Sec or Tat. The nomenclature used here is that of the *P. aeruginosa* T2SS general secretory pathway (Gsp) throughout. The T2SS machinery is made up of three distinct protein sub-complexes spanning the bacterial membranes. At the inner membrane a multi protein platform contains several proteins (GspCEFLM), and a cytosolic traffic ATPase (GspE), associates with this platform (Camberg and Sandkvist, 2005). A second sub-complex, the pseudopilin, is homologous to the long Type IV pilus structure, but does not extend to the exterior of the cell (Durand et al., 2003). The third sub-complex is the secretin GspD, which forms an outer membrane channel providing substrates with access to the external environment.

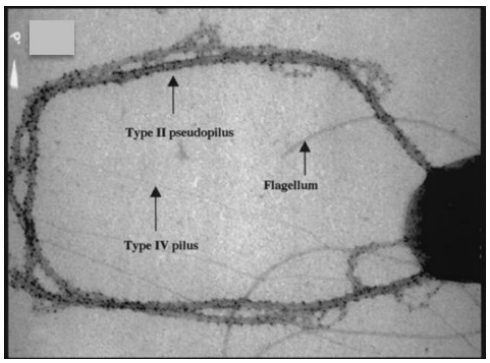


Figure 1.13 The *P. aeruginosa* T2SS pseudopillus
Transmission electron micrograph of *P. aeruginosa* PAO1 overexpressing GspG, following immunogold labelling with antibodies raised against the pilin subunits. The location of the flagellum, type IV pilus and T2SS pseudopilus are indicated by arrows. Reproduced with permission from (Durand et al., 2003)

The similarity between T2SS and type IV pilus assembly is shown by the fact that six of the 12 conserved T2SS genes encode proteins dedicated to the formation of pilus-like structure known as the pseudopilus, however this structure is much shorter than the Type 4 pilus counterpart (Bally et al., 1992). Five of these genes encode pseudopilins (GspG-K), which are subunits of the pseudopilus. GspG is the most abundant pseudopilin and is co-translationally recognised by SRP and utilises the sec pathway for inner membrane translocation (Arts et al., 2007). The sixth gene encodes a prepilin peptidase, GspO, involved in processing of the pilins and pseudopillins (Nunn and Lory, 1991). When

GspG, the major pseudopilin, is overproduced it is able to form long fibre-like structures resembling the type IV pilus, observed by transmission electron microscopy, shown in Figure 1.13 (Durand et al., 2003). Overexpression of the four minor pseudopilins do not result in the production of comparable structures, but GspK is proposed to be the tip of the pseudopilus, preventing its extension beyond the cell envelope, since *gspK* mutants produce exposed, long, fibres (Durand et al., 2005).

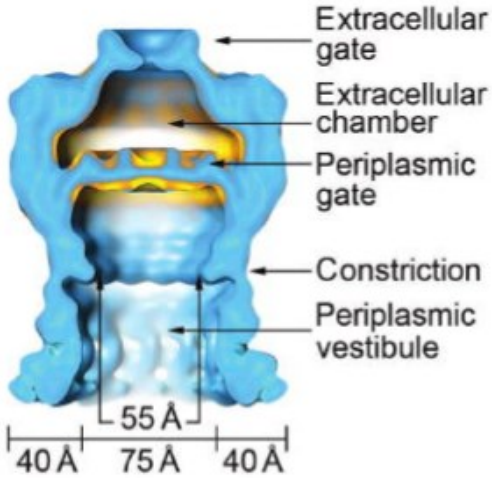


Figure 1.14 The *V. cholerae* GspD secretin
A cut-out side view of the cryo electron microscopy structure of *V. cholera* GspD, revealing the periplasmic vestibule, a constriction, the periplasmic gate (yellow), and an extracellular chamber and gate. The full height of the structure shown is 200 Å, and the width is 155 Å. Reproduced from Nature (Reichow et al., 2010)

The channel formed in the outer membrane is made of dodecamers of the secretin protein, GspD. The secretin from *Vibrio cholerae* has been shown by cryo-electron microscopy to form an elegant cylindrical barrel-like structure with 12 fold symmetry, as shown in Figure 1.14 (Reichow et al., 2010). The *V. cholerae* GspD resembles an upturned cup, and has a large periplasmic vestibule, separated from a small extracellular chamber by a periplasmic gate. The extracellular chamber is itself closed by an extracellular gate (Reichow et al., 2010). The outer membrane secretin is linked to the inner membrane platform by a trans-periplasmic protein, the bitopic inner membrane located GspC. GspC has an N terminal cytoplasmic domain and a C terminal periplasmic domain separated

by a single transmembrane segment. GspC interacts with the OM secretin in *P. aeruginosa*, and thus provides a link between the inner and outer membranes (Gérard-Vincent et al., 2002). The traffic ATPase, GspE, contains conserved ATP binding and hydrolysis domains (Turner et al., 1993), and interacts with the inner membrane platform through interaction with GspL (Ball et al., 1999).

A model is proposed in which T2SS substrates initially access the periplasm via the sec or tat systems, where they are able to interact with the periplasmic domain of the secretin and the pseudopilus tip. This is proposed to stimulate GspE activity to add pseudopilin subunits to the pseudopilus, and the growing tube functions as a piston pushing exoproteins through the secretin channel (Korotkov et al., 2012). This is supported by observations that exoproteins interact with the periplasmic domain of GspD and GspC and components of the pseudopilus tip (Douzi et al., 2011), and that the T2SS secreted cholera toxin is able to bind to the periplasmic side of the *V. cholerae* secretin (Reichow et al., 2011). The T2SS lacks a counterpart for the retraction ATPase found in Type 4 pilus systems, so it is unlikely that the retraction mechanism is conserved (Douzi et al., 2012).

P. aeruginosa T2SS substrates

A recent review described 12 secreted substrates dependant on the *P. aeruginosa* Xcp (extracellular protein) T2SS system, which are listed in Table 1 (Bleves et al., 2010). A second T2SS is encoded on the *P. aeruginosa* genome, known as the Hxc T2SS (Homologous to *Xc*p). The Hxc system has been shown to be active under phosphate limiting conditions, and is responsible for the secretion of a low molecular weight alkaline phosphatase (Ball et al., 2002).

The exoproteins secreted by the Xcp T2SS have a broad spectrum of activity including, but not limited to, proteolytic enzymes and lipid metabolism. The secreted substrates are important in the infection process causing tissue damage, and allowing nutrient acquisition (Jyot et al., 2011). These

include LasA and elastase (LasB), which are able to degrade elastin, a major component of lung connective tissue (Braun et al., 1998). Elastase (LasB), is also able to cleave surfactant protein D, disrupting several immune functions and aiding in *P. aeruginosa* survival during infections (Alcorn and Wright, 2004). LasA has additionally been shown to have specific activity against staphylococci (Kessler et al., 1993).

	Gene	Characteristics	Reference
Proteolytic Enzymes			
LasA	PA1871	Staphylolytic and elastolytic	(Braun et al., 1998)
LasB	PA3724	Metalloproteinase (Elastase)	(Braun et al., 1998)
PaAP	PA2939	Aminopeptidase	(Braun et al., 1998)
PrpL	PA4175	Lysine specific endopeptidase (Protease IV)	(Fox et al., 2008)
Lipid Metabolism			
LipA	PA2862	Triacyl glycerol acyl hydrolase	(Jaeger et al., 1994)
LipC	PA4813	Lipase	(Martinez et al., 1999)
PlcN	PA3319	Non hemolytic phospholipase C	(Voulhoux et al., 2001)
PlcH	PA0844	Hemolytic phospholipase C	(Voulhoux et al., 2001)
PlcB	PA0026	Phosphatidyl-ethanolamine specific phospholipase	(Barker et al., 2004)
Others			
PhoA	PA3296	Alkaline phosphatase	(Filloux et al., 1988)
CbpD	PA0852	Chitin-binding protein	(Folders et al., 2000)
ToxA	PA1948	AB Toxin, ADP-ribosyl transferase	(Lu et al., 1993)

Table 1 *P. aeruginosa* T2SS substrates

The *P. aeruginosa* Xcp T2SS also secretes a range of lipases and phospholipases which are targeted to the eukaryotic membrane. The secreted phospholipases (Plc) are able to cause tissue destruction by hydrolysing phospholipids in the host epithelium or erythrocyte membranes (Schmiel and Miller, 1999). Phospholipases also play a role in disease through over-stimulating the immune response and promoting inflammation (Konig et al., 1997).

The secreted alkaline phosphatase PhoA is capable of liberating free phosphate for utilisation by *P. aeruginosa* (Filloux et al., 1988). A secreted chitin binding protein (CbpD) is degraded by a second T2SS secreted effector, elastase. When bound to chitin, CbpD is protected from proteolysis. Since CbpD is not able to degrade chitin, its role in pathogenicity is proposed to be as a chitin binging

adhesin and not a chitinase, although the precise role of this effector is yet to be shown (Folders et al., 2000).

A well characterised Xcp T2SS substrate from *P. aeruginosa* is the AB toxin exotoxin A (ToxA), which is a highly toxic virulence factor. The catalytic activity of ToxA is responsible for ADP ribosylation of elongation factor 2, which disrupts the protein synthesis of host cells, ultimately leading to cell death (Iglewski and Kabat, 1975). Although the protein is secreted into the extracellular milieu, it is capable of self-targeting to eukaryotic cell membranes. The linear protein sequence can be divided into several domains, including N terminal cell recognition and translocation domains (Theuer et al., 1993), and a C terminal catalytic domain, responsible for the observed toxicity (Siegall et al., 1989).

1.6.4 The Type III Secretion System (T3SS)

The T3SS further extends the secretion capability of *P. aeruginosa*, allowing injection of proteins from the bacterial cytoplasm directly into host cells. The T3SS translocates proteins across the bacterial inner and outer membranes, and the host cell plasma membrane, in a single step. These proteins, called effectors, interfere with host cell signalling. The T3SS apparatus is known as the 'injectisome', and is evolutionarily and structurally related to the flagellum basal body apparatus (Saier, 2004). The observation of a needle like injection apparatus from the T3SS of *Salmonella* spp. (Figure 1.15A) shows the elegant structure of this system (Schraidt et al., 2010).

The T3SS was first characterised in *P. aeruginosa* by Yahr and colleagues (Yahr et al., 1996). A single copy of the T3SS is encoded within the *P. aeruginosa* genome, and the structural components of the system are contained within 5 operons containing 36 genes, clustered within one genetic locus. These structural genes can be categorised into structural components of the needle complex,

proteins involved in the translocation of effectors, regulatory elements and chaperones. T3SS effector genes are scattered throughout the *P. aeruginosa* chromosome.

Components and assembly of the T3SS

The T3SS apparatus is shown in Figure 1.15. The T3SS machinery is composed of a needle like filament, which is a straight hollow tube made up of PscF proteins (*Pseudomonas* secretion component) (Pastor et al., 2005). The observation of PscF needles detached from cells showed they are 7 nm wide and 60-80 nm long (Pastor et al., 2005). The PscF protein polymerises to form these long needles, and are prevented from doing so prematurely by two chaperones, PscE and PscG (Quinaud et al., 2005). The length of the PscF needles is controlled by a molecular ruler, PscP, since deletion of this component in *Yersinia* spp. results in PscF needles varying in size from 45 – 1570 nm, and not the uniform ~ 60 nm observed in the wild type strain (Journet et al., 2003).

The components located at the base of the needle complex are less well characterised. By comparison with components from Yersinia sp. it can be inferred that the PscN component, an ATPase, is responsible for energising the T3SS secretion process (Woestyn et al., 1994). To allow the PscF needle access to the external milieu, a channel is formed in the outer membrane by the secretin protein PscC, which is able to polymerise and form a multimeric pore (Koster et al., 1997).

The first components to be secreted through the assembled T3SS are PopB, PopD and PcrV. These proteins are not true T3SS effectors, but are structural components important in the delivery of effectors to eukaryotic target cells. PopB and PopD contain hydrophobic domains which promote their insertion into the eukaryotic plasma membrane, where they form a pore that is required for the delivery of T3SS effectors (Schoehn et al., 2003). PcrV is an essential component for the assembly of the PopBD pore. PcrV is not a structural component of the pore, as it does not interact

with either of the pore components (Goure et al., 2004). PcrV has been shown to be located at the tip of the PscF needle, and may facilitate the assembly of the PopBD pore acting as a linker forming a continuous conduit between the PscF needle and the PopBD pore (Mueller et al., 2005). Electron microscopy has been used to visualise the PcrV and two homologues (LcrV, from *Yersinia* spp. and AcrV from *Aeromonas salmonicida*). The *pcrV* and *acrV* genes were used to complement *lcrV* deletion in *Y. enterocolitica*, and each produced distinct needle tip complexes as shown in Figure 1.15 (Mueller et al., 2005).

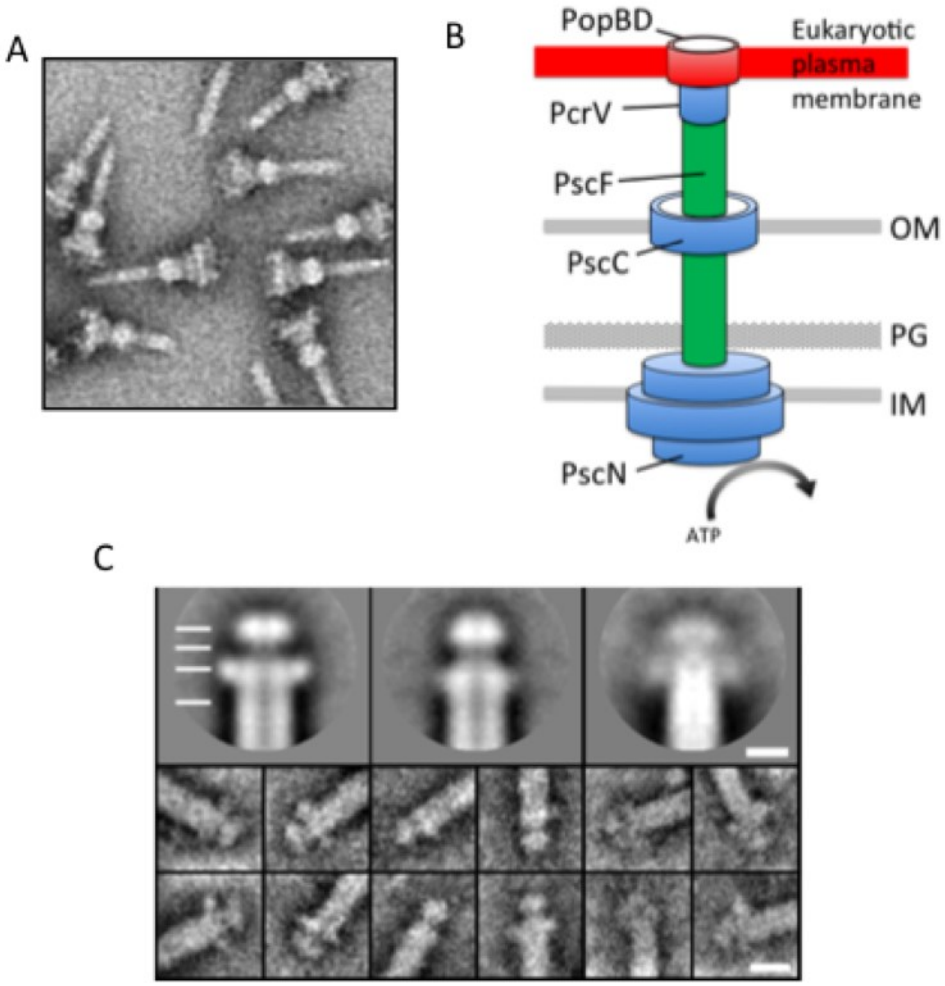


Figure 1.15 The T3SS

A. Electron micrograph of the T3SS needle complexes from *Salmonella typhimurium,* reproduced from (Schraidt et al., 2010). B. Model of the T3SS from *P. aeruginosa*, showing the PscF needle extending from the bacterial cell into a neighbouring eukaryotic cell. Components of the T3SS are described in the text. C. The tip structure formed by LcrV in the T3SS needle from *Yersinia enterocolitica* (left). *pcrV*+ (middle) and *acrV*+ (right) genes from *P. aeruginosa* and *Aeromonas salmonicida* respectively were used to complement a *Y. enterocolitica lcrV* mutant. Reproduced from (Mueller et al., 2005) with permission from AAAS.

The *P. aeruginosa* T3SS toxins

There are four identified T3SS effectors in *P. aeruginosa* strains, known as exoenzymes; ExoS, ExoT, ExoU and ExoY. These effectors are targeted in an unfolded state to the cytoplasm of eukaryotic cells, and play an important role in *P. aeruginosa* pathogenicity. The nature of the T3SS signal is controversial, and proposed to be a non-cleavable N-terminal signal sequence. However, the signal may be encoded within the corresponding mRNA molecule (Blaylock et al., 2008). The trigger for T3SS effector delivery is contact with eukaryotic cells. T3SS can be induced in-vivo under conditions of low Ca^{2+} availability, which results in the secretion of T3SS effectors into the supernatant (Vallis et al., 1999). *P. aeruginosa* strains typically carry only three T3SS effectors, normally ExoT and ExoY, and either ExoS or ExoU (Wolfgang et al., 2003a). The subset of T3SS effectors encoded by a *P. aeruginosa* strain determines its infectious potential. Strains carrying *exoU*, encoded within a pathogenicity island (e.g. PA14), are cytotoxic and cause rapid host cell lysis (Schulert et al., 2003). Strains carrying *exoS* (e.g. PAK, PAO1), cause delayed cell death (Kaufman et al., 2000).

ExoS and ExoT are 48 kDa and 49 kDa respectively, and share 76% amino acid identity and a common domain architecture. The extreme amino termini of these two effectors encode amino acids required for targeting to the T3SS apparatus (Yahr et al., 1996), and a membrane localisation domain for eukaryotic plasma membranes follows the signalling domain (Zhang and Barbieri, 2005). Both ExoS and ExoT are bifunctional enzymes, and have GTPase activating (GAP) domains and ADP ribosyl transferase activity (ADPRT). The ExoS and ExoT GTPase activating proteins (GAP) domains target Rho, Rac and CDC42 host cell proteins, which are small GTPases (Goehring et al., 1999). These proteins normally switch between a GTP-bound active state and a GDP-bound inactive state. ExoS and ExoT render these proteins inactive in the GDP-bound state (Wurtele et al., 2001). As GTPases normally regulate intracellular actin dynamics, the activity of ExoS and ExoT on GAPs disrupts the

actin cytoskeleton, leading to cell rounding and decreased internalisation into epithelial cells and macrophages (Garrity-Ryan et al., 2000).

The ADPRT domain activity is located towards the C termini of ExoS and ExoT. This domain requires the binding of a eukaryotic co-factor, thus self-toxicity in the bacterial cytoplasm is prevented. The ADPRT domains are able to catalyse the transfer of ADP-ribose to specific target host proteins, interfering with signalling pathways involved in actin processes (Ganesan et al., 1999; Maresso et al., 2004). The end results include cell rounding, inhibition of vesicular trafficking and inhibition of endocytosis (Barbieri et al., 2001). Both ExoS and ExoT result in cell death, however, ExoS is a more potent toxin, mediating cell death in 2-5h compared to 10h for ExoT (Shafikhani et al., 2008).

ExoY is a 42 kDa secreted protein with adenylyl cyclase activity (Yahr et al., 1998). Injection of ExoY into host cell leads to an accumulation of cAMP, a global regulator of gene expression. This cAMP accumulation leads to the differential expression of multiple genes, resulting in disruption of the actin cytoskeleton, inhibition of bacterial uptake, and increased membrane permeability (Cowell et al., 2005; Ichikawa et al., 2005). Expression of ExoY in PAO1 promotes the formation and trafficking of *P. aeruginosa* to respiratory epithelial membrane blebs, dependent on the adenlylate cyalase activity of this protein. ExoY adnelyate cyclase activity also promotes *P. aeruginosa* survival in mice cornea (Hritonenko et al., 2011).

The largest *P. aeruginosa* T3SS effector is ExoU, which is a 74 kDa protein and a potent phospholipase (Sato et al., 2003). ExoU is able to cause rapid death of eukaryotic cells within 1-2h due to the rapid loss of plasma membrane integrity (Finck-Barbancon et al., 1997). Comparison to the other Exo proteins suggests it also has a secretion signal at its extreme N terminus. The catalytic activity of this toxin is mediated by a patatin like phospholipase A2 domain, capable of the hydrolysis of a wide range of substrates (Banerji and Flieger, 2004). This domain is followed by a C terminal

domain which has no homology to known motifs, but is essential for the cytotoxicity of the protein (Finck-Barbancon and Frank, 2001).

Three classes of chaperones are associated with the T3SS. Some of the *P. aeruginosa* T3SS toxins interact with chaperones prior to secretion, which facilitate storage of effectors in the bacterial cytoplasm and correct delivery to the secretion apparatus, known as Class I chaperones (Parsot et al., 2003). ExoS and ExoT share the SpcS chaperone, which is required for their maximal secretions (Shen et al., 2008), while SpcU is the cognate chaperone of ExoU (Finck-Barbancon et al., 1998) and both interact with the effectors via interaction with the chaperone binding domain within the first 100 amino acids of the effector. No chaperone has yet been identified for ExoY. Class II chaperones neutralise the potentially damaging effects of the hydrophobic translocators, such as PcrH binding of PopB and PopD (Schoehn et al., 2003). Class III chaperones bind to structural subunits and prevent their polymerisation by masking polymerisation domains, known as PscE and PscG in *P. aeruginosa*, which bind to the PscF subunit of the T3SS needle in a 1:1:1 ratio (Quinaud et al., 2005).

Regulation of T3SS in *P. aeruginosa*

The T3SS is under global regulatory control of cAMP, quorum sensing and two component systems as described in Section 1.8. The master regulator of T3SS is a transcriptional activator known as ExsA in a mechanism illustrated by Figure 1.16. ExsA is a member of the AraC family of transcriptional activators (Hovey and Frank, 1995; Yahr and Frank, 1994; Yahr et al., 1995). ExsA has an N terminal domain involved in self-association, and a c terminal helix-turn-helix DNA binding domain recognising a nucleotide consensus sequence in the promoter regions of T3SS operons (Brutinel et al., 2008; Brutinel et al., 2009). The mechanism of regulation of ExsA occurs by a partner switching mechanism involving three additional proteins, ExsD, ExsC and ExsE (Brutinel and Yahr, 2008). The activity of ExsA is inhibited by ExsD, an anti-activator which inhibits T3SS expression by disrupting

the self-association and DNA binding activities of ExsA (Brutinel et al., 2010). ExsC is an anti-anti-activator capable of disrupting the ExsA-ExsD interaction, binding and sequestering ExsD (Dasgupta et al., 2004). However, ExsC is prevented from doing so due to a higher affinity binding to ExsE when secretion by the T3SS is not active (Dasgupta et al., 2004; Zheng et al., 2007). Increased transcription of T3SS genes is coupled to secretion due to the export of ExsE, which is exported through the T3SS needle (Urbanowski et al., 2007). This reduces bacterial ExsE levels, freeing ExsC to bind ExsD. Under these conditions, ExsA is free to activate transcription of T3SS genes by binding to an ExsA box, leading to a rapid increase in the production of T3SS components under conditions where secretion is active.

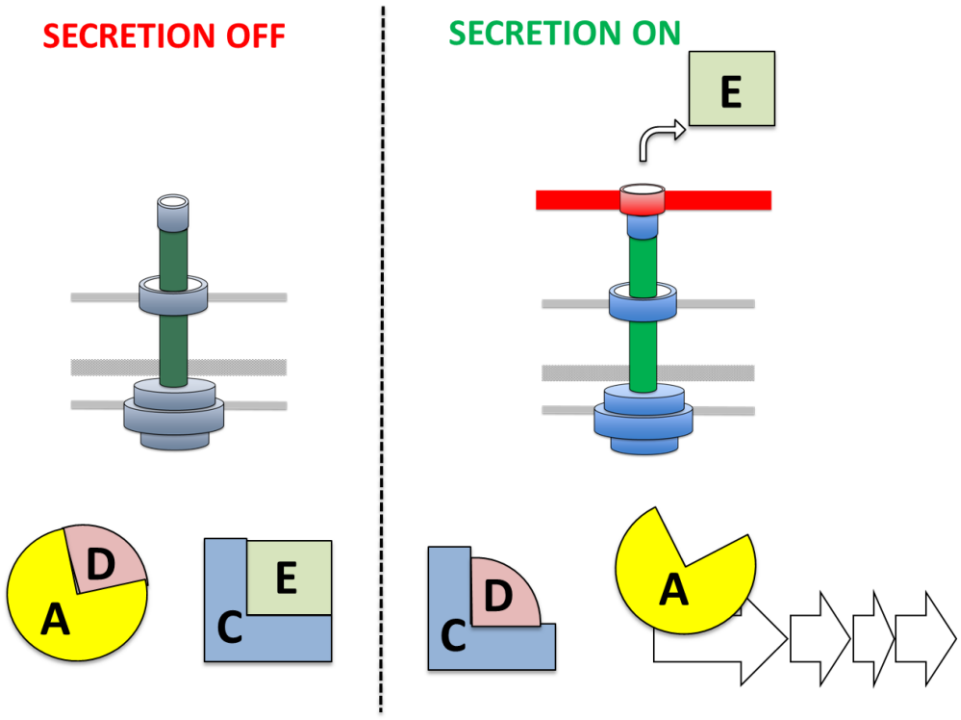


Figure 1.16 ExsE mediated regulation of the *P. aeruginosa* T3SS
Off and on states of the *P. aeruginosa* T3SS mediated by the master regulator ExsA. ExsA (**A**) is a transcriptional activator. ExsD (**D**) is an anti-activator. ExsC (**C**) is an anti-anti activator, and ExsE (**E**) is a secreted anti-anti-anti activator. The mechanism of regulation is described in the text.

The expression of the T3SS is also regulated by the Ret/Gac/Rsm cascade which is described in more detail in Section 1.9. Briefly, a *retS* mutant is defective in T3SS, while deletion of *ladS* has a reciprocal effect (Goodman et al., 2004; Ventre et al., 2006). These sensors mediate their effects via the GacS/GacA two component systems.

1.6.5 The Type IV Secretion System (T4SS)

The T4SS is absent in *P. aeruginosa*, and so is not covered in detail here. It is a well characterised secretion system involved in virulence in other Gram negative bacteria. T4SS are evolutionarily related to systems involved in the conjugative transfer of DNA. The T4SS are versatile, and are involved in both delivery of effectors and genetic exchange (Cascales and Christie, 2003; Christie and Vogel, 2000). The T4SS is important in crown gall disease in plants mediated by the transfer of DNA by *Agrobacterium tumefaciens* (Christie, 1997), as well as delivery of effector proteins into the host by *Legionella pneumophila* (Nagai and Kubori, 2011) Major advances have been made in the study of the T4SS through the elucidation of the structure of the core T4SS complex revealing a structure distinct from other known double membrane-spanning secretion systems (Fronzes et al., 2009)

1.6.6 The Type V Secretion System (T5SS)

The Type 5 secretion system is the simplest in terms of the number of components required for secretion, since the secreted protein and the mechanism required for secretion are either contained within one single or two separate protein(s). T5SS is a two-step process, with secreted substrates utilising the Sec system for inner membrane translocation, and accordingly T5SS substrates encode an N terminal signal sequence. Translocation across the outer membrane is through a channel formed by a β-barrel protein/domain. T5SS substrates can be displayed at the cell surface, or released into the extracellular medium following proteolytic cleavage. There are variations on the

mechanism of type 5 secretion, with different sub-classes being described. Each is described in more detail below.

Autotransporters (T5aSS)

Autotransporters (AT) are single polypeptide molecules capable of auto-secretion without the requirement of any additional components, and have been classified as the T5aSS (Dautin and Bernstein, 2007). AT proteins consist of an N terminal Sec signal peptide, and a C terminal β-strand domain. A passenger domain is located between the signal peptide and C terminal β-domain, which is the secreted part of the protein, containing a catalytic domain. The C terminal β-domain inserts into the outer membrane, forming a 12 stranded β-barrel (Oomen et al., 2004). The formation of the β-barrel results in the exposure of the passenger domain at the cell surface, where it can remain cell-associated or be cleaved for release into the extracellular milieu.

Three ATs are encoded within the genome of *P. aeruginosa* PAO1, (PA0328, PA3535 and PA5112). The best characterised is EstA (PA5112). The passenger domain of EstA has lipolytic activity which can hydrolyse glycerol esters with short or long chain fatty acids (Wilhelm et al., 1999). EstA is not truly a secreted protein since it remains anchored to the cell surface (Wilhelm et al., 1999). Analysis of the structure of the full length EstA autotransporter reveals that the β-channel is too narrow to allow passage of the folded protein, and the translocation across the outer membrane must occur in an unfolded state (van den Berg, 2010). The sequential folding of EstA during secretion may provide the energy required for the translocation of the full length protein (van den Berg, 2010).

More recently two further *P. aeruginosa* ATs have been identified, although their characterisation is not as thorough as that of EstA. AaaA (PA0328) is a surface tethered arginine-specific aminopeptidase of *P. aeruginosa* (Luckett et al., 2012). Deletion of *aaaA* causes attenuation in a

mouse chronic wound infection model, and AaaA provides a growth advantage to *P. aeruginosa* when the sole nitrogen source is peptides with an amino terminal arginine (Luckett et al., 2012). The third *P. aeruginosa* AT is EprS (PA3535), which contains a serine protease motif. Studies on recombinant EprS expressed in *E. coli* confirmed that the protein is a serine protease, and that it is responsible for the cleavage of host inflammatory proteins, activating the host inflammatory response (Kida et al., 2013).

Two partner secretion (T5bSS)

The mechanism of T5bSS is similar to T5aSS, except that the passenger domain and the β-domain are encoded within two separate proteins, TpsA and TpsB respectively (Jacob-Dubuisson et al., 2001). The TpsA protein contains a TPS secretion motif in addition to its functional domain. The TpsB contains the β-domain preceded by a POTRA (polypeptide transport associated) domain. The TPS secretion motif and POTRA domain are important for the interaction and recruitment of TpsA by TpsB (Clantin et al., 2007). Both TpsA and TpsB cross the inner membrane via the Sec system, following which TpsB is inserted into the outer membrane, forming a 16 strand β-barrel (Clantin et al., 2007). TpsA is translocated through the TpsB β-barrel and remains anchored to the cell surface, or is cleaved and released into the extracellular milieu.

Five putative TPSs are encoded within the genome of *P. aeruginosa* PAO1, however they have not all been fully characterised. The LepA/B (TpsA4/B4, PA4540/41) is involved in the outer membrane translocation of LepA, which has a trypsin-like serine protease motif, and demonstrated proteolytic activity (Kida et al., 2008). The CdrA/B (TpsA3/B3, PA4624/25) is required for the outer membrane translocation of CdrA, a putative c-di-GMP regulated adhesin which promotes biofilm formation and bacterial aggregation (Borlee et al., 2010). The remaining putative TPSs (Tps1 (PA2642/43), Tps2 (PA0040/41) and Tps5 (PA0690/92) have not been characterised.

An orphan TpsA-like protein is encoded within the *cupB* gene cluster, which encodes *cup* fimbrial genes (Vallet et al., 2001). The secretion of this TpsA-like protein, known as CupB5, has been shown to be dependent on CupB3, an usher protein involved in assembly of the Cup fimbriae (Ruer et al., 2008). However, further experiments suggest that CupB5 may be secreted by one of the other TpsB proteins encoded elsewhere on the chromosome (Daniela Muhl, Imperial College London, Personal Communication).

T5cSS and T5dSS

Following the classification of the T5aSS and T5bSS, additional systems were discovered which were similar to T5SS, but showed significant differences, resulting in the expansion of the T5SS class. T5cSS was created to describe autotransporter proteins with a β-barrel formed by the assembly of homo-trimers, with each monomer contributing 4 β-strands to the final outer membrane β-barrel (Kajava and Steven, 2006; Meng et al., 2006).

A fourth auto transporter, PlpD, was discovered, which belongs to the lipolytic enzyme family of patatin-like proteins, as does the previously described ExoU, a T3SS secreted effector (Salacha et al., 2010). PlpD is a single polypeptide with a two domain architecture characteristic of T5aSS autotransporters. However, the β-barrel domain is predicted to contain 16 β-strands, characteristic of the T5bSS TPS family. Sequence analysis revealed the presence of a POTRA domain within the PlpD protein, which is also characteristic of the T5bSS family. Due to the mixed nature of PlpD, it represents a novel secretion type known as the T5dSS (Salacha et al., 2010).

1.7 The Type VI Secretion System (T6SS)

The Type Six Secretion System (T6SS) is the most recently characterised secretion system. Despite lacking the T4SS, two T4bSS genes, *icmF* and *dotU*, are conserved in *P. aeruginosa.* *dotU* and *icmF* are systematically found in *P. aeruginosa* and other Gram negative bacteria within gene clusters in which other T4SS homologous genes are not found. Instead, a different set of conserved genes were found, which ultimately became known as the Type Six Secretion System (T6SS) (Mougous et al., 2006; Pukatzki et al., 2006). Three genetic clusters encode T6SS in *P. aeruginosa*. These clusters contain 13 core genes, essential for T6SS function (Zheng et al., 2011). This section describes the components that make up the T6SS, how these components are assembled and the roles and regulation of characterised systems.

1.7.1 Phage-like Components of the T6SS

Components of the T6SS show homology to bacteriophage tail components, which has been instrumental in the advancement of our knowledge of the structure, function and mechanism of this secretion system (Leiman et al., 2009). Bacteriophage are unique among viruses since they possess tails which allow host cell attachment, and the T6SS shows homology to the Myoviridae (Ackermann, 2003). The Myoviridae includes the T4 bacteriophage, which has a long contractile tail, as shown in Figure 1.17. T4 bacteriophage tail is composed of a sheath, an internal tube, a base plate, located at the distal end of the tail, and both long and short tail fibres (Leiman et al., 2010). During T4 infection the long tail fibres are extended and attach to the cell surface, increasing the probability for other fibres to locate host cell receptors, orientating the phage towards the cell surface (Kellenberger et al., 1996). This binding results in conformational changes in the short tail fibres which bind irreversibly to the *E. coli* cell surface. The baseplate changes confirmation, opening like a flower, and

the tail sheath contracts, causing propulsion of the tail tube which punctures the cell membrane (Leiman et al., 2010). Phage DNA is then realises into the host cell cytoplasm through the tube.

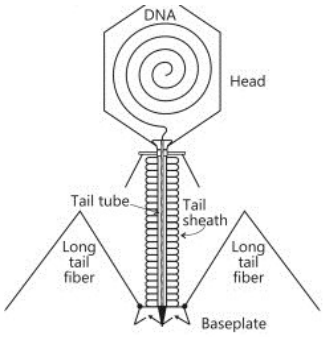


Figure 1.17 The T4 bacteriophage
Schematic representation of bacteriophage T4. The octahedral head containing DNA is shown on a contractile tail sheath, surrounding the tail tube. The baseplate is found at the distal end of the tail tube. Long tail fibres extend from the baseplate and allow recognition of host cells. The mechanism of DNA injection is described in the text. Reproduced from (Leiman et al., 2010).

Many of the T6SS proteins show structural homology to components of bacteriophage, summarised in Figure 1.18, which shows key structural data confirming bacteriophage-like homologies. Two key proteins with structural homology to bacteriophage components are Hcp and VgrG, which were also the first secreted substrates of the T6SSto be characterised. The localisation of Hcp and VgrG in the supernatant of *V. cholerae* cultures is dependent on a complete T6SS cluster (Pukatzki et al., 2006), and their presence in the culture supernatants is a hallmark of a correctly assembled T6SS apparatus. Despite initially being considered as secreted substrates, Hcp and VgrG are now considered as structural components of the system.

The homology to the bacteriophage tail tube has been observed for two Hcp homologues from *P. aeruginosa* (Hcp1 and Hcp2) (Mougous et al., 2006; Osipiuk et al., 2011). The Hcp proteins contains a fold similar to the major tail protein of bacteriophage λ gene product V (gpV) (Pell et al., 2009), and are evolutionarily related to the gp19 of T4-phage (Leiman et al., 2009). Hcp forms hexameric rings as shown in Figure 1.18A which, in the crystal lattice, assembles into nanotubes composed of

hexamers arranged head to head (Hcp2), or head to tail (Hcp1). These tubes have an external diameter of 80 – 90 Å and an internal diameter of 35 – 40 Å (Ballister et al., 2008; Jobichen et al., 2010; Osipiuk et al., 2011). Formation of these nanotubes in vitro can be achieved by engineering disulphide bridges at the hexamer interface, as shown in Figure 1.18C, but their formation *in vivo* is yet to be confirmed, (Ballister et al., 2008).

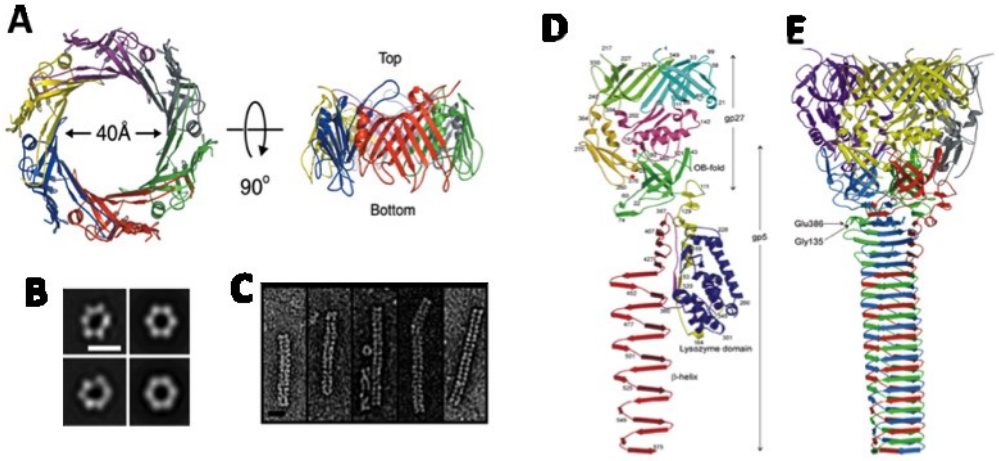


Figure 1.18 T6SS phage-like components: Hcp and VgrG
A. Top and side views of a ribbon representation of the crystallographic Hcp1 hexamer, each monomer is coloured differently. B. Single particle analysis of Hcp1. C. Electron micrograph of representative Hcp1 nanotubes prepared from reduced subunits of tailored Hcp1 subunits. D. structures of gp5 and gp27 monomers, extracted from the $(gp5)_3(gp27)_3$ complex. E. Model of a prototypical VgrG created from the $(gp5)_3(gp27)_3$ complex, and removing the lysozyme domain. Panels A and B reproduced from (Mougous et al., 2006) with permission requested from AAAS. Panel C reproduced from (Ballister et al., 2008), and Panels D and E reproduced from (Leiman et al., 2009).

The structure of the N terminus of VgrG from *E. coli* CF T073 also resembles a bacteriophage component (Leiman et al., 2009). Modelling of full length VgrG proteins shows structural homology to the trimeric $(gp27/gp5)_3$ complex of the bacteriophage T4 (Figure 1.18 D and E). While gp27 and gp5 are two separate proteins in the bacteriophage, they are a fused multi-domain protein in the T6SS. The function of the trimeric bacteriophage $(gp27/gp5)_3$ complex is to puncture the bacterial cell envelope, allowing insertion of the bacteriophage tail tube, and allowing injection of

bacteriophage DNA (Kanamaru et al., 2002). It is proposed that this membrane puncturing function is conserved in the T6SS and mediated by trimeric VgrG (Leiman et al., 2009). Mutlimerisation of VgrGs from *P. aeruginosa* has been observed, indicating that they may trimerise to perform their puncturing function (Hachani et al., 2011).

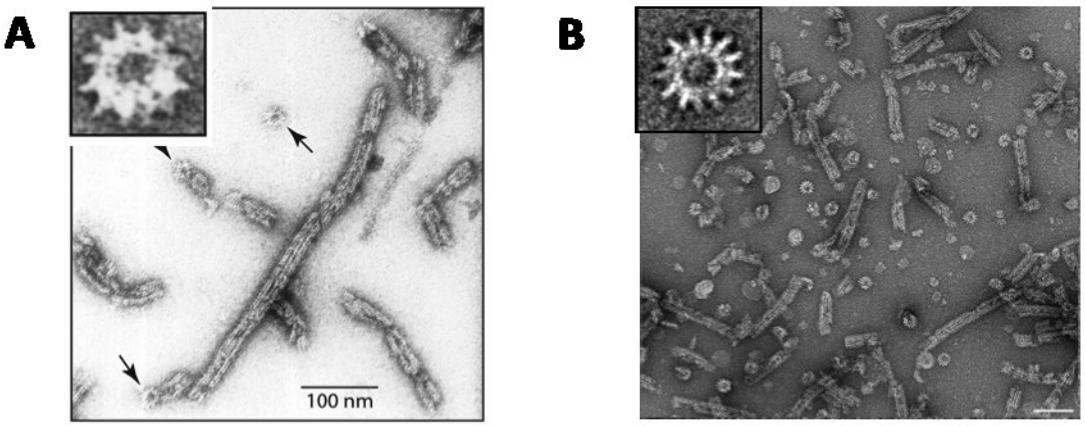


Figure 1.19 The T6SS phage sheath-like component
Cryo-electron micrographs of VipA/VipB complexes from *V. cholerae* (A) and TssB/TssC complexes from *P. aeruginosa* (B). Inserts show the cogwheel cross section of the sheath-like tubules. Panel A. reproduced from (Bonemann et al., 2009) with permission from Nature Publishing Group. Panel B. reproduced from (Lossi et al., 2013).

A protein complex composed of two core T6SS proteins, VipA and VipB from *V. cholerae*, homologous to *P. aeruginosa* TssB and TssC (type six secretion) also shows structural homology to a bacteriophage structure. Cryo-electron microscopy analysis of the VipA/VipB complex from *V. cholerae* shows that they form structures with cogwheel like cross sections, similar in architecture to the T4 viral tail sheath, encoded by gp18 (Figure 1.19A) (Bonemann et al., 2009). The tubes formed have an external diameter of 300 Å, and an internal diameter of 100 Å (Bonemann et al., 2009). The T4 tail sheath is made up of over 100 gp18 products, helically assembled into a sheath which accommodates viral tail tube proteins, and contraction of this outer tube provides the energy needed to push the phage needle through the target cell outer membrane (Aksyuk et al., 2009). Analysis of TssB/TssC from *P. aeruginosa* have shown that they also form a comparable structure

observable by cryo-electron microscopy, shown in Figure 1.19B, indicating that this is a conserved component of the T6SS (Lossi et al., 2013).

The advances in structural biology of key T6SS components (Hcp, VgrG and VipA/VipB) have allowed the development of a model for T6SS mechanism based on that known for bacteriophage cell puncturing devices, illustrated by Figure 1.20A (Leiman et al., 2009). The VgrG tipped Hcp tube can be accommodated within the tail sheath made of TssB/C. Contraction of the sheath proteins provides the energy required for propulsion of the VgrG tipped Hcp tube across the bacterial membranes, in a one-step process. This mechanism has been compared to an inverted bacteriophage, delivering proteins from the inside of the cell to the outside environment, as opposed to the delivery of DNA in the opposite direction by bacteriophage (Figure 1.20B). This model has been supported by direct visualisation of sheath like tubules in *Vibrio cholerae* (Basler et al., 2012). Time-lapse fluorescent microscopy and cryo electron microscopy was used to visualise tube like structures spanning the bacterial cells, existing in extended or contracted conformations, connected to the inner membrane (Basler et al., 2012). Fluorescent microscopy was performed on strains expressing a *vipA-gfp* fusion which formed long, straight, sheath like structures, which were not visible in a *vipB* mutant, indicating that these structures are the hypothesised sheath like components (Bonemann et al., 2009; Leiman et al., 2009). These structures were highly dynamic, cycling through states of assembly, contraction, and disassembly in the bacterial cytoplasm, with assembly occurring at a speed of 20 – 30 μms^{-1}, contraction taking less than 5 ms, and disassembly occurring over 30 – 60 s (Basler et al., 2012).

TagJ is a non-essential component of the T6SS, which is only found in the H1-T6SS of *P. aeruginosa*, absent from H2- and H3-T6SS (Lossi et al., 2012). TagJ interacts with an alpha helix at the N-terminus of TssB, a component of the sheath-like complex, suggesting that TagJ might modulate the incorporation of TssB into the mature T6SS (Lossi et al., 2012).

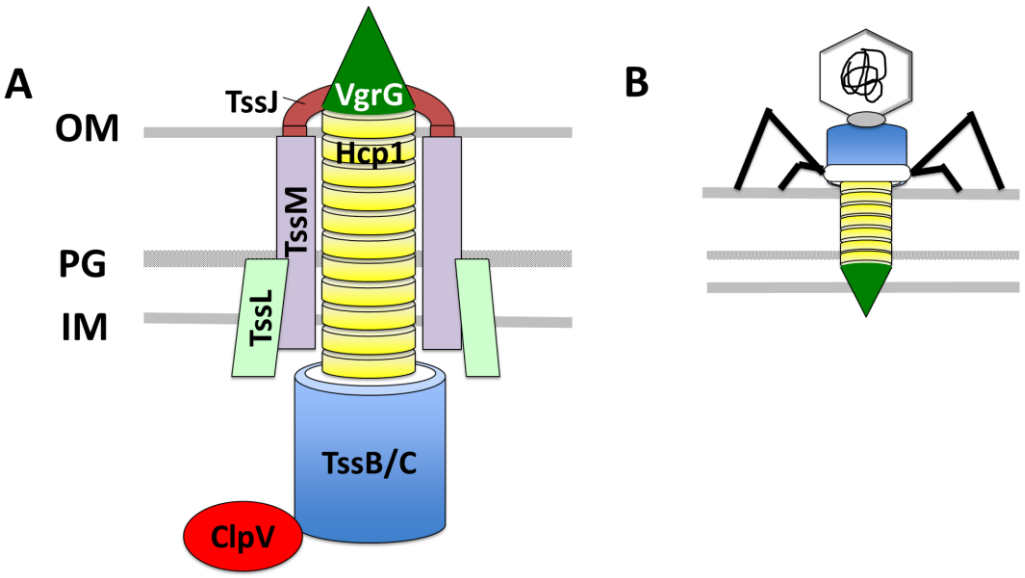


Figure 1.20 T6SS assembly model

A. model showing the predicted assembly of T6SS components. The Hcp1 tube (yellow) is topped by trimeric VgrG (green), which sits inside the TssBC contractile sheath (blue). TssJ is an outer membrane lipoprotein, while TssL and TssM form an inner membrane complex. ClpV is a cytoplasmic protein. B. The homologous components from the T4 bacteriophage are shown in the same colour as the T6SS components from Figure 1.20A, and shows the inverted orientation of the T6SS.

1.7.2 Other components of the T6SS

Unlike the components described thus far, ClpV is not evolutionarily related to known phage components and is implicated in the dynamics of sheath contraction. It is a cytoplasmic protein, and a member of the Hsp100/Clp family of AAA+ ATPases, with the ability to form oligomeric rings (Schlieker et al., 2005). Using the energy of ATP hydrolysis, this family of proteins is able to unfold target proteins. ClpV forms discreet foci within *P. aeruginosa* cells, observed by fluorescent microscopy of strains carrying a *clpV1-gfp* fusion (Mougous et al., 2006). ClpV selectively interacts with contracted VipA/VipB tubules and disassembles the sheath structure allowing the subunits to be recycled and reused in another T6SS event (Basler et al., 2012; Bonemann et al., 2009). ClpV-gfp fusion protein was visualised in *V. cholerae* using time-lapse fluorescent microscopy, and was observed to assemble into short structures that disappeared in tens of seconds (Basler and

Mekalanos, 2012b). These ClpV structures were dependent of the presence of VipA, a component of the sheath-like structure, since in a *vipA* mutant, ClpV-gfp was evenly distributed in the bacterial cytosol. Co-visualisation of VipA-gfp and ClpV-mCherry showed that ClpV colocalised only with the contracted sheath, which were disassembled in a ClpV dependent manner (Basler and Mekalanos, 2012b).

Several of the remaining core T6SS components assemble a membrane complex of the T6SS. TssL and TssM are prominent features of the T6SS, and show homology to DotU and IcmF proteins. In the T4bSS these two proteins have been shown to interact and form a complex that stabilises the apparatus of this secretion machine, and the same function is proposed to be conserved in the T6SS counterparts (Nagai and Kubori, 2011). TssM is the IcmF homolog, and is an integral inner membrane protein carrying three transmembrane segments. Part of TssM is located in the periplasm, consisting of a helical domain and a C terminal beta domain, which has been shown by yeast two hybrid to interact with the outer membrane located lipoprotein TssJ (Felisberto-Rodrigues et al., 2011). A cytoplasmic TssM domain usually contains ATP-binding and hydrolysis Walker A motifs, but conflicting reports have been made regarding the requirement of these domains in the T6SS process. The Walker A motifs are essential for assembly of T6SS machinery in *Agrobacterium tumefaciens*, but their mutation has no effect on T6SS activity in *Edwardsiella tarda* (Ma et al., 2009c; Ma et al., 2012; Zheng and Leung, 2007a). The cytoplasmic Walker A motifs are absent in many TssM homologues, indicating they are not an essential feature of the T6SS.

TssL is homologous to DotU, and is also an integral inner membrane protein, with a single transmembrane segment. The protein has a large C-terminal periplasmic domain encoding a peptidoglycan binding (PGB) motif of the OmpA family. If this domain is absent from TssL an accessory protein, TagL (type six associated gene), is present which compensates by carrying the

PGB motif and interacting with TssL (Aschtgen et al., 2010b). This PGB motif is likely to be responsible for tethering the T6SS apparatus to the cell wall (Aschtgen et al., 2010a).

TssJ is a lipoprotein anchored to the outer membrane (Aschtgen et al., 2008). TssM has been shown to interact with both TssL and TssJ, which are located at the IM and OM respectively, thus TssM is likely to span the periplasm and link the IM and OM. It has been suggested that the role of the TssJLM membrane complex is to form a multimeric ring like structure, producing a channel spanning the cell membranes, and allowing the bacteriophage-like components to gain access to the external milieu (Silverman et al., 2012).

TssE is a gp25-like component, essential for secretion by the H1-T6SS (Lossi et al., 2011). Despite gp25 components of bacteriophage having lysozyme activity, no such activity could be detected for TssE (Lossi et al., 2011). TssK is a trimeric cytoplasmic protein which interacts with TssL, Hcp and TssC, and is suggested to have a role in linking the phage-like complex with the trans-envelope complex of the T6SS (Zoued et al., 2013). The remaining core components, namely TssA, TssF, and TssG are not characterised, but are likely to be homologues of the phage baseplate components.

1.7.3 T6SS toxin delivery

Several examples of T6SS mediated secretion have been described, but the exact method of effector delivery remains unclear. While the nature of characterised effectors are described in the following sections, the proposed strategies used for their secretion are summarised in Figure 1.21.

Scenario A depicts toxins that travel within the Hcp tube and are delivered following cell puncturing by VgrG. The Hcp tube conduit is wide enough to carry globular proteins of less than 50 kDa, but the VgrG trimer is too narrow to allow proteins to pass through (Silverman et al., 2012). In this scenario

the VgrG trimer must dissociate before toxin delivery, as occurs in bacteriophage DNA delivery. There is currently no experimental evidence to support this hypothesis.

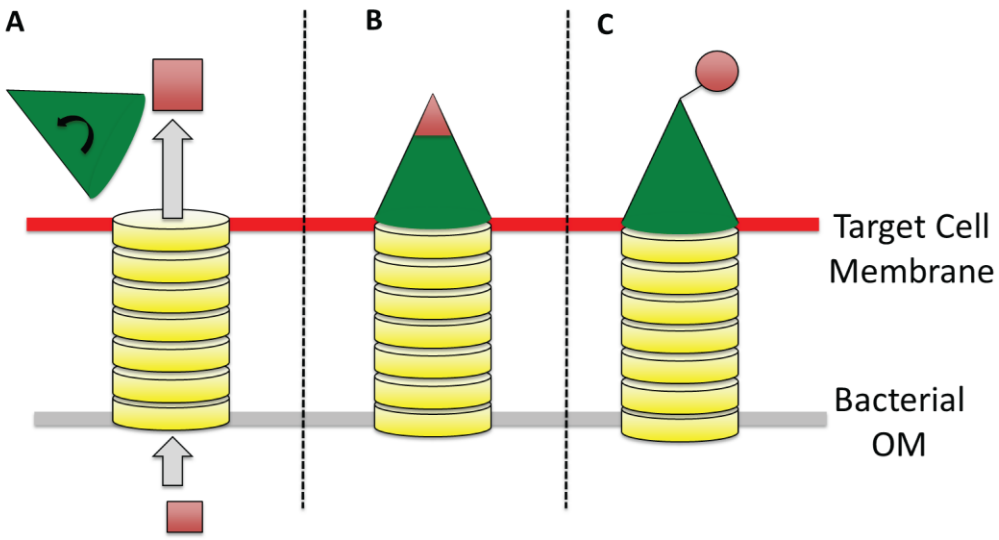


Figure 1.21 T6SS toxin delivery
Three predicted scenarios of T6SS toxin (red) delivery, as described in the text. A. Delivery of toxins into target cells mediated by VgrG (green) cell puncturing and toxin delivery through the Hcp tube (yellow). B. Delivery of toxin domains covalently linked to VgrG. C. delivery of toxins through non-covalent interaction with VgrG.

Scenario B shows toxins being delivered at the tip of the needle, covalently linked to the VgrG. There are several examples of VgrG carrying these C terminal extensions, encoding diverse activities, and they have been named evolved VgrGs. Limited examples exist of evolved VgrG targeted towards eukaryotic cells and bacterial cells, which are described further in the following sections.

Scenario C shows toxins encoded by non-*vgrG* genes and interacting in a non-covalent manner with the VgrG tip. This could allow one VgrG to deliver various toxins in a modular fashion. A superfamily of PAAR proteins (proline-alanine-alanine-arginine repeat) has been shown to be an important part of the T6SS in a role as proposed by this scenario. The crystal structures of PAAR proteins bound to VgrG partners have been solved, and the PAAR form a sharp conical extension to the VgrG spike (Shneider et al., 2013). Inactivation of the two PAAR encoding genes in *V. cholerae* resulted in a 100-

fold decrease in T6SS-dependednt killing of *E. coli*, and a 70% reduction in Hcp secretion, while inactivation of the three PAAR encoding genes from *Acinetobacter baylyi* led to a 10,000-fold decrease in T6SS-dependednt killing of *E. coli* and a 90% reduction in Hcp secretion (Shneider et al., 2013). PAAR genes modified to carry epitope tags fully restored T6SS mediated killing of *E. coli* in PAAR knockout strains of *A. baylyi*, suggesting that other effector proteins fused to PAAR domains could be targeted for secretion by binding to the VgrG tip, which could be decorated with multiple effectors which are delivered simultaneously (Shneider et al., 2013). It is proposed that VgrGs which do not carry large C terminal extensions may carry shorter regions that act as adapter proteins for interacting with PAAR or other proteins to allow co-injection.

1.7.4 T6SS effectors targeted into eukaryotic cells

Many examples exist of T6SS required for or contributing to virulence and interaction with eukaryotic cells. In most cases the molecular mechanism of virulence is not known, more particularly because the nature of the secreted effector is not known. These include T6SS from plant, animal and human pathogens. Table 2, reproduced from a recent review, lists the experimentally characterised roles for eukaryotic targeted T6SS (Coulthurst, 2013).

Organism	T6SS	T6SS-dependent phenotype	Reference
Aeromonas hydrophila	Vas	Cytotoxicity and inhibition of phagocytosis in cultured macrophages and epithelial cells; full virulence in septicaemic mouse model	(Suarez et al., 2008)
Avian pathogenic E.coli		Virulence in chick; adhesion to and actin rearrangement in cultured epithelial cells	(de Pace et al., 2011)
Burkholderia cenocepacia	Bcs	Resistance to *Dictyostelium* predation; virulence in rat model of chronic lung infection; induction of cytoskeletal rearrangements in cultured macrophages; inflammasome activation	(Aubert et al., 2008; Gavrilin et al., 2012; Rosales-Reyes et al., 2012)
Burkholderia mallei	T6SS-1	Virulence in Syrian hamster model; intracellular growth, actin polymerisation and mononuclear giant cell formation in cultured macrophages	(Burtnick et al., 2010; Schell et al., 2007)
Burkholderia pseudomallei	T6SS-1	Virulence in Syrian hamster model; growth and mononuclear giant cell formation in cultured macrophages; intercellular spread in cultured cells	(Burtnick et al., 2011; French et al., 2011)
Burkholderia thailandensis	T6SS-5	Virulence in mouse acute pneumonia model of melioidosis; mononuclear giant cell formation and intercellular spread in cultured cells	(French et al., 2011; Schwarz et al., 2010)
Campylobacter jejuni		Colonisation of mouse; adhesion to and invasion of cultured colonic epithelial cells	(Lertpiriyapong et al., 2012)
Edwardsiella tarda	Evp	Virulence in blue gourami fish; virulence in several other fish models; invasion of cultured carp epithelial cells	(Wang et al., 2009; Zheng and Leung, 2007b)
Escherichia coli K1	Evf	Adhesion to and invasion of human brain microvascular endothelial cells	(Zhou et al., 2012)
Francisella tularensis	Igl	Growth, phagosomal escape and cytopathogenicity in cultured macrophages; virulence in mouse model	(Broms et al., 2009; Broms et al., 2012)
Francisella novicida	Igl	Intracellular growth in cultured murine macrophages	(de Bruin et al., 2011; de Bruin et al., 2007)
Helicobacter hepaticus		Anti-inflammatory response in cultured intestinal epithelial cells and limitation of intestinal colonisation in mice (and perhaps therefore promotion of a tolerant, balanced relationship with host)	(Chow and Mazmanian, 2010)
Pectobacterium atrosepticum		Virulence in potato (stem and tuber infection models)	(Liu et al., 2008)
Pseudomonas aeruginosa	H1-,H2-, H3-T6SS	Virulence in rat model of chronic respiratory infection. Virulence (slow killing) in *Caenorhabditis elegans* model; enhancement of bacterial uptake into non-phagocytic cultured cells. Either H2- and H3-T6SS needed for full virulence in mouse lung and acute burn model; both for wild-type infection of Arabidopsis	(Lesic et al., 2009; Potvin et al., 2003; Sana et al., 2012)
Pseudomonas syringae		Growth suppression of yeast	(Haapalainen et al., 2012)
Rhizobium leguminosarum	Imp	Host-specific inhibition of infection of pea plants	(Bladergroen et al., 2003)
Salmonella enterica serovar Gallinarum	SPI-19	Colonisation of chicken ileum, caeca, liver and spleen	(Blondel et al., 2010)
S. enterica serovar Typhimurium	Sci	Contribution to pathogenesis and systemic dissemination in a mouse model of typhoid, intracellular replication in macrophages	(Mulder et al., 2012)
Vibrio cholerae	Vas	Cytotoxicity towards *Dictyostelium* and cultured mammalian macrophages; inflammatory response, diarrhoea and actin cross-linking in mouse intestine	(Ma et al., 2009a; Ma and Mekalanos, 2010; Pukatzki et al., 2006)
Vibrio parahaemolyticus		Adhesion to cultured cell monolayers	(Yu et al., 2012)

Table 2 Eukaryotic targeted T6SS

Table reproduced from (Coulthurst, 2013)

The T6SS was initially characterised in pathogenic *V. cholerae* and *P. aeruginosa*, which led to the hypothesis that the T6SS was important in virulence. This appears true for the T6SS of *V. cholerae*, which targets eukaryotic cells. *V. cholerae* encodes three VgrG, one of which has a C terminal extension which is similar to the actin cross-linking domain (ACD) of the cholera RtxA toxin, and is thus quoted an evolved VgrG (Sheahan et al., 2004). The activity of this C terminal extension was shown by incubating purified recombinant VgrG1 with lysates from eukaryotic cells, which showed that VgrG1 was capable of cross-linking actin, as shown in Figure 1.22 (Pukatzki et al., 2007). Infection of J774 macrophages with a *vgrG1* mutant showed that VgrG1 was required for actin cross-linking in vivo, and that this phenotype was dependant on the T6SS cluster. This confirms the role of VgrG1 as a T6SS-dependent effector (Pukatzki et al., 2007). The actin crosslinking activity was localised to the C terminus of VgrG1 and deletion of this region abolished T6SS mediated toxicity while not effecting secretion (Ma et al., 2009a).

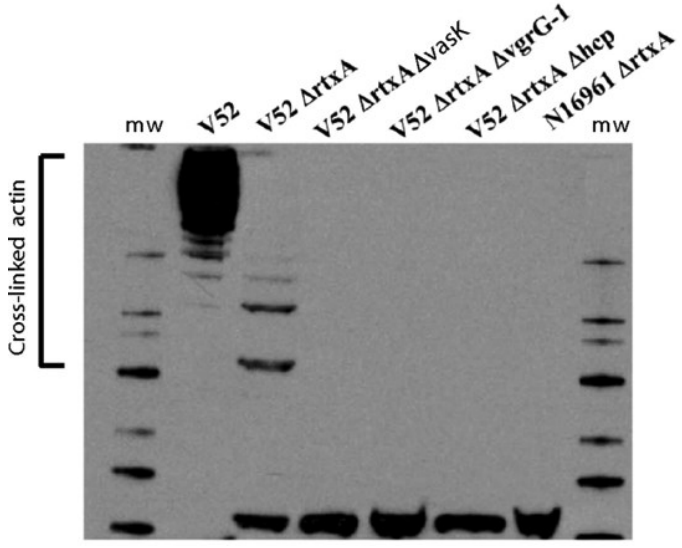


Figure 1.22 *V. cholerae* T6SS actin cross-linking activity
Extracts of cultured J774 macrophage, previously infected with the indicated strain, were separated by SDS-PAGE and probed with actin antiserum. Molecular weight markers are indicated on both sides of the blot. The V52 strain is responsible for significant actin cross-linking, which is reduced by deleting *rtxX*, encoding the RtxA toxin (V52 Δ*rtxA*). The residual actin cross-linking activity is abolished by deletion of *vasK* (*icmF* homolog), *vgrG1*, or *hcp*. An *rtxA* mutant of a pandemic strain (El tor 01 N16961) did not cross-link actin. Reproduced from (Pukatzki et al., 2007).

Direct observation of the delivery of the evolved VgrG1 from *V. cholera* into target cells was observed using β-lactamase fusions to the ACD, or by replacing this domain with β-lactamase. These reporter fusions were detected inside the host cell, dependent on the N terminal region of the VgrG. This confirmed the role of the VgrG as a component of the secretion system, while the C terminus encodes the effector domain (Ma et al., 2009a). An interesting observation in this study was that the T6SS activity of *V. cholerae* is dependent on internalisation into host cells, and therefore the T6SS may protect phagocytosed *V. cholerae* from macrophage killing (Ma et al., 2009a). The pathogenic role of the *V. cholerae* T6SS was confirmed as the VgrG1 ACD causes inflammation and associated pathology in the intestines of mice (Ma et al., 2009a).

A second example of T6SS involvement in pathogenicity is seen with the T6SS system from *Aeromonas hydrophila,* which also exerts its effect through the rearrangement of actin (Suarez et al., 2010). Supernatants from *A. hydrophila* were subjected to 2D gel electrophoresis, and using mass spectrometry a VgrG protein secreted in a T6SS dependent manner was detected. The protein contains a vegetative insecticidal protein (VIP-1) domain at its C terminus, and is another example of an evolved VgrG. Production of this VgrG in HeLa cells leads to disruption of the actin cytoskeleton and a decrease in cell viability (Suarez et al., 2010).

The two examples from *V. cholerae* and *A. hydrophila* are the only known eukaryotic targeted evolved VgrG. Many other VgrG carry predicted evolved VgrG with diverse activities, as summarised in Table 3. Evolved VgrG are not restricted to eukaryotic targeting, as shown by the experimentally demonstrated example of the peptidoglycan targeted VgrG3 from *Vibrio cholerae*, discussed in more detail in the next section (Brooks et al., 2013; Dong et al., 2013).

There are a limited number of non-VgrG effectors targeted towards eukaryotic hosts. An example is VasX, a T6SS secreted effector of *V. cholerae* is required for virulence towards *Dictyostelium discoideum* by an unknown mechanism (Miyata et al., 2011). For the other examples indicated in Table 2 the impact on virulence and T6SS-dependent secretion are yet to be characterised.

Organism	Activity	Reference
Experimentally Demonstrated Evolved VgrG		
Aeromonas hydophila	ADP ribosylation of actin	(Suarez et al., 2010)
Vibrio cholerae	Actin crosslinking	(Pukatzki et al., 2007)
V. cholerae	Peptidoglycan binding / hydrolysis	(Brooks et al., 2013; Dong et al., 2013)
Predicted Evolved VrgG		
Burkholderia cenocepacia	Pe/ppe domain	(Pukatzki et al., 2007)
Erewinia tasmaniensis	Chitosanase	(De Maayer et al., 2011)
P. aeruginosa	Zinc metalloprotease	(Pukatzki et al., 2007)
Pseudomonas entomophila	YidB-like/Fibronectin-like domain	(Pukatzki et al., 2007)
Salmonella enterica	S-type pyocin	(Blondel et al., 2009)
Yersinia intermedia	Mannose binding domain	(Pukatzki et al., 2007)
Yersinia pestis	Tropomyosin-like domain	(Pukatzki et al., 2007)
Y. pestis	YadA-like and pectactin-like domain	(Pukatzki et al., 2007)

Table 3 Experimentally demonstrated and predicted evolved VgrG

1.7.5 Bacterial targeted T6SS toxins

Genetic clusters encoding T6SS have been found in 25% of sequenced Gram negative bacterial genomes (Bingle et al., 2008). T6SS are found in bacterial pathogens and symbionts. However, non-pathogenic, non-symbiont environmental bacteria also encode T6SS. It is clear that the role of the T6SS is wider than pathogenesis alone. Several T6SS have been experimentally shown to target competing bacteria by the direct injection of toxins which are discussed in detail below. The currently known bacterial targeted T6SS toxins are listed in Table 4.

Toxin	Organism	Activity	Reference
Peptidoglycan Targeting			
Tse1	*P. aeruginosa*	Peptidoglycan amidase	(Hood et al., 2010)
Tse3	*P. aeruginosa*	Peptidoglycan muramidase	(Hood et al., 2010)
Tae2^{BT}	*Burkholderia thailandensis*	Peptidoglycan amidase	(Russell et al., 2012)
Ssp1, Ssp2	*Serratia marcessecens*	Peptidoglycan amidases	(English et al., 2012)
Lipid Targeting			
Tle1^{BT}	*B.thai*	Phospholipase A2	(Russell et al., 2013)
Tle5^{PA}	*P. aeruginosa*	Phospholipase D	(Russell et al., 2013)
TseL/Tle2^{VC}	*Vibrio cholerae*	Phospholipase A1	(Dong et al., 2013)
Other			
Tse2	*P. aeruginosa*	Cytoplasmic toxin	(Hood et al., 2010)
Ssp3-6	*S. marcessecens*	Antibacterial toxins	(Fritsch et al., 2013)

Table 4 Experimentally demonstrated T6SS toxins

Peptidoglycan targeting T6SS toxins

Despite the initial assumption that the H1-T6SS of *P. aeruginosa* was involved in host cell interaction, it is now clear that this system targets bacterial cells. A mass spectrometry approach was used to compare the secretome of *P. aeruginosa* strains with a constitutively active or disrupted H1-T6SS, and yielded three putative effectors, which were named Tse1-3 (type six effector) (Hood et al., 2010). Tse1-3 are encoded distantly to the H1-T6SS, but are co-regulated by RetS.

Further characterisation of Tse2 showed that production of this protein is toxic to yeast, mammalian cells and bacteria when produced within those cells (Hood et al., 2010). Attempts to delete a gene downstream of Tse2 were unsuccessful in strains where Tse2 was intact, and thus this downstream gene was proposed to encode an antitoxin protein, preventing self-toxicity, named Tsi2 (type six immunity). Eukaryotic cells were not affected by *P. aeruginosa* cells expressing Tse2, indicating that it is not translocated into eukaryotic hosts. Conversely, *P. aeruginosa* cells lacking Tse2/Tsi2 were

disrupted and outcompeted in co-culture with Tse2 producing cells (Hood et al., 2010). Production of Tsi2 within target cells relieved the inhibition, confirming that Tsi2 is an antitoxin. Tse2 is a bacteriostatic toxin, targeted to the cytoplasm of target cells where it affects their proliferation, but the exact mechanism of toxicity is not known (Hood et al., 2010).

Unlike Tse2, the mechanism of toxicity mediated by Tse1 and Tse3 is characterised. Tse1 and Tse3 are lytic enzymes which degrade peptidoglycan. Tse1 has amidase activity, and Tse3 has muramidase activity, each targeting a different bond within the peptidoglycan molecule (Russell et al., 2011). Ectopic expression of Tse1 and Tse3 in the cytoplasm of *E. coli* did not affect the cells. Expression of periplasmic forms of Tse1 and Tse3 caused lysis of *E. coli* following induction (Russell et al., 2011), showing that Tse1 and Tse3 have a periplasmic target, but have no inherent means of accessing the periplasm. Tse1 and Tse3 also have periplasmic immunity proteins encoded downstream of the toxin genes, which co-immunoprecipitate specifically with the cognate toxin, and act to neutralise the toxin activity (Russell et al., 2011). These immunity proteins are delivered to the periplasm where they protect against injection of Tse1 and Tse3 from neighbouring cells, and not necessarily against toxins produced in the same cell.

Other studies isolated a secreted effector from *Burkholderia thailandensis*, subsequently named $Tae2^{BT}$ for reasons described later (Russell et al., 2012). $Tae2^{BT}$ is responsible for the intoxication of neighbouring bacteria in a T6SS dependent manner, and is a peptidoglycan amidase, confirming $Tae2^{BT}$ as a T6SS delivered toxin (Russell et al., 2012). Russell and colleagues showed that $Tae2^{BT}$ was encoded adjacent to a specific immunity protein. $Tae2^{BT}$ was thus a conserved T6SS amidase found in an organism other than *P. aeruginosa*.

Following the discovery of peptidoglycan targeting proteins in *P. aeruginosa* and *B. thai* it was shown that these proteins were part of a phylogenetically diverse superfamily of T6SS-associated

peptidoglycan-degrading effectors (Russell et al., 2012). Using parameters derived from the experimentally validated Tse proteins, the authors identified 51 putative peptidoglycan-degrading effectors in four distinct families, each family having a different specificity. Thus Tae2^{BT} was named due to its characterisation as a type VI amidase effector of the second family, from *B. thai*. This work identified a large number of putative peptidoglycan targeting toxin and antitoxin pairs in a plethora of organisms, indicating that peptidoglycan is a common and conserved target of the T6SS.

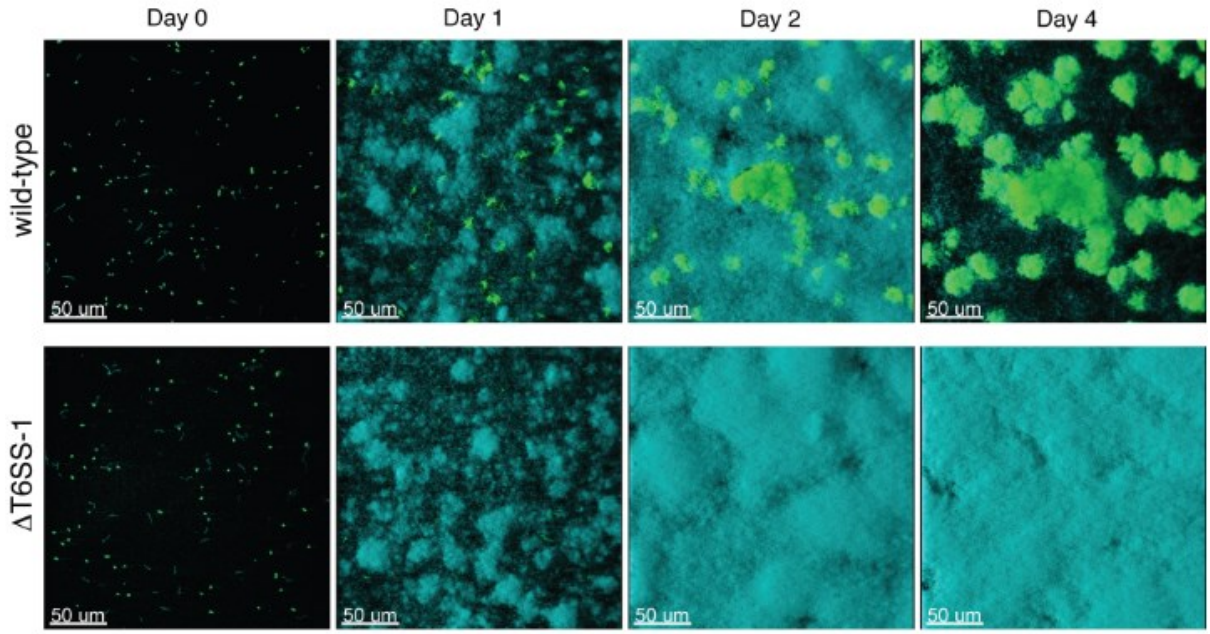


Figure 1.23 *B. thai* biofilm competition assay
Fluorescence confocal microscopy images of *B. thai* (Green) and *Pseudomonas putida* (cyan) mixed biofilm formation in flow chambers at daily intervals following seeding with a 11 mixture of *P. putida* with either wild type *B. thai* (upper panels) or a *B. thai* ΔT6SS-1 mutant (lower panels). Reproduced from (Schwarz et al., 2010)

Burkholderia thailandensis (*B. thai*) is an interesting model for the study of T6SS as it carries five T6SS clusters within its genome. Only one of these systems, the T6SS-5, is required for virulence in a mouse model, while the remaining four are dispensable for virulence (Schwarz et al., 2010). The T6SS-1 of *B. thai* is involved in antibacterial interactions, providing *B. thai* with a growth advantage in

mixed culture with other bacteria. In a flow-cell biofilm assay, *B. thai* predominates when equal amounts of *Pseudomonas putida* and wild type *B. thai* are inoculated. However, inoculation with a *B. thai* strain carrying a deletion in T6SS-1 allows the *P. putida* strain to dominate, as shown in Figure 1.23 (Schwarz et al., 2010). These observations indicate the importance of T6SS in the formation of complex communities within mixed biofilms. The work carried out in *B. thai* serves as an important example since it shows multiple T6SS within one organism having distinct roles.

Studies of *Serratia marcescens* have yielded two small secreted proteins, Ssp1 and Ssp2, which are two further members of the Tae family (English et al., 2012). Ssp1 and Ssp2 belong to the fourth Tae family, and have accordingly also been named Tae4.1^{SM} and Tae4.2^{SM}. These two toxins are encoded within the *S. marcescens* T6SS cluster, and were identified in the culture supernatant by immunoblotting, and by assessing their dependency on a functional T6SS. They carry no sequence or structural homology to peptidoglycan hydrolases, but carry adjacent immunity proteins (English et al., 2012). Despite not demonstrating peptidoglycan degrading activity, it was shown that non-immune strains were susceptible to toxicity mediated by both Ssp1 and Ssp2.

Lipid targeting T6SS toxins

Alternative approaches have led to the discovery of T6SS effectors from different families. A genomic analysis performed by Barret and colleagues showed that orphan VgrG, not associated with T6SS clusters, were often associated with additional genes encoded within genomic islands (Barret et al., 2011). Each orphan *vgrG* was always associated with specific additional genes termed 'cargo genes' in all strains of *P. aeruginosa* carrying that *vgrG* (Barret et al., 2011). Subsequent characterisation revealed that genes encoding predicted lipases/esterases were frequently linked to *vgrG* islands, with 34 genes encoding lipases or esterases found in the vicinity of 118 orphan *vgrG* across all of the genomes interrogated (Barret et al., 2011). One of these *vgrG* islands encodes PldA,

and phospholipase activity has previously been shown for this protein (Wilderman et al., 2001). The discovery of lipase genes within VgrG islands suggested that lipases were a novel family of T6SS secreted effectors. It is also feasible that many of the other genes within *vgrG* islands encode diverse T6SS effectors, which are yet to be characterised but hold enormous potential to further our understanding of the T6SS.

Experimental validation of the observations made by Barret *et al.* was provided by the discovery of phospholipases as a family of T6SS delivered antibacterial effectors (Russell et al., 2013). In this work, Russell and colleagues extended the observations made by Barret and colleagues to uncover 377 putative lipases from five families, named as type VI lipase effectors, Tle(1-5). Each *tle* gene was encoded adjacent to an immunity gene to protect from self-toxicity, indicating bacterial targeting of the lipase activity. Tle5 from *P. aeruginosa* was shown to have phospholipase D activity catalysing the in vitro release of choline from phosphatidylcholine, a component of the bacterial membrane (Wilderman et al., 2001). *P. aeruginosa tle5*/*tli5* mutants, lacking both the Tle5 effector and cognate immunity protein, were susceptible to killing by a wild type *P. aeruginosa* competitor. *P. aeruginosa tle5*/*tli5* mutants survived incubation with *P. aeruginosa tle5* deletion mutant, confirming that Tle5 is an antibacterial toxin against *P. aeruginosa* strains lacking the cognate immunity (Russell et al., 2013). Inactivation of the H2-T6SS by deleting *clpV2* abrogated the $Tle5^{PA}$ dependent toxicity, indicating that the H2-T6SS was responsible for the delivery of $Tle5^{PA}$. $Tle5^{PA}$ is thus the first toxin to be shown to depend on the second *P. aeruginosa* T6SS cluster.

In addition to the characterisation of Tle5 from *P. aeruginosa*, a lipase effector from B. *thai*, from the 1st class of lipases, $Tle1^{BT}$, was also characterised (Russell et al., 2013). $Tle1^{BT}$ antibacterial activity was shown to be dependent on the T6SS-1, and was shown to have phospholipase A2 activity (Russell et al., 2013). This work confirmed that phospholipases are a conserved effector family of

the T6SS, exerting their activity through targeting the cell membrane. The authors identified a large number of putative T6SS lipase effectors.

A third Tle effector from *V. cholerae* was studied by Russell *et al.* (2013), and had previously been identified as an effector by Dong *et al.* (2013). A high throughput analysis made use of transposon mutagenesis and subsequent sequencing to identify T6SS immunity proteins. The authors used a screening approach to identify immunity genes by the fact they would be essential for survival in a wild type strains, but dispensable in a T6SS mutant (Dong et al., 2013). This approach was successful in identifying three immunity proteins, and thus three adjacent effectors. Two of these, VasX and VgrG3, were already known *V. cholerae* T6SS effectors, targeted towards eukaryotic cells as previously discussed (Miyata et al., 2011; Pukatzki et al., 2006). The third candidate, named TseL (type six effector lipase), is a novel effector. TseL was shown to be secreted in a T6SS dependent manner, and shown to be as T6SS dependent antibacterial effector (Dong et al., 2013). TseL contained a predicted lipase domain, and site directed mutagenesis within the catalytic domain abolished the toxic activity of this protein. Dong *et al.* were unable to detect lipase activity for TseL, but subsequent analysis showed that TseL, alternatively known as $Tle2^{VC}$, has phospholipase A1 activity (Russell et al., 2013). An interesting observation is that TseL is able to act as an effector in both amoeba and interspecies bacterial competition, and it cannot be excluded that T6SS lipases are targeted to, and impact the viability of, eukaryotic cells.

Bacterial targeted evolved VgrG

Analysis of *V. cholerae* VgrG3, known to be a T6SS secreted effector, shows a predicted peptidoglycan binding domain (Pukatzki et al., 2007). In a subsequent study, VgrG3 was shown to be a T6SS dependent antibacterial effector (Brooks et al., 2013; Dong et al., 2013). Overexpression of *vgrG3* under the control of a pBAD promoter in *E. coli* resulted in cell rounding and lysis. Site

directed mutagenesis of the predicted catalytic residues abolished this toxicity (Dong et al., 2013). This is an interesting example of an evolved VgrG targeted towards the bacterial cell wall. VgrG3 is also protected by a cognate immunity protein (Brooks et al., 2013).A striking observation by Dong et al. was the direct interaction between TseL and VgrG3 by immunoprecipitation, strongly suggesting secretion and delivery of these components as a multi-protein complex.

It is interesting to note that the T6SS encoded by *V. cholerae* targets both bacterial cells (TseL, VgrG3), but also eukaryotic cells (VasX, VgrG1). This is a unique example of one T6SS targeting multiple hosts, and is in contrast to *B. thai* which has multiple T6SS which appear to have distinct eukaryotic or bacterial targets (Schwarz et al., 2010).

Other characterised T6SS bacterial toxins

Using a sensitive quantitative mass spectroscopy approach four novel T6SS effectors have been identified in *Serratia marcescens* (Fritsch et al., 2013). This approach also identified the previously characterised Ssp1 and Ssp2, as well as Hcp and VgrGs, in the supernatant. The four novel effectors discovered, Ssp3-6, severely impacted *E. coli* growth when expressed from an arabinose inducible promoter (Fritsch et al., 2013). Ssp3-6 are protected by an adjacent, specific, immunity pair. Ssp3 was shown to act in the cytoplasm and Ssp4 in the periplasm, while the location of activity of the other effectors is unknown. These newly discovered T6SS effectors show no similarity at the amino acid sequence level to previously characterised T6SS effectors, and carry no predicted functional domains (Fritsch et al., 2013). The mechanism of their toxicity remains unclear, but is likely to reveal novel classes of T6SS dependent effectors.

1.7.6 Regulation of T6SS

Ferric uptake regulator

Several environmental signals have been shown to regulate the expression and activity of T6SS clusters. The ferric uptake regulator (Fur) is involved in gene expression under iron limitation conditions, and is involved in the expression of some T6SS clusters. When bound to iron, the Fur protein dimerises and represses transcription by binding to consensus DNA sequences known as Fur boxes located in target promoter regions (Carpenter et al., 2009). Fur is involved in the regulation of genes involved in iron acquisition and metabolism, but also in virulence gene expression.

Fur represses the expression of the T6SS in the fish pathogen *E. tarda* (Chakraborty et al., 2011). Fur confers iron-dependent repression of the production of the Hcp homologue, and directly binds to a Fur box in the promoter region of the T6SS gene cluster. Under the iron-limiting conditions experienced during infection, the expression of the T6SS is activated due to the alleviation of Fur-dependent repression.

Fur is also involved in the regulation of the Sci1-T6SS of the emerging enteropathogenic bacterium EAEC (Brunet et al., 2011). Two Fur boxes and three DNA adenine methyltransferase (Dam) catalysed methylation sites are located in the Sci1-T6SS promoter region. Under low iron conditions, Fur dissociates, allowing RNA polymerase access to the promoter. Loss of Fur binding also allows Dam methylation, which prevents Fur re-association, generating a stable T6SS 'on-state'.

In *P. aeruginosa*, two Fur boxes have been identified upstream of the H2-T6SS, and the system is negatively regulated by iron, with expression of the cluster induced 2.2 fold in the absence of Iron (Sana et al., 2012).

Transcriptional Regulation: σ^{54}

Sigma 54 dependent activator proteins, otherwise known as bacterial enhancer binding proteins (bEBPs) are encoded within many T6SS clusters. Many T6SS clusters also contain Sigma 54 binding sequences located in their promoters, suggesting a general role for bEBPs and σ^{54} in the regulation of T6SS (Bernard et al., 2011). σ^{54} factors modulate the activity of RNA polymerase and drive the expression of a distinct subset of genes. σ^{54} recruitment by RNA polymerase forms a closed complex, and the transition to a transcription competent open complex requires a bEBP protein (Rappas et al., 2007).

Studies on bEBPs from a subset of T6SS have confirmed their role in σ^{54} dependent transcriptional activation (Bernard et al., 2011). The bEBP encoded within the T6SS of *V. cholerae*, VasH, is a key regulator of this system (Kitaoka et al., 2011), and is required for the T6SS mediated killing of *E. coli* by *V. cholerae* (MacIntyre et al., 2010).

The H2-T6SS and H3-T6SS clusters of *P. aeruginosa* T6SS also encode bEBPs, and have consensus σ^{54} binding sequences in their promoter regions, which suggests that expression of these clusters also depends on σ^{54}. However, this is yet to be demonstrated experimentally.

Quorum Sensing

Quorum sensing (QS) is a major regulatory mechanism for T6SS, with many diverse clusters showing QS dependent regulation. The *P. aeruginosa* T6SSs are regulated by a complex quorum sensing system which is described further in Section 1.8.

Post-translational Regulation: Surface Association

Early in the study of T6SS it was realised that growth on a solid medium was conducive to T6SS activity. Subsequently, it has become clear that these conditions are favourable to the contact-dependent mechanism of toxin delivery, now directly observed by Mekalanos and colleagues (Basler et al., 2013). H1-T6SS from *P. aeruginosa* is post translationally activated by a threonine phosphorylation pathway in response to surface growth (Silverman et al., 2011). Post translational regulation allows a rapid response to stimuli through the recruitment of ready-made components. This pathway involves the activity of PpkA, an inner membrane serine/threonine kinase, and Fha1, a forkhead associated domain. Fha1 is activated by phosphorylation through Stk1, which promotes T6SS assembly and effector secretion (Mougous et al., 2007). Cells grown on an agar surface assemble an activated apparatus and have elevated levels of phosphorylated Fha1 (Silverman et al., 2011). A cognate serine/threonine phosphatase antagonises the role of PpkA by dephosphorylation of Fha1 (Mougous et al., 2007). The fact that around 30% of T6SS contain components of a threonine phosphorylation pathway suggests that this may be a general mechanism of T6SS regulation (Boyer et al., 2009).

Additional accessory genes encoded by the *P. aeruginosa* H1-T6SS cluster, TagQRST have been shown to participate in the post translational activation of the H1-T6SS (Casabona et al., 2013). TagR has already been characterised as a positive regulator of PpkA, promoting the phosphorylation of Fha1 (Hsu et al., 2009). TagS, TagT and TagQ also act upstream of PpkA and are required for the optimal secretion of Hcp1, as well as for Tse1. This effect is due to the requirement of TagS, TagT and TagQ for phosphorylation of Fha1, and the subsequent assembly and activity of the T6SS. While TagQ is an outer membrane lipoprotein, and TagR is associated with the OM, TagT and TagS form an inner membrane complex with ATPase activity (Casabona et al., 2013). It is proposed that the

TagQRST is involved in the response to an unidentified signalling mechanism, resulting in activation of the T6SS.

Post-transcriptional Regulation: Gac/Rsm

The expression of the T6SS is also regulated by the Ret/Gac/Rsm cascade. Briefly, a mutant in the *retS r*egulatory protein has a hyperactive H1-T6SS, while deletion of the *ladS* regulator has a reciprocal effect (Goodman et al., 2004; Ventre et al., 2006). These sensors mediate their effects via the GacS/GacA two component system. This regulatory cascade also regulated the T3SS and biofilm formation, and is described in detail in section 1.9.

1.7.7 Dynamics of the *P. aeruginosa* H1-T6SS

Time-lapse fluorescence microscopy has been used to visualise the dynamics of the H1-T6SS of *P. aeruginosa*, providing remarkable live images of bacterial interaction. Fluorescent microscopy has already been used to visualise the sheath component of the T6SS in contracted and extended conformations, providing insights into the mechanism of T6SS (Basler et al., 2012). This technique has also allowed insights into the spatial and temporal activity of the H1-T6SS from *P. aeruginosa* and other organisms, as well as the interaction between different bacterial species.

P. aeruginosa cells in monoculture respond to T6SS activity in a neighbouring sister cell, which leads to an increase in the T6SS activity of the responding cell at defined foci, as shown in Figure 1.24. These dynamics were visualised by live cell fluorescence microscopy of cells expressing a ClpV-gfp fusion (Basler and Mekalanos, 2012b). Basler and Mekalanos (2012) subsequently used the term 'T6SS duelling' to explain the coincidence of T6SS activity between pairs of *P. aeruginosa* cells. This duelling activity led to the hypothesis that a signal was being transferred between cells at the

location of T6SS attack (e.g. membrane perturbation, peptidoglycan hydrolysis of protein phosphorylation) which allowed *P. aeruginosa* to recognise the T6SS activity and launch a counterattack (Basler and Mekalanos, 2012b) . This T6SS duelling behaviour is specific to *P. aeruginosa* since the same could not be observed for *V. cholerae*, which appears to have higher background levels of T6SS activity originating randomly within the cell (Basler and Mekalanos, 2012b).

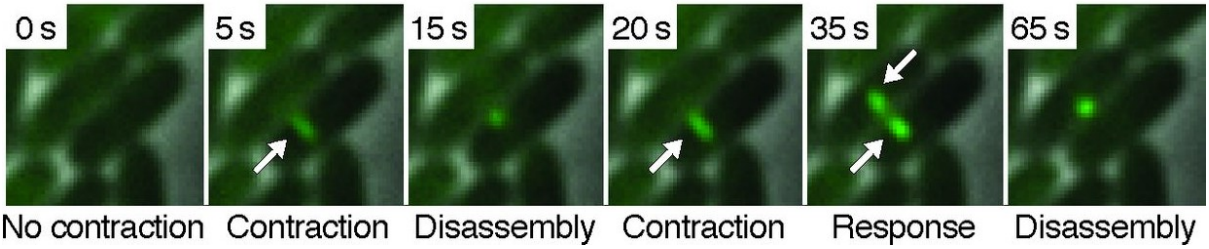


Figure 1.24 *P. aeruginosa* T6SS duelling
P. aeruginosa Δ*retS* expressing *gfp* tagged ClpV1, visualised by time-lapse fluorescent microscopy. *P. aeruginosa* cells are shown to respond to T6SS activity in a neighbouring sister cell (5s and 20s) with an increase in their own T6SS dynamics (35S). Reproduced from (Basler and Mekalanos, 2012a) with permission from AAAS.

The visualisation of T6SS dynamics between different species of bacteria has expanded our knowledge of *P. aeruginosa* T6SS activity, and yet again provided some remarkable live images of bacterial interactions (Basler et al., 2013). The H1-T6SS killing activity of *P. aeruginosa* against *V. cholerae* cells is greatly increased by T6SS activity in those prey cells. *P. aeruginosa* was observed to assemble its T6SS and counter attack at the point of attack from a T6SS+ prey cell, observed by imaging ClpV-gfp and ClpV-mCherry dynamics in the different bacterial species. A *P. aeruginosa* mediated T6SS attack resulted in *Vibrio* cell rounding, dependent on the VipA protein, one of the sheath-like components (Basler et al., 2013). These *V. cholerae* morphological changes occurred predominantly in *Vibrio* cells directly in contact with *P. aeruginosa*. The fact that rounding of *Vibrio* cells was absent when *P. aeruginosa* cells were incubated with T6SS negative *V. cholerae* (Δ*vipA*) led to the conclusion that the T6SS activity of a heterologous organism can trigger the duelling response

of *P. aeruginosa*. This was confirmed by quantitative analysis of bacterial survival, since 100 times fewer surviving T6SS$^+$ *Vibrio* were recovered in a competition assay compared to T6SS$^-$ cells competing against T6SS$^+$ *P. aeruginosa*. Thus, a T6SS duelling response is directed specifically towards T6SS+ *Vibrio* which have first initiated an attack towards *P. aeruginosa* (Basler et al., 2013). The evolutionary significance of this effect is proposed to allow *P. aeruginosa* to eradicate aggressive T6SS+ neighbours while co-existing with peaceful bystanders. This is different to the T6SS strategy of *V. cholerae* which fires T6SS constantly, attacking all invading cells, and thus expends much more energy. In *P. aeruginosa* the H1-T6SS is used as a defensive weapon, while the *V. cholerae* T6SS is an offensive weapon (Basler et al., 2013).

1.8 Global Regulation of Pathogenicity in *P. aeruginosa*

The wide variety of cell associated and secreted virulence factors described in previous section allow *P. aeruginos*a to be a prevalent nosocomial pathogen. However, *P. aeruginosa* is also ubiquitous in natural and man-made environments. Expression of these virulence factors in environmental settings must be tightly controlled to prevent their unnecessary production. Additionally, upon entry to a susceptible human host, rapid and specific activation of virulence factors is required for successful colonisation. In addition, virulence mechanisms need to be activated upon detection of eukaryotic predators or bacterial competitors in the environment. Several complex and interconnected regulatory mechanisms have evolved in *P. aeruginosa*, allowing *P. aeruginosa* to launch efficient virulence attack. The most extensively studied and best characterised of these regulation systems are described below.

1.8.1 Quorum Sensing

Quorum sensing (QS) is a mode of cell-cell communication allowing bacterial populations to coordinate gene expression and synchronise behaviours at high cell density. It was discovered in the bioluminescent marine bacterium *Vibrio fischeri*, where QS regulates light production (Engebrecht et al., 1983; Engebrecht and Silverman, 1984). When population density reaches a critical level, light is produced in response to the accumulation of a diffusible signalling molecule (Engebrecht and Silverman, 1984). This system allows *V. fischeri* to assess the density of neighbouring cells, and produce light only when cell density is high. The process involves the regulation of the *lux*CDABE operon by LuxI and LuxR (Engebrecht and Silverman, 1984), which is regarded as the canonical quorum sensing system, shown in Figure 1.25. LuxI is an enzyme responsible for the synthesis of the signalling molecule, which is an acylated homoserine lactone (AHL), namely N-(3-oxohexanoyl) homoserine lactone (Eberhard et al., 1981). AHL is produced at all times and diffuses freely in and out of cells. As the cell density increases, so does the concentration of this autoinducer molecule in the local environment. Once the concentration of AHL is above a critical limit the molecules are able to bind to a cognate receptor, LuxR. LuxR is also a transcriptional activator, but is only when bound to AHL. LuxR has a modular structure containing a DNA binding domain with a helix-turn-helix motif, and an AHL binding domain. Binding of AHL leads to LuxR dimerization, allowing it to bind DNA and activate transcription, producing the enzymes required for light production (Stevens et al., 1994). An auto induction mechanism occurs, in which LuxR controls the expression of LuxI, amplifying the amount of AHL produced when the system is active.

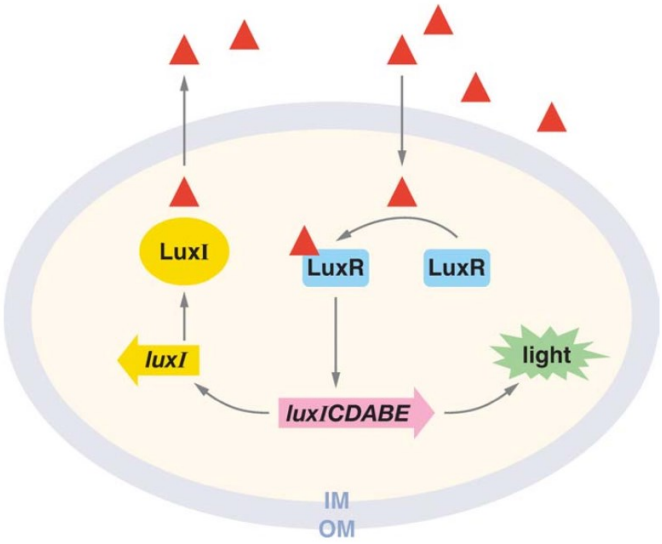


Figure 1.25 The Lux quorum sensing system from *V. fischeri*
Schematic representation of the LuxR/LuxI quorum sensing system from *V. fischeri*. The acylated homoserine lactone (AHL) molecule (red triangle) is produced by the LuxI enzyme. AHL diffuses freely into and out of the cell. AHL binds to the LuxR receptor and activates expression of the *luxICDABE* operon, resulting in the production of light.

A highly complex QS system exists in *P. aeruginosa*, which is likely the most studied and characterised QS system, together with *V. cholerae*. In *P. aeruginosa* QS does not control bioluminescence, but is involved in the regulation of virulence. *P. aeruginosa* has two complete systems homologous to the LuxI/LuxR systems (LasR/LasI and RhlR/RhlI) (Juhas et al., 2005; Schuster and Greenberg, 2006), and together these two systems regulate around 10% of the *P. aeruginosa* genome (Schuster and Greenberg, 2006). An incomplete system composed of an orphan LuxR-like protein QscR also exists in *P. aeruginosa* (Chugani et al., 2001). A third signalling system, PQS, is also involved in *P. aeruginosa* QS (Deziel et al., 2005; Wade et al., 2005). These systems do not act alone, and are inter-linked through regulatory connections.

The LasI/R and RhlI/R quorum sensing systems are summarised in Figure 1.26. These systems are involved in the production of similar yet different acyl-homoserine lactone molecules (Brint and Ohman, 1995; Passador et al., 1993). The AHL synthase LasI produces N-3-oxododecanoyl homoserine lactone (3OC12-HSL) (Pearson et al., 1994) , while RhlI produces N-butyryl-homoserine

lactone (C4-HSL) (Pearson et al., 1995). The presence of both AHL molecules has been shown in samples taken from the lungs of chronically infected CF patients, indicating that these quorum sensing systems are important in infections (Erickson et al., 2002).

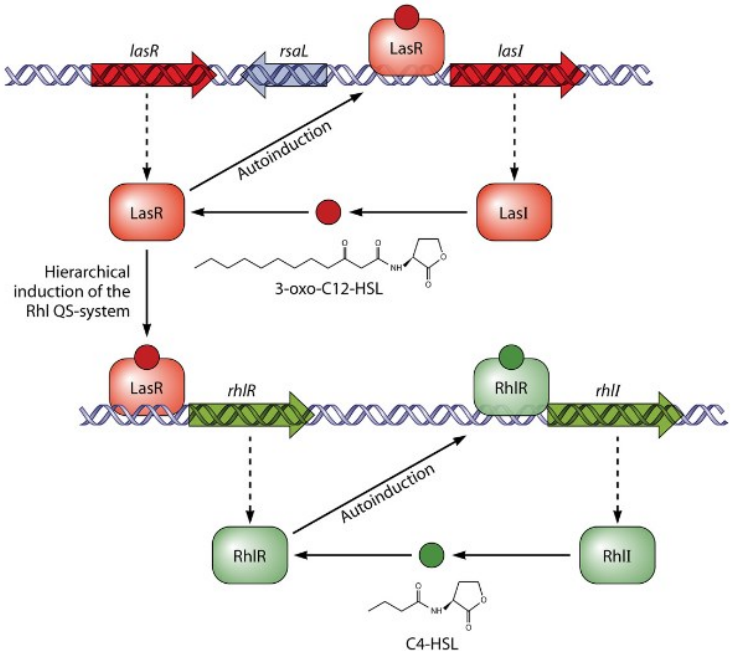


Figure 1.26 The LasI/R and RhlI/R quorum sensing systems from *P. aeruginosa*
3-oxo-C12-HSL is produced by LasI, and once a threshold concentration is reached, the 3-oxo-C12-HSL-LasR complex binds to the promoters of multiple genes, activating their transcription, including the promoter of *lasI*, causing an autoinduction effect. The *rhlR* promoter is also activated, increasing production of the RhlR response regulator, activating the second *P. aeruginosa* AHL quorum sensing pathway. The virulence factors regulated by these two systems are listed in Table 5. Figure reproduced from (Jimenez et al., 2012)with permission

The LasI/LasR system was first discovered as a regulator of the secreted virulence factor elastase or LasB (Gambello and Iglewski, 1991). Once the *P. aeruginosa* population reaches a certain cell density, the LasI product 3OC13-HSL binds to the transcriptional activator LasR, which dimerises and binds to target promoters, activating their transcription (Schuster et al., 2004b). The LasR target promoter sequences precede a number of secreted virulence factors important in the initiation of *P. aeruginosa* infections, listed in Table 5. These include elastase (*lasB*), LasA protease and exotoxin A, already described as virulence factors secreted by the T2SS in section 1.6 (Gambello and Iglewski,

1991; Jones et al., 1993; Passador et al., 1993). Quorum sensing therefore controls virulence in *P. aeruginosa* by regulating the secretion of virulence factors when cell density is high enough to launch an attack. Another target of the LasI-AHL complex is the expression of LasI itself, by an autoinduction mechanism (Seed et al., 1995).

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	

Table 5 *P. aeruginosa* quorum sensing regulated virulence factors
Table reproduced from (Juhas et al., 2005) with permission.

The second *P. aeruginosa* quorum sensing system, RhlI/R, was identified as a regulator of rhamnolipids (Ochsner et al., 1994). The RhlI/R systems is itself controlled by the LasI/R system, since the expression of *rhlI* is a target of LasI-AHL, so the two systems are tightly linked in a hierarchical system (Figure 1.26) (Latifi et al., 1996). The regulon of RhlR-AHL includes the expression of exoproducts already under LasI/R control (Brint and Ohman, 1995) but also a specific set of target genes listed in Table 5. One of these is the gene encoding RpoS, the stationary phase sigma factor, which controls expression of genes for resistance to environmental stresses, and a specific set of virulence genes (Schuster et al., 2004a). The Rhl system regulates rhamnolipid biosurfactant, cyanide, and pyocyanin production (Latifi et al., 1995; Ochsner et al., 1994; Winzer et al., 2000).

The *rhl* system is responsible for the down-regulation of the T3SS (Bleves et al., 2005), which means that at high cell density the secretion of the potent toxins ExoU and ExoS is reduced. This seems at odds with the role of quorum sensing to co-ordinately activate virulence factors. However, this may indicate that T3SS is only required in the early stages of *P. aeruginosa* infection, when only a small number of cells are present. The T3SS may help *P. aeruginosa* to disrupt host cells to allow and infection to establish, but is then down-regulated at later stages of infection (Willcox et al., 2008). There is also growing evidence that the three T6SS clusters encoded by *P. aeruginosa* are differentially regulated by quorum sensing. Expression of the well-characterised H1-T6SS is repressed by LasR and MvfR (also known as PqsR, a regulator of the PQS system described below), while the H2- and H3-T6SS are positively controlled (Lesic et al., 2009). However, the physiological significance of this effect is yet to be characterised. Quorum sensing is important in *P. aeruginosa* biofilm formation, as *lasI* mutants are unable to form mature biofilm structures (Davies et al., 1998).

The orphan LuxR-like protein QscR shares approximately 30% identity to both LasR and RhlR. It contains both an AHL binding domain and a DNA binding domain (Chugani et al., 2001). QscR has been shown to form complexes with C4-HSL and 3O-C12-HSL, or complexes with LasI or RhlR in the absence of AHLs, and thus may regulate quorum sensing by sequestering these molecules (Ledgham et al., 2003). QscR is also a transcriptional regulator, with a regulon of over 400 genes, which overlap with the LasR and RhlR regulons (Lequette et al., 2006). QscR directly binds DNA, and requires 3O-C12-HSL to do this, despite being able to bind both AI molecules (Lee et al., 2006).

A third complete quorum sensing system in *P. aeruginosa* is dependent on the auto inducer molecule 2-heptyl-3-hydroxyl-4-quinolone, known as the *Pseudomonas* quinolone signal (PQS) (Pesci et al., 1999). The expression of the PQS biosynthetic operon *pqsABCDE* is positively regulated by a regulator PqsR (corresponding to MvfR in PA14). The synthesis and activity of PQS is mediated by

the *las* and *rhl* systems, linking this system to the hierarchical quorum sensing system of *P. aeruginosa.* LasR regulates PQS production, which is required for the transcription of *rhlR* and *rhlI*, providing a regulatory link between these systems (McKnight et al., 2000; Pesci et al., 1999). PQS is produced maximally during late stationary phase, and around 90 genes have been found to be regulated by the *pqs* system, including genes involved in biofilm formation and several virulence factors including elastase and pyocyanin (Table 5) (Bredenbruch et al., 2006; Deziel et al., 2005). PQS is found in sputum and other samples taken from CF patients (Collier et al., 2002). The *P. aeruginosa* PQS signal influence interspecies behaviour, affecting other Gram negative and Gram positive organisms. In the presence of a PQS precursor molecule, motility was suppressed in a broad range of bacteria, and biofilm formation was repressed in *Bacillus subtilis* and *Candida albicans* (Reen et al., 2011). Other bacteria in the vicinity interact with *P. aeruginosa* signal molecules, with the PQS system modulating polymicrobial communities, and not just influencing *P. aeruginosa* cells (Reen et al., 2011).

Targeting quorum sensing has become an attractive strategy for treatment of *P. aeruginosa* infections. The crystal structure of the PqsR protein from *P. aeruginosa* has been solved, and molecules generated which block the binding site. Several novel potent QS inhibitors were discovered and shown to block virulence gene expression (Ilangovan et al., 2013). Garlic extracts have been shown to inhibit *P. aeruginosa* QS. Fractionation of garlic extracts revealed ajoene was responsible for attenuation of QS-controlled virulence factors, as well as a synergistic antimicrobial effect with tobramycin on biofilm killing and a significant clearing of infecting *P. aeruginosa* in ajoene treated mice (Jakobsen et al., 2012). By targeting and abolishing QS systems by therapeutic treatments, the devastating outcome of infections may be reduced if not eliminated. The fact that bacterial cells are not killed suggests that resistance will not be selected, an advantage given the growing problem of antibiotic resistance.

1.8.2 cAMP

Cyclic adenosine-3,5' monophosphate (cAMP) is a second messenger important in signal transduction in eukaryotic cells, amoeba and bacteria. cAMP is synthesised from ATP by adenylyl cyclase, and activates transcription factors of the CRP (cAMP regulator proteins) family (Harman, 2001). Three adenylyl cyclases are encoded by *P. aeruginosa*. CyaA and CyaB are intracellular adenylyl cyclases (Wolfgang et al., 2003b). The third adenylate cyclase is the T3SS secreted effector ExoY, already described in previous sections, exerting its affect following injection by the T3SS into host cells (Yahr et al., 1998). Cellular cAMP levels are reduced in *cyaA cyaB* mutants, but CyaB is responsible for the majority of cAMP in *P. aeruginosa* (Wolfgang et al., 2003b). A *cyaB* mutant is attenuated in a mouse model of infection, indicating an important role for cAMP in pathogenesis (Smith et al., 2004).

The intracellular levels of cAMP influence gene expression in *E. coli* through interacting with a cAMP-receptor protein, CRP. CRP is a transcriptional regulator involved in carbon metabolism in response to glucose availability in a process known as catabolite repression (Gosset et al., 2004). Binding of cAMP to CRP results in a conformational change allowing binding to DNA, and results in transcriptional activation or inhibition (Botsford and Harman, 1992). Catabolite repression does not occur in *P. aeruginosa*, and CRP is not conserved. Alternatively, a functional CRP homologue is found in *P. aeruginosa*, known as Vfr (virulence factor regulator) which shares 67% sequence identity with CRP (West et al., 1994). Vfr binds cAMP, effecting the expression of over 200 genes, including multiple virulence factors (Suh et al., 2002; Wolfgang et al., 2003b). Exotoxin A, type IV pili and the T3SS are upregulated (Beatson et al., 2002; Wolfgang et al., 2003b), while flagellar gene expression is downregulated by Vfr (Dasgupta et al., 2002). Quorum sensing and Vfr are tightly linked, since the transcription of *lasR* is directly regulated by Vfr binding (Albus et al., 1997). The role of Vfr in

infection is clear, since *P. aeruginosa vfr* mutants are substantially attenuated in a mouse model of infection (Smith et al., 2004).

1.8.3 The Stringent Response

The stringent response allows bacteria to sense and respond to nutrient-poor environments via a pair of second messengers known as ppGpp and pppGpp (guanosine tetra and pentaphosphate respectively). When *P. aeruginosa* encounters cellular stresses (e.g. amino acid starvation), the accumulation of these molecules modulates energetically costly cell processes, including protein translation, to preserve cellular resources (Srivatsan and Wang, 2008). Levels of (p)ppGpp are controlled by RelA and SpoT which synthesises and degrade pppGpp respectively. The stringent response in *P. aeruginosa* has been shown to be important in the regulation of virulence factors, since *relA* mutants show decreased virulence in a *Drosophila melanogaster* model (Erickson et al., 2004). The increase in ppGpp concentrations seen upon *relA* overexpression leads to activation of quorum sensing even in the absence of high cell density (van Delden et al., 2001). An elevated basal level of ppGpp is correlated with an increased tolerance of *relA* and *spoT* mutants to quinolone antibiotics (Viducic et al., 2006). Thus, (p)ppGpp may act as a messenger, mediating the response of *P. aeruginosa* to stress conditions encountered upon host colonisation, promoting survival and virulence.

1.8.4 c-di-GMP

c-di-GMP is a secondary messenger involved in signalling in many species from all kingdoms. The levels of this intracellular messenger are modulated by two classes of enzymes, diguanylate cyclases (DGC) and phosphodiesterases (PDE), which synthesise and degrade c-di-GMP respectively. These activities are mediated by conserved motifs, known as GGDEF motifs for DGCs and EAL or HD-GYP

domains for PDE, based on amino acid conservation. The *P. aeruginosa* genome encodes 39 proteins with DGC, PDE or both domains, although this number varies in different strains (Kulasakara et al., 2006). Proteins have also been identified with degenerate GGDEF domains, which are not catalytically active, suggesting they act as allosteric sites or c-di-GMP receptors (Christen et al., 2005; Newell et al., 2009). The levels of c-di-GMP influence a plethora of phenotypes in diverse bacterial species, including, but not limited to, biofilm formation, motility and cytotoxicity.

There are several examples describing the effect of c-di-GMP on virulence phenotypes of *P. aeruginosa*. One of the best characterised is WspR, a DGC linked to the chemosensory *wsp* operon. Loss of the *wsp* operon from *P. aeruginosa* results in the loss of swimming and swarming motility and a hyper-aggregative phenotype. Deletion of *wspF* results in high c-di-GMP levels and increased biofilm formation, which could be restored to wild type upon *wspR* deletion (Hickman et al., 2005). This effect is mediated through higher expression of *pel* and *psl* operons, as well as *cupA* fimbrial genes, contributing to increased biofilm formation. WspF is a negative regulator of the Wsp signalling, and it is proposed that loss of *wspF* leads to accumulation of phosphorylated, active WspR, leading to increased c-di-GMP (Hickman et al., 2005).

Additional DGCs and PDEs are involved in *P. aeruginosa* biofilm formation. One of these, SadC, has DGC activity and is important in the transition of cells from reversible to irreversible attachment. Deletion of *sadC* results in a hyper-swarming phenotype defective in biofilm formation (Merritt et al., 2007). Deletion of the genetic region encoding BifA, which contains both DGC and PDE domains, results in an increase in the cellular pool of c-di-GMP, production of Pel polysaccharide, and abolishment of swarming activity (Kuchma et al., 2007).

Twitching motility is also affected by c-di-GMP. FimX is required for type IV pili formation, which are essential for twitching motility and adherence (Huang et al., 2003). FimX contains both EAL and

GGDEF domains, however this GGDEF is degenerate and likely to perform as a c-di-GMP binding motif without catalytic activity. FimX is able to degrade c-di-GMP via its EAL domain, but unable to synthesis c-di-GMP. Binding of c-di-GMP to the degenerate GGDEF exerts an allosteric effect, controlling the function of FimX. Deletion of *fimX* results in diminished twitching motility, and this effect could also be achieved by mutagenesis of either the EAL or degenerate GGDEF domains (Kazmierczak et al., 2006).

A further example of the importance of c-di-GMP in the virulence of *P. aeruginosa* is seen in its effects on the T3SS and T6SS. The RetS sensor (see section 1.9) is responsible for switching between T3SS and T6SS active phenotypes (Goodman et al., 2004). This switch is correlated with c-di-GMP levels. Low levels of c-di-GMP correlate with an active T3SS and poor biofilm formation, while high levels of c-di-GMP cause a switch to high biofilm formation and an active T6SS. These phenotypes can be influenced by artificial modulation of c-di-GMP levels, but specific DGCs or PDEs involved in this process are yet to be fully characterised (Moscoso et al., 2011).

1.8.5 Global analysis of *P. aeruginosa* gene expression

The complex regulatory mechanisms described here allow *P. aeruginosa* to respond adaptively to its environment. To understand these responses, techniques have been developed to examine gene expression on a global scale. Transcriptome studies allow a snapshot of an organisms response based on gene expression, by analysing the entire set of RNA transcripts produced by a population of cells at a given time. Large scale genome sequencing, and the publication of the *P. aeruginosa* PAO1 genome (Stover et al., 2000) allowed the transcriptome of *P. aeruginosa* to be studied for the first time on a global level using microarray analysis. Commercially available tools, such as the *P. aeruginosa* gene chip from affymetrix, allows the 5570 PAO1 open reading frames to be probed.

Genome-wide transcription analyses have provided important information on the physiology of *P. aeruginosa*. Analysis of gene expression in *lasI rhlI* quorum sensing mutants have allowed genes regulated by quorum sensing to be discovered, providing an insight into the importance of quorum sensing in the regulation of virulence factors (Schuster et al., 2003; Wagner et al., 2003). Comparison of planktonic and biofilm cells have revealed that only 1% of the *P. aeruginosa* genome is differentially regulated in cells within biofilms (Whiteley et al., 2001), and that the expression profiles of developing biofilms is similar to that of log phase cultures, while mature biofilms have an expression profile similar to stationary cells (Whiteley et al., 2001). This analysis has shown that type 4 pili are down regulated in mature biofilms, indicating that pili are not essential in the late stages of development. Global analysis is clearly a powerful tool in elucidating the molecular mechanisms of *P. aeruginosa* involved in pathogenicity.

Transcriptome analyses have also been applied to investigate the response of human lung epithelial cells to *P. aeruginosa* infection. Analysis showed that 25% of 1500 human genes probed were differentially regulated upon *P. aeruginosa* infection (Ichikawa et al., 2000). This experiment also discovered a novel signalling pathway involved in activation of the host immune response, highlighting the importance of transcriptome studies in elucidating the mechanism of pathogen-host interaction.

1.9 *P. aeruginosa* lifestyles; regulatory aspects and mechanisms

As previously described, *P. aeruginosa* is both an ubiquitous environmental organism, and a prevalent opportunistic pathogen. In the environment *P. aeruginosa* can exist in a planktonic lifestyle or within multicellular biofilm communities (section 1.4). Similar dual lifestyles are also thought to mediate the different outcomes of infections within a human host.

Acute *P. aeruginosa* infections are rapid in progression, and multiple virulence factors are expressed. Examples include the *P. aeruginosa* mediated breakdown of lung tissue in acute pneumonia, and dissemination into the blood stream (Driscoll et al., 2007). The result is systemic infection, septic shock and death within days. In such acute infections, *P. aeruginosa* undertakes an invasive and cytotoxic lifestyle, mediated by the production of a range of secreted proteins, including the T3SS secreted effectors, as described in section 1.6.4.

Conversely, *P. aeruginosa* can also cause chronic lung infections, the characteristics of which are vastly different to acute pneumonia. *P. aeruginosa* can persist for decades in the CF lung, and chronic inflammation ultimately leads to death (O'Sullivan and Freedman, 2009). In these infections, *P. aeruginosa* remains non-invasive, non-cytotoxic and systemic infections do not occur. *P. aeruginosa* undergoes phenotypic changes including the overproduction of alginate and mutations resulting in the loss of toxin secretion and motility (Jain et al., 2004; Mahenthiralingam et al., 1994). The production of biofilms is thought to be important in chronic CF lung infections due to the fact that the quorum sensing profile and signalling molecules detected in CF sputum are more similar to that of cells grown in in-vitro biofilms than to planktonic cells, and images of CF sputum shows polysaccharide-encased bacteria (Singh et al., 2000).

In addition to the global virulence regulators already described in section 1.8, a dedicated and complex switch exists in *P. aeruginosa* to regulate the expression of genes involved in acute or chronic lifestyle and influence the outcome of infection. This is mediated by the Gac/Rsm cascade, which is conserved among Gram-negative bacteria, and two additional components, LadS and RetS, specific to *Pseudomonas* species. This section describes the components of this cascade, and the mechanisms employed to influence the outcomes of *P. aeruginosa* infections.

1.9.1 Conserved Gac/Rsm pathways

The Gac/Rsm signal transduction pathway controls gene expression through modulating the translation of target small RNAs (sRNAs). The components and mechanism of the pathway are described in more detail in the following sections, but a central component is an RNA binding protein of the CsrA/RsmA family. CsrA/RsmA is capable of translational activation and repression. Homologs of the Gac/Rsm pathway are found in many of the γ-proteobacteria, and CsrA/RsmA regulate numerous unrelated biological pathways (Lapouge et al., 2008).

In *E. coli* the RNA binding protein CsrA (carbon storage regulator) regulates central carbon pathways. CsrA positively regulates energy consumption through glycolysis, while negatively regulating energy storage by gluconeogenesis and glycogenesis (Sabnis et al., 1995). CsrA is conserved in *Salmonella* Typhimurium, but its targets are different since it regulates maltose and acetate metabolism (Lawhon et al., 2003).

CsrA and its homologues have been implicated in the regulation of social behaviour in bacterial cells, influencing phenotypes such as quorum sensing, biofilm formation and motility. In *Vibrio* and *Pseudomonas* spp. CsrA interacts with quorum sensing by negatively regulating the production of AHL through an unknown mechanism (Lenz et al., 2005; Pessi et al., 2001). In *E. coli* the formation of PGA, a linear homopolymer involved in attachment during biofilm formation, is negatively regulated by CsrA (Wang et al., 2005).

CsrA/RsmA is also involved in the control of virulence. In *P. fluorescens* the production of hydrogen cyanide synthase subunit A (*hcnA*) required for the synthesis of the antifungal exoproduct hydrogen cyanide, is under the direct control of RsmA (Lapouge et al., 2007), and was the only known direct target until subsequent publications by the Lory group (Brencic and Lory, 2009). The role of *P.*

aeruginosa RsmA (regulator of secondary metabolism), and the mechanism of this regulation and its effect on the lifestyle of *P. aeruginosa* are discussed in more detail in the following sections.

1.9.2 The *P. aeruginosa* Gac/Rsm pathway

An overview of the *P. aeruginosa* Gac/Rsm pathway is shown in Figure 1.27. Briefly, the RsmA translational regulator positively influences phenotypes associated with acute infections (motility and T3SS), while repressing phenotypes associated with chronic infection (biofilm formation), and repressing the T6SS. The activity of RsmA can be controlled by regulatory sRNAs RsmY and RsmZ which are able to bind and sequester RsmA. The levels of these regulatory sRNAs are controlled by a two component system composed of GacA, a response regulator, and GacS, a membrane bound sensor kinase. Two additional sensor kinases, RetS and LadS, specific to *Pseudomonas,* also influence this cascade. The characteristics of each component and the molecular details of this cascade are described below.

1.9.3 The Translational Regulator RsmA

The *P. aeruginosa* translational regulator RsmA has a regulon of over 500 genes discovered by microarray analysis of gene expression in an *rsmA* mutant compared to the wild type strain (Brencic and Lory, 2009). In the microarray the expression of a third of these 500 genes was positively affected by the mutation, the remainder negatively. Expression of genes encoding structural and regulatory components of the T3SS, associated with acute infections, were decreased in the *rsmA* mutant (section 1.6.4). Genes which showed increased expression in the *rsmA* mutant included genes of *pel* and *psl* polysaccharide biosynthetic operons important in production of the biofilm matrix, associated with chronic infections (section 1.4), and genes of the H1-T6SS operon (Section 1.7).

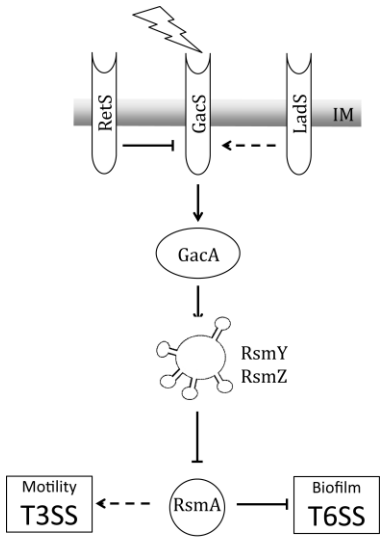


Figure 1.27 The *P. aeruginosa* RetS/Gac/Rsm cascade
Schematic representation of the RetS/Gac/Rsm signalling cascade which antagonistically controls the phenotypic switches for biofilm/motility and T6SS/T3SS. RetS, GacS and LadS are inner membrane (IM) located sensor, GacA is a cytoplasmic response regulator, RsmY and RsmZ are small regulatory RNAs, and RsmA is a translational regulator. Pointed arrows indicate a positive effect, while blocked arrows indicate a negative effect. Dashed lines indicated an indirect interaction. An unknown activation signal is represented by the lightning bolt.

The mechanism of negative regulation by CsrA has been shown for the CsrA/RsmA family protein from *Pseudomonas fluorescens*, known as RsmE in this case. The crystal structure of RsmE bound to a 20 nucleotide sequence from a target RNA from the *hcnA* gene has been solved (Schubert et al., 2007). The *hcnA* gene encodes hydrogen cyanide synthase subunit A, which is translationally repressed by RsmE (Blumer et al., 1999). The work by Schubert *et al.* (2007) provides a direct visualisation of the mechanism of CsrA/RsmA repression, as shown in Figure 1.20. A 20 nucleotide region of the *hcnA* RNA was produced, and was predicted to form a short hairpin, with an unpaired GGA motif in the loop region essential for the binding to CsrA/RsmA proteins (Figure 1.28A) (Liu et al., 1997). This nucleotide section is seen bound as a stem loop within the RsmA molecule (Schubert et al., 2007). Within this loop, embedded within the RsmA protein, are five nucleotides of the Shine-Dalgarno sequence of *hcnA*. The negative regulation mediated by RsmA is clearly achieved at the post-transcriptional level through the interruption of the ribosome-mRNA interaction, blocking translation of specific mRNA molecules.

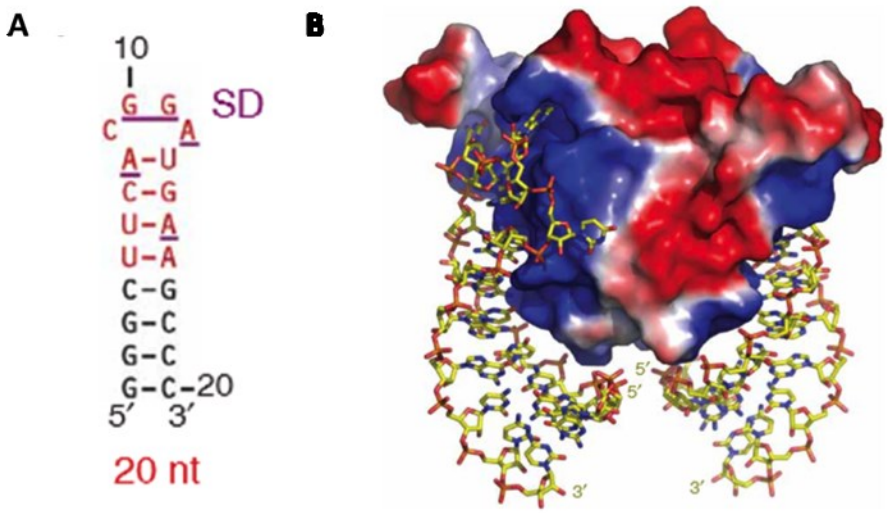


Figure 1.28 Binding of hcnA RNA to the translational repressor RsmE
A. Predicted secondary structure of the 20 nucleotide hcnA sequence used for structure determination of the RsmE-RNA complex, with the location of the hcnA shine-dalgarno sequence underlined in purple. B. NMR solution structure of the RsmE-*hcnA* RNA complex. Surface representation of RsmE dimer in complex with the *hcnA* 20 nucleotide RNA. Blue colour indicates positive electrostatic potential, while red colour indicates negative electrostatic potential. Reproduced with from (Schubert et al., 2007) with permission.

In *P. aeruginosa* Brencic & Lory (2009) were able to co-purify RsmA bound to mRNA corresponding to the H1-T6SS genes, expanding the known targets for CsrA/RsmA binding. The mRNA sequences recovered contained the characteristic GGA motifs required for RsmA binding, as shown in Figure 1.29a. They confirmed that the negative regulation of RsmA on the H1-T6SS was due to the direct interaction of this protein with the leader sequence of the mRNA by performing gel shift assays on radiolabelled mRNA molecules as shown in Figure 1.29b (Brencic and Lory, 2009).

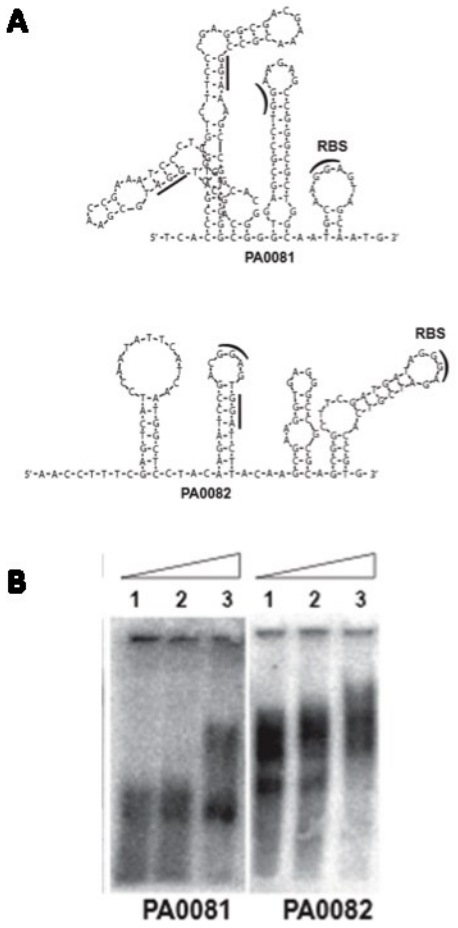


Figure 1.29 Direct binding of RsmA to H1-T6SS promoters

A. secondary structures of leader sequences of H1-T6SS genes identified as targets of RsmA, predicted by M-fold (Zuker, 2003). GGA sequences are underlined, and the RBS is indicated in each case. B. RNA mobility shift assay with purified RsmA-H_6, binding to the leader sequences of transcripts encoding PA0081 and PA0082 (*tssA1* and *fha1*). Transcripts were radiolabelled and incubated in the absence or presence of various concentrations of RsmA-H_6 for 30 minutes at room temperature, prior to analysis on 8% native polyacrylamide gel. Lanes 1-3 contained 10 nM of radiolabelled RNA, and 0, 1 and 3 µM of RsmA-H_6 respectively. Reproduced from (Brencic and Lory, 2009) with permission.

CsrA/RsmA proteins are also responsible for the positive regulation of genes associated with the acute lifestyle of *P. aeruginosa* (including the T3SS). The precise mechanism of positive regulation by this protein is not characterised. Positive regulation of T3SS gene expression by RsmA is likely to be indirect, since RsmA bound to the mRNA of targets that were positively regulated were not isolated in the study by Brencic & Lory (2009). More recently it has been shown that CsrA can lead to the activation of expression of flagella synthesis genes in *E. coli*. CsrA binds to two regions in the leader

sequence of *flhDC* mRNA and protects the mRNA from cleavage by RNaseE (Yakhnin et al., 2013). In *P. aeruginosa*, RsmA exerts a positive regulatory effect on ExsA, the master regulator of T3SS genes. RsmA controls expression of ExsA at the post-transcriptional level, with an *exsA-lacZ* translational fusion showing a 2-fold reduction in an *rsmA* mutant, dependent on a specific region of the leader sequence. RsmA therefore relieves a block on ExsA translation, leading to higher T3SS activity (see section 1.6.4.3) (Timothy L. Yahr (University of Iowa), Poster Presentation, American Society for Microbiology 113th General Meeting, 2013).

1.9.4 Small Regulatory RNAs RsmY and RsmZ

The activity of the transcriptional regulator RsmA can be antagonised by non-coding small regulatory RNA molecules (sRNA). sRNAs can be between 50 to 500 nucleotides and have important roles in bacterial gene expression. They mostly act post transcriptionally to activate or repress translation initiation, or by stabilising / destabilising target mRNAs. This effect is achieved by one of two mechanisms.

The antisense mechanism involves base pairing interactions between the non-coding RNA and target mRNA, thereby inhibiting translation. Anti-sense RNAs can repress the synthesis of many proteins. RyhB is one such anti sense RNA produced under iron limiting conditions in *P. aeruginosa*. RyhB base pairs with the mRNAs from multiple operons encoding iron-binding proteins, leading to rapid degradation of the molecule by RnasE (Masse et al., 2003; Wilderman et al., 2004), reducing the cellular need for iron. Multiple sRNAs are involved in the control of quorum sensing in *V. cholerae*. Multiple quorum sensing signals converge on a transcriptional regulator LuxO, which leads to negative regulation of another transcriptional regulator LuxR. Mutant screens identified Hfq, an RNA chaperone, in the transduction of signals between LuxO and LuxR, suggesting that small RNAs were involved in the process. Four homologous small RNAs were subsequently found in a

computational search, which were shown experimentally to each be sufficient to transduce the signals through the pathway, suggesting that they are redundant (Lenz et al., 2004).

Within the Gac/Rsm cascade, sRNAs are able to sequester CsrA/RsmA RNA binding proteins. The global transcriptional regulator of carbon metabolism (CsrA) from *E. coli* was co-purified by nickel-affinity chromatography and shown to be a stable complex of around 20 CsrA subunits bound to a single 350-nucleotide sRNA molecule, subsequently named CsrB. By generating cDNA clones of this RNA molecule and determining the nucleotide sequence they were able to identify the genetic locus encoding this molecule (Liu et al., 1997). The genetic locus of *csrB* contained no predicted open reading frames, indicating that CsrB was not a messenger RNA, and RNA structure predictions show it contains multiple GGA motifs in stem loop structures (Liu et al., 1997). A second sRNA, CsrC, was subsequently found in *E. coli*, which also binds and antagonises CsrA at a lower affinity than CsrB (Weilbacher et al., 2003).

An RNA molecule bound to the purified RsmA protein from *P. fluorescens* was identified and named RsmZ (regulator secondary metabolism), which is conserved in *P. aeruginosa* (Heeb et al., 2002). A second small regulatory RNA, RsmY, was subsequently detected in *Pseudomonas* sp. in silico and shown to be required for Gac/Rsm dependent regulation (Valverde et al., 2003). RsmY is a 118 nucleotide molecule, while RsmZ is 127 nucleotides, and each has multiple GGA motifs, located in predicted stem-loop structures. Multiple single stranded GGA motifs are a common feature of sRNAs that bind RsmA/CsrA proteins, and are essential for the binding of RsmY to RsmA in *P. aeruginosa* (Sorger-Domenigg et al., 2007). In the *P. fluorescens* RsmY homolog discrete complexes are formed with RsmA in gel mobility assays, and these complexes are lost when the GGA motifs are disrupted by mutation (Figure 1.30) (Valverde et al., 2004). Once bound to RsmY or RsmZ, RsmA is sequestered and unable to bind to target mRNAs, thus relieving any positive or negative effect on translation of the target mRNA molecule. A third sRNA was found in *P. fluorescens*, RsmX, but is

absent from *P. aeruginosa* (Kay et al., 2005). In *P. aeruginosa* the two sRNAs RsmY and RsmZ are functionally redundant for the repression of T3SS genes, and deletion of both is required to observe a phenotype (Bordi et al., 2010).

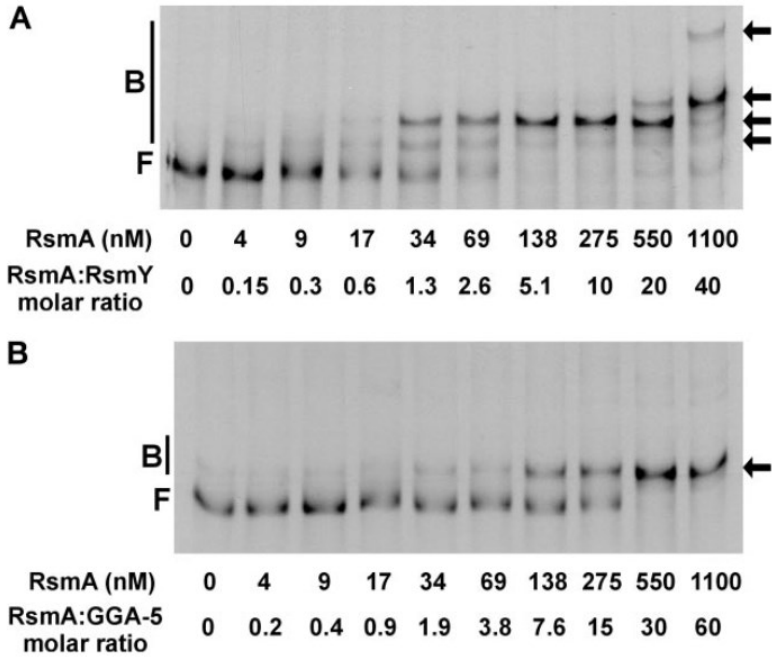


Figure 1.30 Interaction between RsmA and GGA motifs of *Pseudomonas fluorescens* RsmY
RsmY and a RsmY mutant in which the conserved RsmY GGA motifs were modified (GGA-5) were synthesised in the presence of radiolabelled UTP. RsmY (A) and GGA-5 (B) were incubated with different concentrations of purified RsmA-$_{His6}$ before running on native gels and autoradiography. The postilion of free (F) and bound (B) RNA is indicated. Arrows indicate RNA-protein complexes with reduced mobility when compared to free RsmY or GGA-5. Reproduced with from (Valverde et al., 2004) with permission.

The mechanism of RsmY and RsmZ in *P. aeruginosa* has been shown by gel shift assays. As described previously, Brencic & Lory (2009) were able to show that RsmA binds directly to the promoter regions of H1-T6SS specific genes, repressing their translation. RsmY and RsmZ are able to compete with H1-T6SS RNA for RsmA binding, as shown in Figure 1.31. Incubation of RsmA with RNA specific to the promoter region of PA0082 (the first gene in the H1-T6SS cluster) causes a band shift, indicative of direct binding. Titration with either RsmY or RsmZ inhibits this binding, indicating that

RsmA preferentially binds to RsmY or RsmZ. Thus, when present, RsmY and RsmZ sequester RsmA from T6SS target genes, relieving its repression (Brencic and Lory, 2009).

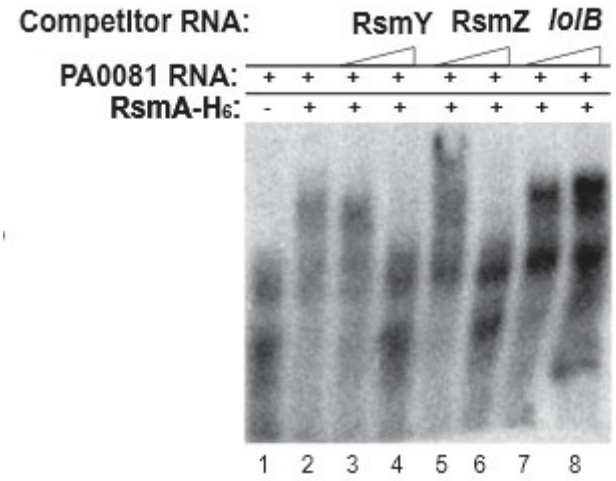


Figure 1.31 RsmY and RsmZ compete with target mRNA for RsmA binding
RNA mobility shift assay with purified RsmA-H_6, binding to the leader sequences of transcripts encoding PA0081 (*fha1*). 10 nM of labelled PA0081 transcript and 0 (lane 1) or 2.5 µM (lanes 2-8) of purified RsmA-H_6 monomer. Lane two shows the reaction between RmsA-H6 and PA0081 RNA in the absence of competitor RNA. Reactions shown in lanes 3-8 were incubated in the presence of unlabelled transcripts of RsmY (lanes 3 and 4), RsmZ (lanes 5 and 6) and *lolB* (lanes 7 and 8) at two folds (lanes 3,5,7) or 200 fold (lanes 4,6,8) excess relative to the labelled transcript. Reproduced from (Brencic and Lory, 2009) with permission.

1.9.5 Two component systems: GacS/GacA

The expression of RsmY and RsmZ is controlled by a two-component system made up of GacS and GacA. Two component systems (TCS) are signal transduction pathways that allow bacteria to sense their external environment and respond accordingly by modulating their behaviour. The characteristic components of TCS are illustrated in Figure 1.32. The two central components are known as a sensor kinase, and a cognate response regulator. Sensor kinases contain an input domain, activation of which leads to the autophosphorylation of a conserved histidine residue following ATP hydrolysis. This phosphoryl group is subsequently transferred to a conserved aspartate residue on the receiver domain of a response regulator. Phosphorylation of the response

regulator causes a conformational change within this protein, promoting its activity, usually as a transcriptional regulator. The incorporation of one or more transmembrane domains in the sensor kinase molecule allows periplasmic localisation of the sensing domain and sensing of external stimuli, although sensor kinases are often cytoplasmic. Characterised examples of sensor kinases respond to diverse signals. The PhoP-PhoQ system regulates virulence and stress response in *E. coli*, and the sensor kinase PhoQ senses divalent cations (Cheung et al., 2008). The sensor kinase CitA from *Klebsiella pneumonia* regulates the transport and anaerobic metabolism of citrate in response to its extracellular concentration, and directly binds citrate (Sevvana et al., 2008). Oxygen can also be sensed by binding to heme groups within proteins, as seen for the DosT histidine kinase (Podust et al., 2008).

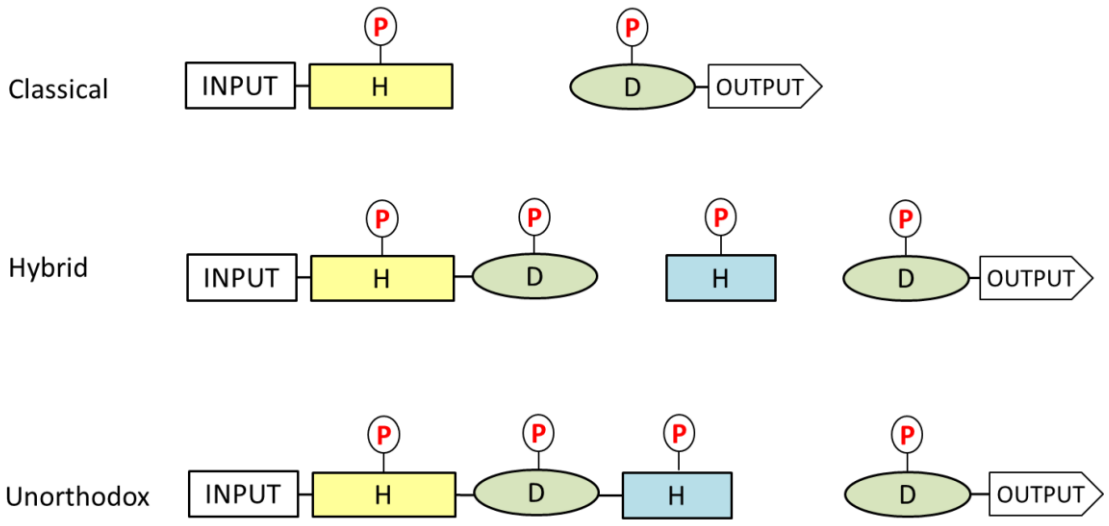


Figure 1.32 Canonical two component systems
Classical, hybrid and unorthodox two component systems. Response regulators are shown, each with input domains (left). Phosphate groups are indicated by P, conserved histidine residues by H, and receiver domains with conserved aspartate residues by (green oval, D). P(Hpt) are shown in blue. The differences between each type of system is outlined in the text. Response regulators are shown with output domains (right).

Adaptations to the classical two component systems have resulted in complex phosphorelay systems, where hybrid histidine kinase molecules autophosphorylate and transfer the phosphoryl group to an internal receiver domain (Figure 1.32). Since phosphotransfer must occur subsequently from histidine to aspartate, a shuttle molecule known as histidine phosphotransfer domain (Hpt) is required to shuttle the signal from hybrid sensors to the cognate response regulator. In unorthodox sensor kinases, this Hpt domain is fused to the sensor kinase protein (Figure 1.32). These phosphorelay pathways allow fine-tuning of responses, allowing many sensors to converge into the activity of a single response regulator, or the divergence of signal sensing from one sensor kinase into the activity of several output domains, resulting in complex, multicomponent signalling pathways.

An archetypal two-component system is the *E. coli* EnvZ-OmpR system, involved in osmoregulation *in E. coli*. This system responds to osmotic change and regulates the expression of outer membrane porins, OmpF and OmpC. Upon changes in osmolality, the inner membrane sensor kinase EnvZ is activated and autophosphorylated at a conserved histidine residue. This phosphate is transferred to a conserved aspartate residue on OmpR, a cytoplasmic response regulator and transcription factor. OmpR-P is then able to bind upstream regions of *ompF* and *ompC* promoters, activating their transcription (Comeau et al., 1985). Examples of two component systems in bacterial virulence include the PhoP-PhoQ system, which is a master regulator of virulence in *Salmonella* (Groisman, 2001). The periplasmic concentration of Ca^{2+} and Mg^{2+} is detected by the membrane sensor kinase PhoQ, and results in PhoP autophosphorylation. PhoP-P is able to act as a transcriptional regulator, activating the expression of genes involved in Mg^{2+} uptake and reducing the Mg^{2+} requirement of the cell. Since the ionic concentration of the phagosome is low, it is thought that the PhoP-PhoQ system has evolved to detect internalisation of *Salmonella* by eukaryotic cells (Groisman, 2001). Salmonella encodes a T3SS which functions only inside eukaryotic cells. The expression of this system depends on a second two component system, SsrB/SpiR. PhoPQ controls intramacrophage expression of this

T3SS by directly regulating the transcription of the response regulator SsrB, and post translationally controlling SpiR (Bijlsma and Groisman, 2005).

Specifically within the *P. aeruginosa* GacS/GacA system, GacS is an unorthodox sensor kinase, composed of an input domain and a phosphotransferase domain, and has two transmembrane domains (Figure 1.33). The phosphotransfer domain is paired with a response regulator protein, GacA. The first evidence that GacS and GacA form a TCS came from a genetic study in *Pseudomonas syringae*, which showed that GacA was important for lesion formation and production of extracellular products while showing similarity to known response regulators (Rich et al., 1994). Work on the homologous BarA/UvrY system in *E. coli* confirmed that BarA was able to auto phosphorylate, and pass on this phosphoryl group to UvrY, but not to other response regulators (Pernestig et al., 2001). Direct transfer of a labelled phosphate from GacS to GacA has since been demonstrated in vitro (Goodman et al., 2009). Once phosphorylated, the GacA response regulator is able to bind to a conserved upstream element, the predicted GacA box ($TGTAAGN_6CTTACA$) in the promoter region of GacA targets (Kulkarni et al., 2006).

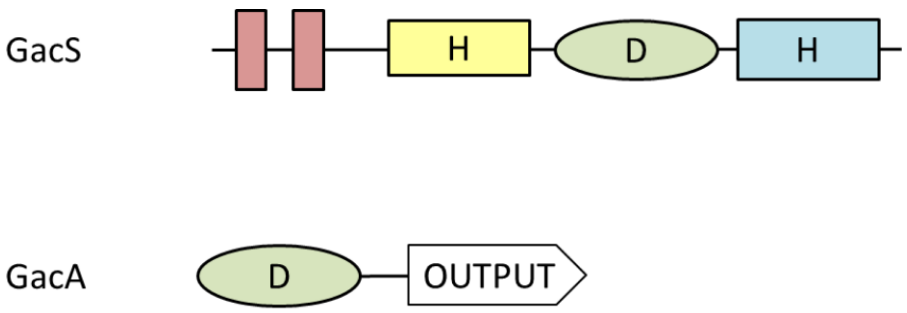


Figure 1.33 The GacS /GacA Two Component System
The sensor kinase (GacS) and response regulator (GacA) are shown. GacS contains two transmembrane domains (red), a conserved histidine (H, yellow), receiver domains with conserved aspartate residues (D, green) and a Hpt domain (Blue) required for phosphotransfer. The GacA response regulator has a conserved aspartate (D, green), which receives a phosphate group from GacS, which modulates the activity of the output domain.

In *P. aeruginosa*, the targets of the phosphorylated GacA response regulator have been shown to be exclusively *rsmY* and *rsmZ,* via binding to conserved GacA boxes (Brencic et al., 2009). Due to the fact that the genes regulated in a *gacA* microarray showed a near complete overlap with those regulated in the microarray of an *rsmYZ* mutant, the authors suggest that the regulon of GacA is dependent on RsmYZ alone, thus RsmY and RsmZ are the sole targets of GacA. This was confirmed using a genome wide DNA-protein interaction analysis where only two genomic regions, located upstream of *rsmY* and *rsmZ* open reading frames, interacted with GacA (Brencic et al., 2009).

1.9.6 Additional sensor kinases RetS and LadS

In *P. aeruginosa*, a higher level of control on the Gac/Rsm pathway is mediated by two proteins of the sensor kinases hybrid family, known as RetS and LadS, which are not conserved in other Gac/Rsm pathways. LadS and RetS are both members of the hybrid family of sensor kinases, shown in Figure 1.34, and share a very similar domain architecture. The N terminus of both proteins contains 7-transmembrane and 7-transmembrane associated extracellular domains (Anantharaman and Aravind, 2003). These are followed by characteristic histidine kinase domain characteristic of sensor kinases. The C termini of LadS and RetS contain one or two fused response regulator domains, respectively (Ventre et al., 2006). There are no genes for cognate response regulators encoded in the proximity of either *retS* or *ladS*, so they are termed orphan sensor kinases, indicating that their activity is mediated via signal transduction integrated into another pathway or through orphan response regulators encoded elsewhere on the chromosome (Goodman et al., 2009; Ventre et al., 2006). It is has become clear that the latter scenario is unlikely due to the evidence supporting the integration of RetS and LadS signals into the Gac/Rsm cascade, as described below.

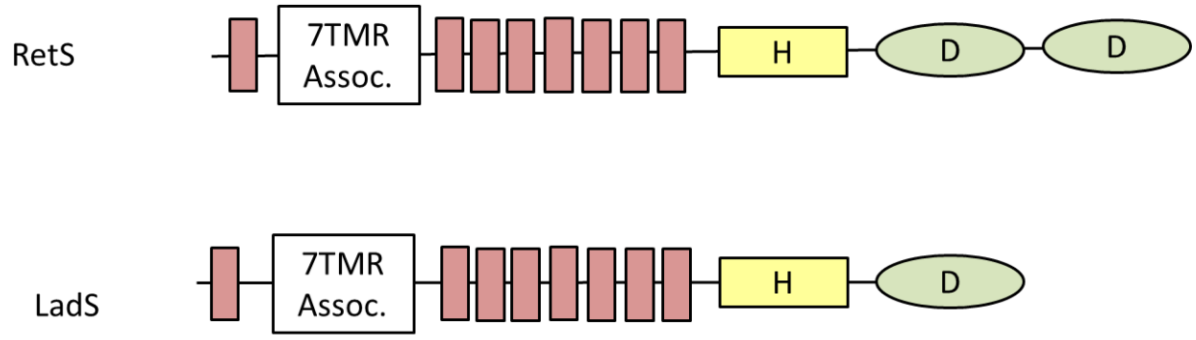


Figure 1.34 RetS and LadS: Additional Sensor kinases influencing the Gac/Rsm cascade.
The RetS and LadS sensor kinase each have multiple transmembrane domains (red) and a 7 transmembrane associated domain (white rectangle). RetS has one conserved Histidine residue, followed by two receiver domains with conserved aspartate residues. LadS has a similar organisation, but has only one receiver domain.

RetS was discovered in a transposon mutagenesis screen of 39 genes encoding predicted response regulators (Goodman et al., 2004). Disruption of a gene encoding what became known as RetS caused a hyperbiofilm phenotype, as well as decreased cytotoxicity shown by reduced levels of lactate dehydrogenase released from Chinese hamster ovary cells. This was due to a reduction in expression of the T3SS by measuring the activity of a *lacZ* fusion to a known T3SS toxin gene, *exoS*. A *retS* mutant was also attenuated in a mouse model of chronic disease (Goodman et al., 2004). The discovery of RetS was concurrently reported by Kasmierczack and colleagues, with RetS being named RtsM in their work. They reported that RtsM was necessary for expression of the T3SS, and identified RtsM as being a two-component signalling protein (Laskowski et al., 2004). *retS* mutants were shown to have delayed disease development and altered pathology in a murine model of corneal infection, which coincided with loss of ExsA-regulated T3SS secretion, reduced epithelial adherence and invasion, and reduced intracellular survival (Zolfaghar et al., 2005).

To comprehensively characterise the effect of *retS* deletion Goodman and colleagues performed a microarray, comparing global gene expression in a wild type *P. aeruginosa* PAK strain to that of a *retS* deletion mutant. Of the 5678 genes included in the array, 397 were significantly altered by the

retS deletion, including many virulence factors (Goodman et al., 2004). Genes which were downregulated by *retS* deletion included all 40 structural and regulatory genes of the T3SS, eight genes encoding components of the *xcp* T2SS machinery, as well as *lipA* and *toxA*, toxins secreted by the T2SS (section 1.6.3). Many genes associated with the expression of type IV pili were also downregulated under these conditions. Upregulated genes included exopolysaccharide biosynthetic operons, responsible for the production of biofilm matrix components, including both *pel* and *psl* operons (section 1.4). The involvement of the *psl* operon in the *retS* mediated biofilm phenotype was confirmed by the observation that biofilm formation was drastically reduced in a PAK Δ*retS* Δ*psl* mutant. Given these observations, the name Regulator of exopolysaccharide and Type 3 Secretion (RetS) was given. Following the discovery of the T6SS it was noticed that the H1-T6SS was also upregulated in a *retS* mutant (Mougous et al., 2006). The effect of *retS* on the biofilm, T3SS, and T6SS phenotypes of PAK are shown in Figure 1.35.

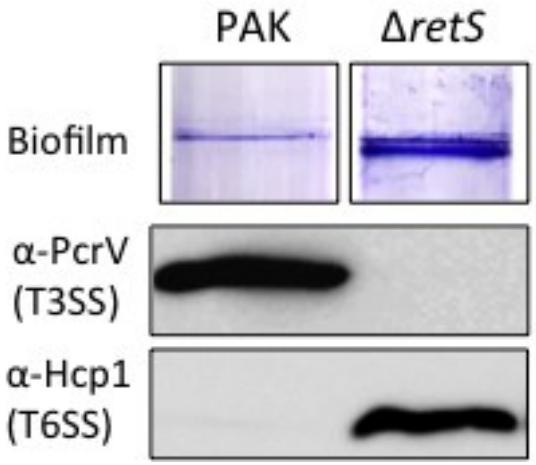


Figure 1.35 Phenotypes of a PAK Δ*retS* strain
The effect of *retS* deletion on PAK biofilm formation, T3SS and T6SS are shown. Crystal violet solution is used to stain biofilm biomass in glass test tubes. Antibodies directed against the tip of the T3SS needle (anti – PcrV) and against the T6SS tail tube (anti Hcp1) are used as reporters for T3SS and T6SS activity respectively. Images and blots were produced in our laboratory by Joana Moscoso.

A transposon mutagenesis strategy was used to screen for suppressors of hyperadhesion and of T3SS repression mediated by *retS* deletion. Goodman et al (2004) selected transposon mutants in the *retS* background which were unable to adhere to the walls of culture flasks, but which exhibited

blue colonies on X-gal plates through the activation of an *exoS-lacZ* fusion. Their screen yielded four insertion mutants in the promoter of *gacS*, seven insertions in the promoter of *gacA* and two located within the *rsmZ* gene, strongly suggesting that the RetS sensor exerts its effect by incorporation into the Gac/Rsm pathway (Goodman et al., 2004). A further suppressor mutation mapped to PA4332, which encodes the *sadC* gene. SadC is involved in production of c-di-GMP, which links the biofilm deficient phenotype of a *sadC* mutation to the Gac/Rsm cascade (Merritt et al., 2007) (section 1.8.4), and may also play a role in the other c-di-GMP responsive phenotypes dependent on this pathway (Moscoso et al., 2011).

A second hybrid sensor kinase, known as LadS, has also been shown to be important in the switch between chronic and acute lifestyles of *P. aeruginosa*. This loss of adherence sensor was discovered in a transposon screen for strains unable to form biofilm in the absence of type IV pili (Ventre et al., 2006). PAKΔ*ladS* has a biofilm deficient phenotype, the opposite of a PAKΔ*retS* mutant. Overexpression of LadS leads to thick bacterial aggregates associated with exopolysaccharide over production. As with RetS, the LadS biofilm phenotype involves the *pel* operon as the activity of a *pel-lacZ* fusion is reduced four fold in a PAK Δ*ladS* strain compared to the wild type parent (Ventre et al., 2006). In contrast to RetS, LadS is a positive regulator of biofilm formation.

In addition to regulating biofilm formation, LadS also regulates the T3SS. Consistent with the inverse regulation by RetS and LadS on biofilm formation, these two sensors also inversely regulate the T3SS, which LadS represses (Ventre et al., 2006). This is shown by the increase in the activity of an *exoS*-lacZ fusion in PAKΔl*adS*, and additionally an increased abundance of the secreted toxin ExoS in the supernatant of this *ladS* mutant strain (Ventre et al., 2006). In terms of cytotoxicity a *ladS* deleted strain is hyper-cytotoxic, correlating with the data collected on the activity of the T3SS. It is interesting to know that the PA14 strain of *P. aeruginosa* is a spontaneous mutant of *ladS*, displaying

reduced levels of biofilm formation, and has increased cytotoxicity towards eukaryotic cells (Mikkelsen et al., 2011a).

The LadS sensor has also been implicated in the Gac/Rsm cascade. The promoter of *rsmZ* was fused to a *lacZ* reporter, and the activity of this promoter analysed in strains carrying deletions in components of the Gac/Rsm cascade (*retS*, *ladS*, *gacS*, and *rsmA*), as shown in Figure 1.36. Deletion of *retS* led to a significant increase in the expression of the *rsmZ-lacZ* fusion, which is consistent with the observations made by Goodman et al. (2004) (Ventre et al., 2006). Deletion of *ladS* abolished the activity of the *rsmZ-lacZ* fusion, as does deletion of g*acS* and *gacA*. This incorporates LadS into the Gac/Rsm cascade, and also provides a molecular explanation for the effects on biofilm formation and T3SS (Ventre et al., 2006).

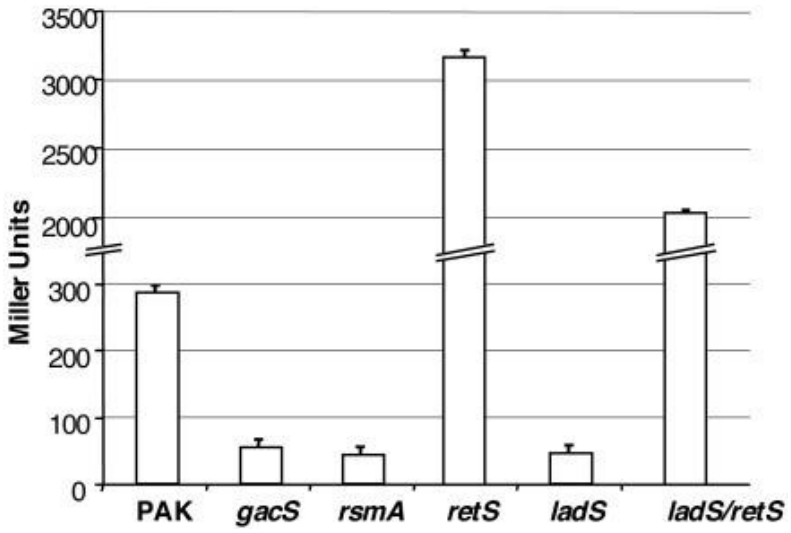


Figure 1.36 *rsmZ-lacZ* fusion activity in PAK and mutants of the Gac/Rsm regulatory cascade.
The activity of the *rsmZ-lacZ* fusion was measured in different PAK backgrounds, as indicated below the graph. Levels of β-galactosidase were measured in cultures of each strain. Due to the high levels in a retS mutant, the y axis scale is broken between 300 and 2000 Miller units. Reproduced from (Ventre et al., 2006).

Comparison of the microarray data from a *P. aeruginosa* PAK *ΔretS* mutant with PAK *ΔladS* reveals that a substantial amount of genes are reciprocally regulated in the two data sets, but also that a

number of genes are regulated independently by both proteins. Of the 79 genes significantly affected by deletion of *ladS*, 49% were oppositely regulated by deletion of *retS*. Similarly, although only 27% of the genes affected by deletion of *retS* were also affected in the *ladS* microarray, 91% of these were reciprocally regulated (Ventre et al., 2006).

1.9.7 Signalling and Sensing by the RetS/LadS sensors

The precise nature of the environmental signals activating the RetS, LadS and GacS sensor kinases are not known, but significant advances have been made in deciphering the mechanism of signalling and sensing in this cascade. Following from their work identifying the RetS sensor, Goodman and colleagues further characterised the interaction of RetS with GacS. Following incubation of GacS with radiolabeled ATP, autophosphorylated GacS was observed by SDS-PAGE and autoradiography. Phosphotransfer from GacS to GacA was shown, since an accumulation of phosphorylated GacA was observed after five minutes of incubation of GacA with radiolabelled GacS (Goodman et al., 2009). A direct interaction between RetS and GacS was shown by co-purification experiments, and two hybrid analysis showed that the core kinase domains of GacS and RetS were sufficient for their interaction (Goodman et al., 2009). Incubation of RetS and GacS with radiolabelled ATP leads to a significant decrease in the autophosphorylation of GacS, in a manner that did not require any of the classical phosphorelay domains of RetS, or result in phosphorylation of RetS. The authors conclude that RetS regulates its downstream targets by a novel mechanism in which it forms heterodimers by interacting with GacS, blocking its autophosphorylation independent of the conserved RetS phosphorelay residues. This is supported by the fact that mutation of the conserved RetS phosphorelay domains does not affect the levels of *rsmZ-lacZ* fusions (Goodman et al., 2009).

Advances were made in the understanding of RetS signalling by the determination of the crystal structure of the RetS periplasmic sensing domain (RetSp) (Jing et al., 2010; Vincent et al., 2010). The

sensing domain crystal structure of RetS has been solved, and predictions of a β-sandwich fold, similar to a family of carbohydrate binding domains, were confirmed. The proposed carbohydrate binding domain found in RetSp displays an additional alpha helix which is located directly above the β-sandwich fold (Jing et al., 2010). Despite being able to map a predicted ligand binding site through comparison with known carbohydrate binding proteins the natural ligand binding to RetSp is not known, and its interactions with RetSp could not be assessed.

Additional insights into RetS sensing have been obtained by observations that the RetSp sensing domain is able to form multimeric complexes. A dimer was observed in the crystal environment for RetSp, which was formed by interactions between β-sheet regions of the two monomers. The formation of a RetSp dimer was confirmed by crosslinking experiments (Jing et al., 2010). In their work, Jing et al (2010) propose that in the absence of a ligand at the binding site, RetS is likely to dimerise, thus RetS-GacS interactions are prevented. Upon ligand binding, RetS dimers are disrupted and RetS-GacS interactions are preferred, blocking the downstream phosphorelay of GacS (Goodman et al., 2009).

The dimer observed by Jing et al. (2010) was also observed by Vincent et al. (2010), who observed further multimeric forms of RetSp. A second dimeric form was observed which constituted a domain-swap between two RetSp monomers, where the final three β-strands are exchanged between monomers. A tetrameric form was also observed in the crystal environment which was shown to be present in small amounts under physiological solution conditions (Vincent et al., 2010).

The analysis of the RetSp crystal structure also revealed the presence of a second putative substrate-binding site for an unknown ligand. It is proposed due to the conservation of aromatic residues on the surface of RetSp. This binding site is located at the opposite face of the carbohydrate binding domain, but is located at the RetSp dimerization interface (Vincent et al., 2010). In an update to the

previous model, it is proposed that binding of an unknown molecule at this dimer interface may have the effect of destabilising RetS homodimers and favouring RetS-GacS heterodimers, and the subsequent blocking of the phosphotransfer, as shown in Figure 1.37. This model takes into account that the alpha helix found above the β-sandwich fold may block the carbohydrate binding site, which may not be accessible, but may become accessible following conformational changes.

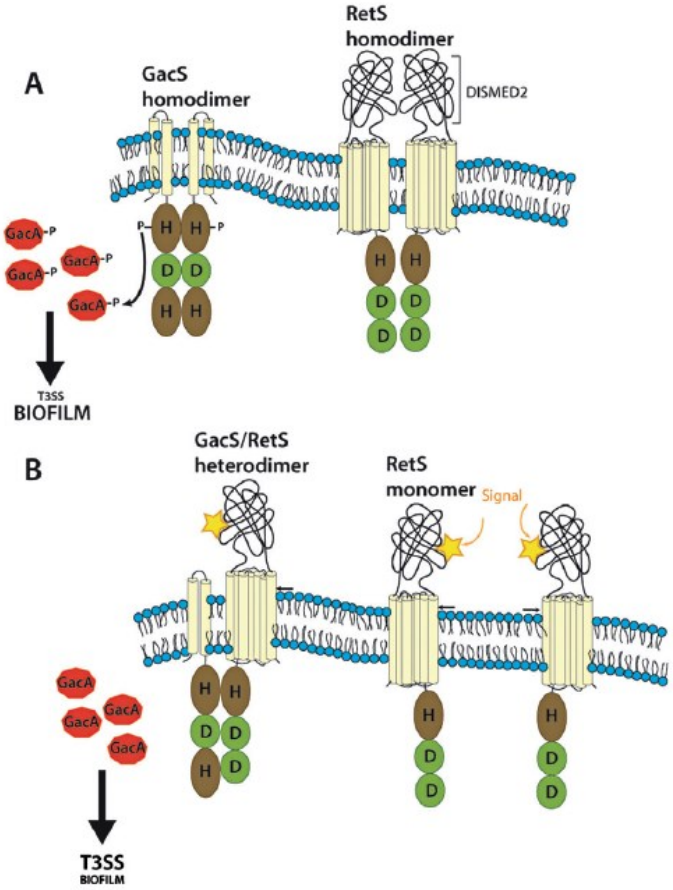


Figure 1.37 A model for RetS signalling mechanism

Membrane bound GacS and RetS sensors are shown with histidine kinase (brown) and response regulator receiver domains (green). A. In the absence of an activating signal, RetS is found as a homodimer, leading to phosphorylated GacS and subsequent phosphorylation of GacA (red), resulting in increased biofilm formation and repression of the T3SS. B. An unknown activating signal (yellow star) binds to the second ligand binding site within the RetS sensing domain, disrupting RetS homodimers and promoting RetS/GacS heterodimers, which prevents GacS and subsequent GacA phosphorylation. The resulting phenotype is an increase in T3SS activity, and repression of biofilm formation, the opposite of A. Reproduced from (Vincent et al., 2010) with permission.

Although the structure of the LadS sensing domain has not been solved, it has a similar domain architecture and 35% sequence identity to RetS, and also exerts its effects via the Gac/Rsm pathway.

Vincent et al. (2010) were able to model the structure of the LadS sensing domain using the solved structure of RetSp. The model shows that LadS may form a similar overall fold, and that the proposed carbohydrate binding motif is conserved. The second binding site at the dimer interface is lacking in the LadSp model. Due to these differences, the mechanism of LadS signalling is likely not the same as that proposed for RetS (Vincent et al., 2010). Further investigations into the mechanism of LadS may provide insights into the inverse effect of these two sensors on the Gac/Rsm cascade and resultant phenotypes. Additionally the characterisation of the exact molecules binding to the RetS and LadS ligand binding sites will complete our understanding of this mechanism.

1.9.8 Additional components influencing the cascade

Additional components have been shown to modulate the RetS/LadS/Gac/Rsm pathway and may be important in the regulatory cascade. The HptB signalling pathway controls biofilm and T3SS, and intersects at the GacA response regulator, exclusively regulating *rsmY* expression (Bordi et al., 2010). Additionally, a further hybrid sensor kinase encoded by PA1611 directly interacts with RetS and controls the expression of genes associated with acute and chronic infections (Kong et al., 2013). This is only the second observation of heterodimerisation of sensors used for regulatory control. Expression of PA1161 represses T3SS and promotes biofilm formation, via *rsmY* and *rsmZ*. The multimerisation state of RetS may therefore be modulated by PA1611, affecting its ability to interact with GacS (Kong et al., 2013). These examples show that regulation via the Gac/Rsm cascade is highly complex, with many components and multiple inputs influencing the outcome of the regulatory cascade.

1.10 Conclusions

P. aeruginosa is a highly versatile organism, capable of growth in most natural environments. It is also prevalent in man-made environments, and is a successful nosocomial pathogen. These infections range from self-limiting otitis media, to long-term infection of the CF lung, associated with high mortality.

The ability of *P. aeruginosa* to cause such devastating infections is due to its large arsenal of cell-associated and secreted virulence factors. *P. aeruginosa* can form either acute or chronic infection, which mirror closely the environmental lifestyles of this organism, being either planktonic or sessile. The secretome of *P. aeruginosa* is large, and it possesses five out of six of the characterised Gram-negative secretion systems. The secreted effectors of these systems are largely studied, but the newly discovered T6SS clusters of *P. aeruginosa* remain only partly characterised.

The success of *P. aeruginosa* as a pathogen is associated with its ability to tightly control the expression of potent virulence factors. Many regulatory mechanisms are encoded and allow regulation of different virulence factors. These systems are often intertwined, affecting each other, resulting in highly complex regulatory cascades. *P. aeruginosa* is able to sense and respond to many environmental signals and tailor its response specifically to different environmental stimuli. While the mechanisms of these regulatory systems are generally well understood, the nature of specific stimuli is often unknown.

Chapter 2 Materials and Methods

2.1 Bacterial strains, culture conditions and reagents

2.1.1 Bacterial strains

Pseudomonas aeruginosa PAK or *P. aeruginosa* PA14 were used as model organisms in this study, with PAO1 occasionally used for comparison. Isogenic deletion mutants and other genetic modifications were generated in these backgrounds. A full list of *P. aeruginosa* strains used and generated in this work is presented in Table 6. *P. aeruginosa* strains were routinely grown in tryptone soya broth (Oxoid) with agitation at 37 °C, or on LB agar (Merck) plates incubated at 37 °C. *P. aeruginosa* strains were isolated on *Pseudomonas* Isolation Agar (BD Difco). *E. coli* strains and plasmids used in this work are listed in Table 7. *E. coli* strains were routinely grown in Luria Broth (Merck) with shaking at 37 °C or on LB agar (Merck) plates incubated at 37 °C.

2.1.2 Planktonic cultures

Planktonic growth curves were produced by following the growth of bacterial cultures initially inoculated at an OD_{600nm} of 0.1 in sterile glass Erlenmeyer flasks. Growth medium was supplemented with antibiotics or other reagents as required, and incubated with agitation. Growth was analysed by taking measurements of the optical density (OD_{600nm}) at 1-2 h intervals using a handheld spectrophotometer and plotting $\log_{10} OD_{600nm}$ against time.

Strain Name	Characteristics	Source/Ref.
PA14	Wild type *P. aeruginosa* PA14	Lab collection
PA14-DP	PA14 carrying divergent arabinose inducible pBAD promoters upstream of *hsiA2* and *hcp2*, interspaced by a single copy of the araC regulator.	This study
PA14-DP ΔH2	PA14-DP strain carrying a deletion of H2-T6SS from mid-*hsiA2* to mid-*hcp2*	This study
PA14-DP vgrG14-v5	PA14-DP producing a V5-Hisx6 tagged version of VgrG14 (PA14_43080) following the chromosomal introduction of the required sequence.	This study
PA14-DP rhsG14-v5	PA14-DP producing a V5-Hisx6 tagged version of Rhs14 (PA14_43100) following the chromosomal introduction of the required sequence.	This study
PA14::*pscC*	PA14 carrying a transposon insertion in PA14_42350 (*pscC*)	Liberati *et al*. 2006
PA14 Δ*pscC*	A clean deletion of PA14_43250	This study
PA14 Δ*pscC* ΔH2	A clean deletion of PA14_43250 and deletion of the H2-T6SS cluster	This study
PA14-DP Δstp2 vgrG14-v5	PA14-DP producing a V5-Hisx6 tagged version of VgrG14 in a Δ*stp2* background	This study
PA14-DPΔstp2 rhsG14-v5	PA14-DP producing a V5-Hisx6 tagged version of Rhs14 in a Δ*stp2* background	This study
PAK	Wild type *P. aeruginosa* PAK	Lab collection
PAK	Wild-type *P. aeruginosa*	Laboratory collection
PAK ΔH1-T6SS	In-frame deletion of H1-T6SS from mid HsiA1 (PA0082) to mid VgrG1a (PA0091) in *Pseudomonas aeruginosa* PAK wild type strain	This study
PAK Δ*retS*	In-frame deletion of *retS* (PA4856) in *P. aeruginosa* PAK wild-type strain	Goodman *et al.* (2004)
PAK Δ*ladS*	In-frame deletion of *ladS* (PA3974) in PAK	Ventre *et al.* (2006)
PAK Δ*gacS*	In-frame deletion of *gacS* (PA0928) in PAK	Bordi *et al.* (2010)
PAK Δ*gacA*	In-frame deletion of *gacA* (PA2586) in PAK	Bordi *et al.* (2010)
PAK Δ*rsmYZ*	In-frame deletions of *rsmY* (PA0527.1) and *rsmZ* (PA3621.1) in PAK	Bordi *et al.* (2010)
PAK::pCTX-PA0082-lacZ	PAK PA0082 Transcriptional fusion	Brencic & Lory (2009)
PAK ΔrsmA ::pCTX-PA0082-lacZ	PAK Δ*rsmA* PA0082 Transcriptional fusion	Brencic & Lory (2009)
PAK::pUC18-mini-Tn7-PA0082-lacZ20	PAK PA0082 Translational fusion	Brencic & Lory (2009)
PAK ΔrsmA ::pUC18-mini-Tn7-PA0082-lacZ20	PAK Δ*rsmA* PA0082 Translational fusion	Brencic & Lory (2009)
PAO1	Wild type *P. aeruginosa* PAO1	Lab collection

Table 6 *P. aeruginosa* strains used in this study.

Name	Characteristics	Source
E. coli		
One-shot® TOP10	Used for interbacterial competition assay.	Invitrogen
CC118 λpir	Host strain for pKNG101 replication	Lab collection
1047	Carrying conjugative plasmid pRK2013, helper strain for conjugation	Figurski & Helinski (1979)
BL21 DE3	Host strain for expression of pET vectors	Lab collection
Plasmids		
pSB302	Based on pMP220 containing 211 bp region of *exoT* promoter-*lacZ* reporter.	Bleves *et al.* (2005)
pSB305	Based on pMP220 containing 219 bp region of *pcrD* promoter-*lacZ* reporter	Bleves *et al*. (2005)
pCdrA::gfp^{S}	pUCP22Not-PcdrA-RBS-CDS-RNaseIII-gfp(Mut3)-T0-1, Amp^{r}Gm^{r}	Rybtke *et al*. (2012)
pCR2.1-*araC*pBAD	cloning vector containing the pBAD araC region from the arabinose inducible pJN105 vector	Mikkelsen et al. (2013)
pCR2.1-pBAD*araC*pBAD	cloning vector containing divergent pBAD promoter regions interspaced by a single copy of the *araC* gene.	This study
pCR2.1 v5-hisx6	Cloning vector containing the v5-hisx6 sequence from pDEST42	This study
pET28a *hsiB2*	Plasmid for over expression of his tagged HsiB2	This study
pDEST42-*hcpA*	Plasmid for over expression of PA1512 (HcpA), identical to Hcp2.	This study

Table 7 *E. coli* strains and plasmids used in this study

The response to antibiotics by *P. aeruginosa* during planktonic growth was assessed by adding various concentrations of antibiotics to cultures at the start of growth experiments. Kanamycin, gentamycin, and tobramycin (Sigma) were prepared in water and filter sterilised. Tetracycline (Sigma) was dissolved in ethanol. Antibiotic stocks were stored at -20 °C until use.

Growth media was supplemented with arabinose (Sigma) for induction of gene expression in strains carrying genes under the control of pBAD promoters. Arabinose was prepared as a 20% w/v stock, filter sterilised, and used routinely at a concentration of 2% (w/v) unless otherwise stated.

2.2 Bioinformatics and computational approaches

P. aeruginosa DNA and protein sequences were obtained from the *Pseudomonas* genome database at pseudomonas.com (Winsor et al., 2011). Geneious pro 5.4.5 software (Biomatters Ltd) was used for routine sequence analysis and to design cloning strategies. Sequence alignments were performed using ClustalW (Larkin et al., 2007), and similarity searches performed using NCBI basic nucleotide blast or protein blast.

Protein sequences were analysed for conserved domains using ExPASy prosite (Artimo et al., 2012), or using the pfam database (Punta et al., 2012). Protein secondary structure predictions were made using psipred (Buchan et al., 2010). The Phyre 2.0 server was used to predict protein structural homology (Kelley and Sternberg, 2009). Phylogenetic trees were built using the 'one-click' method at phylogeny.fr (Dereeper et al., 2008).

2.3 PCR and cloning

2.3.1 Purification of *P. aeruginosa* gDNA

Genomic DNA from *P. aeruginosa* strains was prepared using a PureLink genomic DNA kit (Invitrogen). 1 ml of *P. aeruginosa* overnight culture was used for purification, following the manufacturers guidelines, and gDNA was eluted in 100 µl of molecular biology grade water. gDNA was stored at -20 °C and used as a template for cloning reactions.

2.3.2 Cloning PCR

Cloning of genes and genetic regions from *P. aeruginosa* genomic DNA was performed using high fidelity DNA polymerases. The Expand High Fidelity Plus PCR system (Roche) was used for generating PCR products with overhanging residues, and KOD DNA polymerase (Novagen) was used for the generation of blunt ended PCR products. Reactions were performed as per manufacturer's instructions, using dNTP master mix (Eurogentec) and the addition of betaine (Sigma) at a final concentration of 1 M to disrupt the formation of secondary structures (Henke et al., 1997). Custom primers were obtained from Eurogentec and are listed in Table 8.

Table 8 Cloning primers used in this project

ID	Primer Purpose	Sequence (5'-3')
Divergent Promoter Engineering		
OAL771	5' pBAD region duplication	GGAATTCCATATGTCAGAGAAGAAACCAATTGTCCATATTG
OAL772	3' pBAD region duplication	GGAATTCCATATGAAAACGGGTATGGAGAAACAGTAGAG
OAL601	5' Upstream of H2-T6SS Promoter	GGGCCCGATCGGTACGTTCTCGT
OAL586	3' Upstream of H2-T6SS Promoter	GCAGCTAGCTTTCATATGGTTAAGATATTCATTGGCGCAC
OAL587	5' Downstream H2-T6SS Promoter	AACCATATGAAAGCTAGCTGCGAGGGGTGGTCCAACC
OAL588	3' Downstream H2-T6SS Promoter	ACTAGTTCGTCGGAGCCGGAG
OAL589	5' OUT H2-T6SS Promoter	CTTCAGCCACCAGGCG
OAL590	3' OUT H2-T6SS Promoter	CTGCCTGGCGCGGG
OAL591	pBAD promoter screening primer	CGCGTAACAAAAGTGTC
Cloning of V5-hisx6 region		
OAL599	5' amplify *V5-hisx6* encoding region	GCTAGCCCATTCGAAGCTTGAAGGTAAGCCTAT
OAL600	5' amplify *V5-hisx6* encoding region	GCTAGCTCAATGGTGATGGTGATGATGACC
Mutation of *vgrG14* (PA14_43080) stop codon		
OAL602	5' Upstream *vgrG14* stop codon	CACCAACGCCACGACACC
OAL597	3' Upstream *vgrG14* stop codon	TTTGCTAGCTTTGGGAGGTCACAGGCACCGTC
OAL598	5' Downstream *vgrG14* stop codon	CCAAAGCTAGCAAACGCCCTCTCCCCGCG
OAL603	3' Downstream *vgrG14* stop codon	CTTGCACTTCTCGCACTC
OAL808	5' OUT *vgrG14* stop codon	GCTACAACGAGCTGCGCATCGAGG
OAL809	3' OUT *vgrG14* stop codon	GCAGGTTGTCGATAGCGGTAGTCG
PCR screen for presence of *arr*		
OAL1085	5' *arr* probe (PA2818)	AGCGCATCACCCCCAGCAAC
OAL1086	3' *arr* probe (PA2818)	CGCCAAGTGCGAGCCACTGA
Mutation of *rhs14* stop codon		
OAL1136	5' Upstream *rhs14* stop codon	AATAAAACTCGCTGTCCCGAAGCATTAG
OAL1137	3' Upstream *rhs14* stop codon	CATGAGTTTTCTGCTAGCGGGTCTATTACTTCGAAGTCTATTAGGTTCTTGTTG
OAL1138	5' Downstream *rhs14* stop codon	GAAGTAATAGACCCGCTAGCAGAAAACTCATGAAAACCATTTATAACTTCAAACAGCG
OAL1139	3' Downstream *rhs14* stop codon	TTCATTTGCTTCTGTCTTTTGGTTTTTTATATAACC
OAL1160	5' OUT *rhs14* stop codon	CGGGACGCTACCTGACCCCC
OAL1161	3' OUT *rhs14* stop codon	GATGCGTGAACTTCCTGAGCCAGG

***hsiB2* cloning into pET28a**		
OAL1171	5’ hsiB2	GCCATATGGCCAAAGAAGGCTCGGTAGCC
OAL1172	3’ hsiB2	GCCTCGAGGGCGTCCTGGGAGGGGGC
deletion of *pscC*		
OAL1389	5’ Upstream *pscC*	ACGCAACCTGTGCCAGGCACAGG
OAL1399	3’ Upstream *pscC*	CTAATTCCCGCGGCGCATCAGGGACGCC
OAL1400	5’ Downstream *pscC*	ATGCGCCGCGGGAATTAGCATGGCCTGGAAGATCC
OAL1401	3’ Downstream *pscC*	TAGGCGCGCACCCTCGCC
OAL1484	5’ OUT *pscC*	GAGGTCCTCGAATGCCTCTGG
OAL1485	3’ OUT *pscC*	TTGTCCTCCAGGTAGCCGCC
Deletion of H2-T6SS (mid *hsiA2* to mid *clpV2*)*		
OAL996	5’ Upstream H2-T6SS	GACTGGTTGAAAATCCTGGAAAAC
OAL997	3’ Upstream H2-T6SS	TCAGGCGAACGGCCTCCTGCTGGGCGC
OAL998	5’ Downstream H2-T6SS	AGGAGGCCGTTCGCCTGAGGTGGGTGC
OAL999	3’ Downstream H2-T6SS	CAACACGGTATAGGGGTTGTG
OAL1000	5’OUT H2-T6SS	GAATTGTTAAGATATTCATTGGCGCAC
OAL1001	3’OUT H2-T6SS	TCGAGCAGCAGGGTTCCGCCATCCGCG
Deletion of *stp2*		
OAL346	5’ Upstream *stp2*	CTTCTTCGAAACCTACATGCG
OAL347	3’ Upstream *stp2*	TCATTGGCTGCGTTGCATCAGAGCTGC
OAL348	5’ Downstream *stp2*	ATGCAACGCAGCCAATGAACGAACCGC
OAL349	3’ Downstream *stp2*	CGTGGACGTAGGCCAGAA
OAL350	5’OUT *stp2*	TACCTGTATCTCAACCAGCGC
OAL351	3’OUT *stp2*	CAAGCGGGACAAGATGTTAAT
Vector specific primers		
M13 F	sequencing from pCR2.1	TGTAAAACGACGGCCAGT
M13 R	sequencing from pCR2.1	CAGGAAACAGCTATGACC
RpKN	Screening of pKNG constructs	CATATCACAACGTGCGTGGA
UpKN	Screening of pKNG constructs	CCCTGGATTTCACTGATGAG

Table 8 Cloning primers used in this project. Primer categories indicated with * were designed by Ms. Kailyn Hui

PCR reactions were initiated with a 5 minute 95 °C denaturing step, followed by 20 cycles of denaturing (95 °C, 30 sec), annealing (55 °C, 30 sec, depending on the primers used) and elongation (72 °C, 1min/kb). A final extension step at 72 °C for ten minutes was performed.

PCR products were visualised using 1% TAE-agarose gel electrophoresis (TAE: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) containing SYBR safe DNA gel stain (Invitrogen). 1 µl of 6 x DNA loading dye (NEB) was added to 5 µl of the PCR product prior to loading, and a 1 kb DNA ladder (NEB) was used for molecular weight determination. Correctly amplified PCR fragments were purified using a DNA purification kit (Qiagen) and PCR products were routinely subcloned into the pCR2.1 vector for storage and propagation using the TA cloning kit (Invitrogen).

Subcloning was performed by digesting the required insert and vector using appropriate restriction enzymes and buffers (Roche). Following digestion, products were checked by agarose gel electrophoresis and PCR or gel purified using the kits provided by Qiagen. If required, cut vectors were dephosphorylated using calf intestinal alkaline phosphatase (NEB) for 1 hour at 37 °C. T4 DNA ligase (NEB) was used to ligate insert and vector overnight at 16 °C.

Following ligation plasmids were transformed into the required *E. coli* strain. Cells were previously made competent using a calcium chloride based method. 100 µl of cells were added to the ligation mix and incubated on ice for 30 minutes. Cells were then heat shocked for 2 minutes at 42 °C, and returned to ice. 500 µl of SOC medium (Invitrogen) was added and cells incubated at 37 °C for 1 hour. Cells were then plated onto appropriate selective media and incubated overnight.

2.3.3 Colony PCR

Positive clones obtained following transformation were screened by colony PCR to confirm the presence of the required insert. Taq polymerase (Life Technologies) and dNTP master mix (Eurogentec) were used for colony PCR, and DMSO added to minimise secondary structure formation. Plasmid specific or custom primers (Eurogentec), as listed in Table 3, were used.

An isolated colony of the strain being tested was resuspended in 100 µl of molecular biology grade water. The resuspended colony was lysed by one freeze-thaw cycle of freezing at – 80 °C for ten minutes followed by boiling at 95 °C for ten minutes. The lysate was centrifuged at maximum speed in a benchtop centrifuge to pellet insoluble cell debris. The DNA contained in the supernatant was used as a template for subsequent PCR reactions.

A typical reaction set up contained 5 µl of the colony DNA preparation, 3 µl of Taq buffer, 2 µl of each primer (from a 10 µM stock), 2 µl of dNTPs (from a 20 mM stock), 14.3 µl of molecular biology grade water, 0.7 µl of Taq polymerase and 1 µl of DMSO. PCR reactions were initiated with a 5 minute 95 °C denaturing step, followed by 30 cycles of denaturing (95 °C, 30 sec), annealing (55 °C, 30 sec, depending on the primers used) and elongation (72 °C, 1min/kb). A final extension step at 72 °C for ten minutes was also performed. PCR products were visualised by agarose gel electrophoresis to identify positive clones.

Plasmids from positive clones were purified using a plasmid purification kit (Qiagen). The presence and orientation of inserts was confirmed using restriction digest (Roche). 30 - 100 $ng\mu l^{-1}$ of purified plasmid DNA from positive clones was sent to GATC Biotech (Germany) for sequence confirmation.

A similar approach was used to test DNA from *P. aeruginosa* for genetic changes, however this required two freeze thaw cycles to obtain the required template DNA. All subsequent steps were as described above.

2.4 Genetic manipulation of *P. aeruginosa*

2.4.1. Construction of *P. aeruginosa* deletion mutants

Clean deletion mutants of *P. aeruginosa* genes were generated by amplifying 500 base pairs homologous regions either side of the target gene. These regions were fused by overlap extension PCR, generating a 1 kb mutator fragment, cloned into the pCR2.1 vector and confirmed by sequencing. The mutator fragment was subcloned into the broad host range suicide vector pKNG101 (Sarker and Cornelis, 1997), generating the mutator plasmid, which was transformed into *E. coli* cc118 λ*pir* as previously described.

Mutator plasmids were mobilised into *P. aeruginosa* by three partner conjugation using the required *P. aeruginosa* receiver strain, the *E. coli* cc118 donor strain carrying the mutator plasmid, and the *E. coli* 1047 pRK2013 helper strain. 20 µl aliquots of the donor and helper strain from an overnight culture were spotted together onto an LB agar plate and incubated at 37 °C for 2 hours, and 100 µl of the receiver strain was incubated for 2 hours at 43 °C. After 2 hours, 40 µl of the receiver strain was added to the donor and recipient spot, and incubated for a further 4 hours at 37 °C. Following incubation, the spot was resuspended in 100 µl of LB and plated onto *Pseudomonas* isolation agar supplemented with 2000 $\mu g ml^{-1}$ streptomycin.

Due to the non-replicative nature of pKNG101 in *P. aeruginosa*, selection of colonies by streptomycin resistance indicated integration of the mutator plasmid into the chromosome of the

recipient strain by homologous recombination, which was then confirmed by PCR. The backbone of the mutator plasmid was ejected by plating the strain onto LB agar supplemented with 5% sucrose, since the pKNG101 vector carries the levansucrase gene, conferring sucrose sensitivity. A second recombination event allowed ejection of the vector backbone permitting growth on sucrose plates, and the colonies obtained were either wild type or deletion mutants. This was confirmed by PCR using primers external to the mutation site.

2.4.2 *P. aeruginosa* chromosomal insertions

Insertions of genetic sequences at defined regions of the *P. aeruginosa* chromosome were achieved in a similar way to the deletion of open reading frames as described above. 500 base pair regions were amplified either side of the required location of the insertion. These regions were fused by overlap extension, and suitable restriction sites were incorporated into the overlap region allowing DNA regions to be inserted into the overlap region by restriction digest. The homologous regions and inserted sequences were subcloned into the pKNG suicide vector, and conjugated into *P. aeruginosa* as previously described. Selection of two events of homologous recombination subsequently resulted in reversion to wild type or insertion of the required sequences at the desired chromosomal location. Chromosomal insertions were confirmed using a combination of primers specific to the inserted region and a primer specific to a chromosomal location external to the modified region.

2.5 RNA analysis

2.5.1 RNA extraction and purification

Total RNA from *P. aeruginosa* cells was isolated using an RNeasy kit (Qiagen). Cultures were incubated under required growth conditions, and 2ml of culture at the mid-log growth phase were resuspended in 1 ml of RNAlater for preservation of RNA prior to extraction. Prior to extraction, RNA was thawed on ice, and RNA was extracted from a volume of cells corresponding to 1 OD unit of bacteria. Harvested cells were resuspended in 1 mg/ml lysozyme (Sigma) in water and incubated for 13 minutes at room temperature. RNA extraction was performed using the RNeasy kit (Qiagen) following the suppliers bacterial protocol. Following extraction RNA was purified following the 'purification of total RNA from bacterial lysates' protocol.

Following purification, RNA was treated with Turbo DNAse (Ambion). RNA sample volume was adjusted to 100 µl. 10 µl of Turbo DNase buffer and 1.5 µl of Turbo DNase was added prior to incubation at 37 °C. After 30 minutes an additional 1.5 µl of DNase was added and samples were incubated for a further 30 minutes at 37 °C. The reaction was stopped by adding 20 µl of resuspended DNase inactivation agent for 5 minutes at room temperature with occasional mixing. The inactivation agent was sedimented by centrifugation at 10,000 rpm for 1.5 minutes, and the supernatant containing the RNA was transferred to a fresh tube and stored at -80 °C prior to analysis. Prior to freezing, 10 µl was removed for quality control testing.

2.5.2 RNA quality control

RNA concentration was measured using a NanoDrop spectrophotometer, to ensure a concentration of 20 ngµl^{-1} or higher, and a 260/280 ratio above 1.8. RNA samples were also checked by agarose gel

electrophoresis where bands of 23S and 16S ribosomal RNA, but not genomic DNA, were visible. DNA contamination was also tested using primers known to amplify a short region of genomic DNA, a positive result therefore indicated dsDNA contamination.

2.5.3 cDNA generation

cDNA was generated from RNA using SureScript II RNase H-reverse transcriptase (Invitrogen). 200 ng of RNA was used for each reaction, in a total volume of 10 µl RNA free water. RNA secondary structures were disrupted by heating samples to 65 °C for 5 min, and chilling on ice. Reverse transciptase reactions were set up by adding 4.0 µl first strand buffer, 0.1 M DTT, 1.0 µl dNTPs, 0.5 µl RNA guard (Roche), and 1.0 µl random hexamer oligonucleotides (Amersham), 1.5 µl nuclease free water and 1 µl of the reverse transcriptase. Reverse transcriptase negative controls were also set up. Samples were incubated for 5 minutes at 25 °C, 50 minutes at 42 °C and 15 minutes at 70 °C to generate cDNA. Following reverse transcription, cDNA samples were diluted 1/5 with RNAse free water and stored at -20 °C prior to analysis.

2.5.4 q-RT-PCR

Quantitative reverse transcriptase PCR was performed to measure the relative amount of RNA transcripts in different samples. q-RT-PCR reactions were performed in an ABI 7300 real time PCR system machine using the CYBR green reporter. Primers used were designed to probe for the expression of specific genes, yielding 100 base pair amplicons, and are listed in Table 9.

Reactions were set up in ABI prism 96 well-optical reaction plates. Reverse transcriptase negative controls were included to test for contaminating DNA. In each well 5 µl of cDNA was added to 12.5 µl

of ABI SYBR green PCR master mix (Applied Biosystems), 1.25 µl of each primer and 5.0 µl of nuclease free water. Plates were sealed with ABI prism optical adhesive covers.

40 amplification cycles were performed, each consisting of a 95 °C incubation for 15 seconds and 60 °C incubation for 1 minute. Fluorescence measurements were recorded at each cycle and processed to produce threshold values (Ct) for each sample. All samples were normalised to the reference gene *rpoN*.

ID	Target Gene	Sequence (5'-3')
OAL920	fw-stk2	CCGCTGGTAGCATTGAAGCT
OAL921	rv-stk2	GGCGAACTCGCTATAGAGCAA
OAL922	fw-hsiG2	GTGTTCGCTTCGGTTCTGAAC
OAL923	rv-hsiG2	GTGCTCCTCACCCGCAACT
OAL924	fw-hsiF2	ACTACGGGTTGCCCGATCTC
OAL925	rv-hsiF2	GGTTCGTAAGCCTCGATGAAAC
OAL926	fw-PA14_43090	GCCGTCACCTGCTACCGATA
OAL927	rv-PA14_43090	GATCAGGTAACGGCCGAACA
OAL928	fw-rhs14	GACAAGGACGCCAACATCCT
OAL929	rv-rhs14	CGAAAGTATGCAGCAGTTTCAGTT
OAL511	fw-hsiA2	GGTTGACCTGGGCCCTCTAC
OAL512	rv-hsiA2	GATGGATCTCGACCCAATGC
OAL540	fw-hcp2/A/B/C	CCAAGGTCGAGATCCAGTGGTA
OAL541	rv-hcp2/A/B/C	GTAGTCCTTGATGTCGACGATGAT
OAL542	fw-vgrG14	TCACCCCGGCCCAGAT
OAL543	rv-vgrG14	TCTCGCACTCTTCGCAGAAG
OAL820	*fw-rpoD* (reference gene)*	AGGCCGTGAGCAGGGATAC
OAL821	*rv-rpoD* (reference gene)*	TCCCCATGTCGTTGATCATG
OAL538	fw-vgrG5	GCCCGAAGGGTGAGGAA
OAL539	rv-vgrG5	CTCGCGATCCCAGTGGAAT
OAL536	fw-vgrG2b	GGAGCCGGGAAAGACGTT
OAL537	rv-vgrG2b	AGGCTTCCCCGAACTCGTT
OAL721	fw-pelA*	CCTTCAGCCATCCGTTCTTCT
OAL722	rv-pelA*	TCGCGTACGAAGTCGACCTT
OAL822	fw-tse3*	GGCACGCAATGCCTTGAT
OAL823	rv-tse3*	GCAGATGTCGAAGAAGGTGATG

Table 9 qPCR primers used in this project.
Primers indicated by * were designed by Helga Mikkelsen.

2.6 Protein analysis

2.6.1 Preparation of whole cell lysates

P. aeruginosa whole cell lysates were prepared by harvesting 1 ml of planktonically grown cells of a known optical density, by centrifugation at 14,000 rpm for 1 minute. Cells were resuspended to an optical density of 0.1 OD equivalent unit per 10 µl in SDS gel-loading buffer (45 mM Tris pH 6.8, 0.7% SDS, 0.7 mg/ml sucrose, 3.5% beta-mercaptoethanol, trace bromophenol blue) and analysed by SDS page.

2.6.2 Protein Secretion Assay

Supernatants were separated from cells by centrifugation. Overnight cultures of *P. aeruginosa* strains were subcultured to OD_{600nm} 0.1 and inoculated into 30 ml of tryptone soya broth (Oxoid) in 250 ml glass flasks. Cells were incubated with agitation at 37 °C and samples were taken at various points during growth. Before sampling, the optical density of the culture was noted. Cells were harvested for preparation of whole cell lysates as previously described. For supernatant preparation, 10 ml of culture was pelleted by centrifugation at 4000 rpm at 4 °C for 15 min. The uppermost 7 ml of the supernatant was transferred to a fresh tube, and spun again under the same conditions for 20 min. 1.8 ml of the uppermost supernatant was harvested. 200 µl of trichloro acetic acid (Sigma) was added to the supernatant and proteins were precipitated on ice at 4 °C overnight. Following precipitation, supernatant proteins were pelleted by centrifugation at 16,000 x *g* for 30 minutes at 4 °C. Supernatants were removed and discarded, and the pellet washed in 1 ml of cold 90% acetone for 15 minutes on a vortex shaker. After washing, the proteins were pelleted by centrifugation. The supernatant was removed and the pellet air dried for 30 min, prior to

resuspension in SDS-gel loading buffer at a concentration of 1 OD unit per 10 µl. This resulted in 10 x concentrated supernatants, compared to cell extracts.

In experiments involving the T3SS, this system was induced by the chelation of calcium ions. Cells cultures were supplemented with 5 mM EGTA to chelate calcium, and 20 mM $MgCl_2$ (Soscia et al., 2007). Cell extracts and supernatants were prepared as described.

2.6.3 SDS-PAGE

Whole cell and supernatant protein samples were separated by SDS-PAGE. Prior to loading, resuspended samples were boiled for 10 min at 95 °C. Proteins were analysed on 8-12% Tris-glycine SDS-PAGE gels prepared using the min-protean kits (Bio-rad). Resolving gels contained 8-12 % 39:1 acrylamide : bisacrylamide, 375 mM Tris pH 8.8, 0.1 % SDS, 0.5 % APS and 0.7 % TEMED. Stacking gels contained 5 % 39:1 acrylamide : bisacrylamide, 62.5 mM Tris pH 6.8, 0.1 % SDS, 0.1 % APS and 0.24 % TEMED. Gels were immersed in Tris-Glycine-SDS (TGS) electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1 w/v SDS). 10 µl of each sample was loaded onto the gels along with Kaleidoscope protein marker (Bio-Rad) for molecular weight determination, and a constant voltage of 150 V was applied for 1 hour. Following electrophoresis proteins were transferred to nitrocellulose membranes for immunoblotting.

2.6.4 Western blotting

Following separation by SDS-PAGE, proteins were analysed by western blotting. Whatman nitrocellulose membrane and filter paper and the electrophoresed gels were soaked in transfer buffer (20% ethanol, 10% TGS buffer, 70% H_2O). Proteins were transferred using a semi-dry transfer cell (Bio-rad) at a constant voltage of 24V, 3 mAmp per cm^2 for 45 minutes. Following transfer,

membranes were briefly stained with ponceau (Sigma) to visualise proteins. Ponceau stain was removed by washing in TBS Tween (0.5 M Tris, 1.5 M NaCl, pH 8.0, 0.1% Tween20).

Membranes were blocked for one hour at room temperature in blocking buffer (TBS Tween + 5% milk powder). Primary antibodies were added to the membrane at a suitable dilution in blocking buffer for 1-2h. Following incubation with primary antibody membranes were washed three times in TBS Tween. Secondary antibody (anti-mouse or anti rabbit, conjugated to HRP (Sigma)) was added at 1/5000 dilution in blocking buffer and incubated for 45 minutes at room temperature. Membranes were washed three times in TBS Tween prior to detection.

Antibodies were detected using SuperSignal West pico chemiluminescence substrate (Thermo) and a LAS3000 Fuji imager. Equal volumes of the peroxide solution and enhancer solution were mixed and applied to the membrane, and incubated for 2 minutes prior to visualisation.

2.6.5 Antibodies

A list of antibodies used in this project, along with their source, is shown in Table 10. Antibodies generated specifically for use in this project were raised against purified full length proteins carrying his tags. The genes encoding the required proteins were either cloned into pET28a or obtained in pET-Dest42 expression vectors. These constructs were transformed into E. coil BL21 DE3 cells as described, and protein expression induced by addition of 0.5 mM 1-thio-β-D-galactopyranoside when subcultures reached an optical density of 0.6 OD_{600nm}. Following induction, cultures were incubated at 18 °C overnight with agitation. Cells were harvested and resuspended in 50 mM HEPES, 500 mM NaCl, 200 mM imidazole, complete EDTA-free protease inhibitors, pH 7.2 and lysed by French Press. The lysate was centrifuged at 3000 x *g* for 15 minutes. The supernatant was applied to His-trap columns (GE healthcare) for purification by imidazole gradients. Proteins were

subsequently purified by size exclusion chromatography into final buffer conditions of 50 mM Tris, 250 mM $NaCl_2$, pH8. Purified proteins were concentrated and sent for antibody production in rabbits (Eurogentec).

Antibody	Comments	Source / Reference
Primary Antibodies		
α -V5	Commercial antibody against the V5 epitope tag, raised in mice.	Invitrogen
α-RNAP	Commercial antibody directed against the RNA polymerase β subunit of *E. coli* origin, raised in mice	Neoclone
α-Hcp1	Peptide antibody raised in rabbits against Hcp1 peptides,	(Hachani et al., 2011)
α-VgrG1a	Antibody raised against purified full length VgrG1a-V5-his in rabbits	(Hachani et al., 2011)
α-Tse3	Peptide antibody raised in rabbits against Tse3 peptides,	(Hachani et al., 2011)
α-PcrV	Antibody raised against recombinant His6 tagged PcrV in rabbits.	(Goure et al., 2004)
α-LasB	Antibodies against purified *P. aeruginosa* elastase raised in rabbits.	(Jaffar-Bandjee et al., 1995)
α-XcpY	Antibodies raised against a GST-XcpY fusion protein in rabbits.	(Ball et al., 1999)
α-Hcp2	Antibody directed against purified full length Hcp2-V5-his in rabbits	This work
α-HsiB2	Antibody raised against purified full length HsiB2-V5-his in rabbits	This work
Secondary Antibodies		
α-rabbit	anti-rabbit IgG (whole molecule)-peroxidase conjugate	Sigma
α-mouse	anti-mouse IgG (whole molecule)-peroxidase conjugate	Sigma

Table 10 Antibodies used in this project.

2.6.6 Protein localisation studies

For protein localisation studies, cellular proteins were initially separated into soluble and insoluble fractions. An overnight culture of the required strain was subcultured to early stationary phase. A cell sample was removed for preparation of whole cell lysates as previously described. A volume of cells equivalent to 20 OD_{600nm} units was harvested and resuspended in 1 ml 50 mM Tris pH 8.0 supplemented with complete protease inhibitor cocktail (Roche) and 1 mM EDTA. Cells were lysed by sonication (3 x 30 s, amplitude 35%) on ice. Intact cells were removed by three spins at 4000 x *g* for 5 minutes at 4 °C, retaining only the uppermost supernatant each time. The insoluble fraction was separated from the soluble fraction by ultracentrifugation at 100,000 x *g* for 1 h at 4 °C,

following which samples of the supernatant and the pellet, containing soluble and insoluble proteins respectively, were resuspended in SDS gel loading buffer.

Proteins contained in the insoluble pellet were further fractioned into inner and outer membrane proteins. This was achieved by resuspending the insoluble pellet in 500 µl of sodium N-lauroylsarcosinate buffer (2% w/v SLS, 15 mM tris-HCl, pH 7.4) to solubilise proteins from the inner membrane. Samples were incubated for 30 minutes at 4 °C with gentle mixing. Solubilised inner membrane proteins were separated from insoluble outer membrane proteins by ultracentrifugation at 100,000 x *g* for 1 h at 4 °C, following which samples of the supernatant and pellet, containing inner membrane and outer membrane proteins respectively, were resuspended in SDS gel loading buffer.

Periplasmic proteins were isolated through selective disruption of the outer membrane. Cells were subcultured to early stationary phase and a volume corresponding to 10 OD_{600nm} equivalent units was harvested. Cells were washed in 1 ml of ice cold 50 mM Tris pH7.6 and centrifuged at 8000 x *g* for 10 min. The supernatant was removed and pellet resuspended in 0.5 ml spheroplasting buffer (0.2 M $MgCl_2$ Tris pH 7.6), incubated for 30 min at 30 °C, 5 min on ice, then 15 min at room temperature. Following incubation samples were centrifuged at 8,000 x *g* at 4 °C for 10 min. The supernatant was carefully removed, and the cell pellet, containing spheroplasts was resuspended in SDS gel loading buffer. The supernatant was centrifuged twice more, removing the uppermost supernatant each time, to remove all remaining spheroplasts. The resulting supernatant, containing the periplasmic content, was resuspended in SDS gel loading buffer.

Each of the resulting cell fractions were analysed by SDS-PAGE and western blotting, as described.

2.7 Biofilm assays

2.7.1 Quantitative analysis of biofilm production

The ability of *P. aeruginosa* strains to form biofilms was quantitatively measured in 48 well microtitre plates (BD Falcon). Overnight cultures of *P. aeruginosa* strains were sub-cultured to an optical density of 0.1 OD_{600nm} in LB broth, with the addition of antibiotics as required. 700 µl of bacterial culture was added to each well, and plates were placed in a static incubator at 37 °C for 16 hours.

Following incubation, 70 µl of crystal violet solution (VWR) was added to each well, and plates were stained for ten minutes at room temperature. Liquid was removed from wells by aspiration, and the wells were washed three times with water. The remaining stained biofilm was dissolved in 95% ethanol with rocking for ten minutes. The optical density of the dissolved stain was measured by reading OD_{600nm} using a hand-held spectrophotometer.

2.7.2 Visual biofilm assay

Biofilm production was visualised in glass test tubes. Overnight cultures of *P. aeruginosa* strains were sub-cultured to an optical density of 0.1 OD_{600nm} in LB broth, with the addition of antibiotics as required. 3 ml of the required culture was added to each test tube, and tubes were placed in a static incubator at 37 °C for 16 h. Following incubation, 300 µl of crystal violet solution was added, and the tubes were stained for ten minutes at room temperature. Liquid was removed by aspiration and test tubes were washed three times with water. The remaining stained biofilm was photographed using a digital camera.

2.8 Reporter Assays

2.8.1 β-galactosidase assays

β-galactosidase assays were performed on strains containing appropriate *lacZ* reporter fusions, as indicated in Table 6. Cells were cultured as previously described, and at appropriate time points 1 ml of each culture was harvested by centrifugation, and the optical density of the culture recorded. Cell lysates were prepared by resuspending the pelleted cells in 1 ml of Z- buffer + beta-mercaptoethanol (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M MgSO4, 0.05 M beta-mercaptoethoanol), 50 µl of 0.1 % SDS and 100 µl chloroform. Samples were lysed in a vortex mixer for 2 min, and then left to stand for 2 min to allow separation of the chloroform.

Assays were performed in 96-well microtitre plates (BD Falcon). 200 µl buffer Z was added to the first row as a control. To other wells, 180 µl buffer Z and 20 µl cell lysate was added. 40 µl ONPG (4 mg/ml, Sigma)) was added to each well, and plates were incubated at 28 °C until a yellow colour was detectible, at which point the duration of incubation was noted. The reaction was stopped at this point by the addition of 100 µl 1M Na_2CO_3. Absorbance at 405 and 540 nm was measured. Samples were blank corrected, and β-galactosidase activity for each sample was calculated using the following formula.

$$\beta - \text{galactosidase activity} = \frac{1000\ x\ (OD\ 405nm - OD\ 540nm)}{Time\ (mins)\ x\ volume\ of\ cells\ (0.02)\ x\ OD\ 600nm}$$

2.8.2 Measurement of c-di-GMP levels

Relative levels of c-di-GMP were measured using the c-di-GMP responsive *cdrA-gfp* fusion plasmid (Rybtke et al., 2012). The plasmid was mobilised into *P. aeruginosa* strains by electroporation. The reporter plasmid was prepared from the *E. coli* host strain using a QIAgen miniprep kit. 1.5 ml from an overnight culture of the recipient *P. aeruginosa* strain was harvested on ice, and washed twice in 300 mM sucrose, and resuspended in 100 µl 300 mM sucrose. 150 ng of purified plasmid was added to the cells in a chilled, sterile, electroporation cell. The cells were pulsed at 2.5 kV, and 1 ml of SOC media (Invitrogen) was added to cells, which were incubated at 37 °C for 1.5 h and plated onto selective media.

P. aeruginosa strains carrying the reporter plasmid were subcultured for 6h with shaking, and three 200 µl samples were removed into a black 96-well plate with a translucent base (BD Falcon). The absorbance (620 nm, gain 1000) and fluorescence (excitation 485 nm, emission 520 nm, gain 2000) of the cells was measured using a Fluostar Optima plate reader. Relative fluorescence was measured by normalising to 1 ml and by optical density of the original bacterial culture.

2.9 T6SS Bacterial Killing Assay

A visual assay was used to assess T6SS mediated bacterial killing of *E. coli* by *P. aeruginosa*, as previously described (Hachani et al., 2013). *P. aeruginosa* cells were incubated with *E. coli* carrying the pCR2.1 empty vector (Invitrogen) allowing alpha complementation of β-galactosidase. The required *P. aeruginosa* strain was incubated in a mixed patch with equivalent numbers of *E. coli* pCR2.1 cells, on an LB agar plate for 5 h at 37 °C. Following incubation, the mixed patches were recovered and resuspended in 1 ml of LB broth. A dilution series ranging from 10^{0} to 10^{-3} was plated in triplicate on LB plates supplemented with 100 µg/ml X-gal (Invitrogen), and incubated at 37 °C

overnight. A qualitative assessment of LacZ positive *E. coli* survival was achieved by visualisation of a blue colour in the plated dilution series.

2.10 Cytotoxicity Assay

The cytotoxicity of *P. aeruginosa* strains was measured by monitoring the release of lactate dehydrogenase (LDH) from a murine RAW 264.7 macrophage cell line (ATCC TIB-7). RAW macrophages were routinely grown in Dulbecco modified Eagle medium, GlutaMAX I, sodium pyruvate, and phenol red (Gibco), supplemented with 10% foetal bovine serum and non-essential amino acids (Gibco). Macrophages were grown to 80% confluence in 96 well plates and washed with sterile phosphate-buffered saline (PBS), and incubated with RPMI1640 without phenol red for 1 h prior to infection.

Macrophages were infected with late exponential phase *P. aeruginosa* at a multiplicity of infection of 10 for 3 h at 37 °C, in the presence of 5% CO_2. The macrophage infection was synchronised by sedimenting bacteria with a 5 minute centrifugation step at 200 x g.

Following 3h of infection, plates were centrifuged again for 5 minutes at 200 x *g* to sediment cell debris and bacteria. The release of cytosolic LDH into the culture supernatant was measured with the CytotTox 96 non-radioactive cytotoxicity assay (Promega). Cytotoxicity was calculated relative to that of non-infected cells, set to 0% cytotoxicity, and that of cells lysed with 1% triton X-100, set to 100% cytotoxicity.

2.11 Internalisation Assay

Internalisation of *P. aeruginosa* into HeLa cells was assessed by gentamycin protection assay. HeLa cells were cultured to 70% confluence in 24-well plates in Eagle's minimal essential medium (MEM), supplemented with 10% foetal bovine serum (Gibco). The cells were washed twice with sterile PBS and infected with *P. aeruginosa* strains grown to early stationary phase at a multiplicity of infection of 10 in MEM with FBS, to a final volume of 1ml. The infection was synchronised by sedimenting bacteria with a 5 minute centrifugation step at 200 x *g* prior to incubation for 3 h min at 37 °C in the presence of 5% CO_2. Following infection, cells were washed twice with sterile PBS and incubated with MEM supplemented with 300 µg/ml gentamycin (Sigma) for 75 min, to kill extracellular bacteria. Cells were subsequently washed three times with room temperature PBS and osmotically shocked with ice cold sterile water containing 0.05% triton x-100 (Sigma) for 30 min on ice. Eukaryotic cell lysates were collected, and serial dilutions in sterile LB were plated onto *Pseudomonas* isolation agar and colony forming units enumerated following overnight incubation at 37 °C.

Chapter 3 *P. aeruginosa* response to subinhibitory antibiotics

3.1 Introduction

As previously described, *P. aeruginosa* is capable of existing as one of two diverse lifestyles, thriving as a motile organism in a planktonic lifestyle, or establishing multicellular communities known as biofilms. Biofilm cells are embedded in a matrix of exopolysaccharides attached to surfaces, as described in detail in section 1.4. Biofilm formation occurs in the natural environment as well as during infections, and results in persistent infections that are difficult to eradicate. Regulation of biofilm formation is highly complex, and involves the interplay of several regulator components, including two-component systems, small regulatory RNAs and post-transcriptional regulators (Mikkelsen et al., 2011b). Biofilm formation has also been closely linked to the second messenger c-di-GMP (Harmsen et al., 2010). The precise nature of the environmental stimuli which activate these regulatory cascades, leading to biofilm formation, are uncharacterised.

It has been observed that sub inhibitory concentrations of several different antibiotics can induce *P. aeruginosa* biofilms. Hoffman and colleagues were able to show that subinhibitory concentrations of the aminoglycoside tobramycin, a clinically important antibiotic, increased *P. aeruginosa* PAO1 biofilm formation (Hoffman et al., 2005). Incubation of PAO1 with a concentration of tobramycin equivalent to 0.3 times its minimum inhibitory concentration resulted in a 3.4 fold increase in the levels of biofilm formation, compared to incubation without antibiotics. This effect was also observed with subinhibitory concentrations of amikacin, streptomycin, and gentamycin, although the level of induction was not as high using these aminoglycosides.

Incubation with subinhibitory concentrations of polymyxin B, a peptide antibiotic which is cationic like the aminoglycosides and alters membrane permeability, does not induce *P. aeruginosa* PAO1 biofilm formation, indicating that the effect of the aminoglycosides is not related to its cationic nature (Hoffman et al., 2005). Like aminoglycosides, chloramphenicol acts by disrupting protein synthesis, however, subinhibitory concentrations of chloramphenicol do not induce biofilm formation, indicating that the effect of aminoglycosides on biofilm formation is also not related to its role as an inhibitor of protein synthesis (Hoffman et al., 2005). Carbenicillin, a cell wall synthesis inhibitor, also does not induce bacterial biofilm. These observations led the authors to conclude that the effect seen with subinhibitory concentrations of aminoglycosides is not a non-specific response to antibiotic challenge, but a specific response to aminoglycoside molecules.

Subinhibitory aminoglycosides were also shown to induce *E. coli* biofilm formation, and it was proposed that a signalling pathway common to *P. aeruginosa* and *E. coli* was involved in the phenotypes observed. A transposon screen identified a *P. aeruginosa* phosphodiesterase, encoded by *arr*, responsible for tobramycin induced biofilm formation via the modulation of c-di-GMP levels. The discovery of a conserved regulatory pathway suggests that biofilm formation by tobramycin is a specific and defensive reaction to the presence of this antibiotic.

A similar effect is seen with antibiotics which inhibit bacterial DNA replication, since *P. aeruginosa* PAO1 was shown to form biofilms in response to subinhibitory concentrations of quinolone antibiotics (Gotoh et al., 2008). Induction of biofilm formation by quinolones was also suppressed by mutation of *arr*, indicating a broad role for Arr in the biofilm response. A publication by Linares et al. (2006) shows that tobramycin, tetracycline, and norfloaxin can induce biofilm formation at subinhibitory concentrations. Additionally, they show that tobramycin increases bacterial motility and tetracycline triggers expression of the T3SS (Linares et al., 2006). This paper discusses the notion of antibiotics as signalling molecules that regulate bacterial behaviour, and not simply as

weapons used for fighting competitors. These examples show that subinhibitory concentrations of antibiotics can result in beneficial adaptations by *P. aeruginosa*, leading to biofilm formation as a protective strategy. This has obvious implications in the treatment of infections, since sub-lethal antibiotic concentrations or incorrect dose administration may have the unexpected effect of promoting persistent *P. aeruginosa* infections.

The Ret/Gac/Rsm cascade is a key regulatory system involved in *P. aeruginosa* virulence, as described in detail in section 1.9 (Coggan and Wolfgang, 2012). The cascade is involved in a switch between two different lifestyles, which ultimately result in different outcomes of infection. A highly motility lifestyle and active secretion by the T3SS is associated with acute infections, while biofilm formation and high activity of the H1-T6SS are associated with chronic *P. aeruginosa* infections. Both subinhibitory concentrations of aminoglycosides and the RetS/Gac/Rsm cascade regulate biofilm formation (Goodman et al., 2004; Hoffman et al., 2005), however, it is not known if these two regulatory mechanisms interact, or if they represent two distinct regulatory pathways converging on a common phenotype. The precise signals required to activate the RetS/Gac/Rsm cascade are not known. Uncovering the exact signals that are sensed by this cascade will lead to a greater understanding of how *P. aeruginosa* responds to its environment, and mediate different types of infection. Additionally, it is not known if aminoglycosides influence other phenotypes which are controlled by the RetS/Gac/Rsm cascade or if the cascade has a role in biofilm induction by subinhibitory concentrations of aminoglycosides.

3.2 Specific aims of the study

This work presented in this chapter aims to investigate the response of *P. aeruginosa* PAK to subinhibitory antibiotics, focusing on the response to aminoglycosides, which have previously been reported to influence *P. aeruginosa* PAO1 biofilm formation (Hoffman et al., 2005). The effect of

subinhibitory concentrations of antibiotics will be tested on phenotypes known to be regulated by the RetS/Gac/Rsm cascade, including biofilm formation, T3SS, and H1-T6SS. The role of the RetS/Gac/Rsm cascade in the molecular mechanism of the response of *P. aeruginosa* PAK to subinhibitory concentrations of antibiotics will be investigated. The main aims of the chapter are outlined below:

- Investigate the induction of biofilm formation by *P. aeruginosa* PAK in response to subinhibitory concentrations of various aminoglycosides, and other antibiotics.

- Evaluate the role of *arr* in *P. aeruginosa* PAK, and investigate the change in c-di-GMP levels following incubation with subinhibitory concentrations of aminoglycosides.

- Extend the analysis to phenotypes co-regulated with biofilm formation via the RetS/Gac/Rsm cascade by investigating the effect of subinhibitory concentrations of aminoglycosides on the T3SS and H1-T6SS.

- Investigate if the RetS/Gac/Rsm cascade is required for the response of *P. aeruginosa* PAK to subinhibitory concentrations of aminoglycosides.

3.3 Subinhibitory aminoglycosides and biofilm formation

It has been shown that subinhibitory concentrations of antibiotics induce formation of biofilms by *P. aeruginosa*, notably tobramycin and *P. aeruginosa* PAO1 (Gotoh et al., 2008; Hoffman et al., 2005). The first clinically used aminoglycoside was streptomycin, derived from bacteria of the *Streptomyces* genus, and subsequently additional aminoglycosides have been isolated or synthesised. This class of antibiotics is useful in the treatment of infections involving aerobic Gram negative bacteria.

Aminoglycosides are highly potent, bactericidal, broad spectrum antibiotics which act primarily by impairing bacterial protein synthesis by binding to the 30s subunit of the prokaryotic ribosome, causing misreading of the mRNA resulting in premature termination of translation (Mingeot-Leclercq et al., 1999). Since other antibiotics that interfere with protein synthesis, such as tetracycline, are bacteriostatic in their action, it is not thought that the protein synthesis inhibition of aminoglycosides is responsible for the bactericidal activity of the aminoglycosides. It is proposed that aminoglycosides have an additional site of action at the bacterial outer membrane, where they form fissures which lead to leakage of cellular contents (Davis, 1987).

3.3.1 Establishment of subinhibitory aminoglycosides concentration

To investigate the role of aminoglycosides in inducing *P. aeruginosa* PAK biofilm formation, it was necessary to establish suitable concentrations of aminoglycosides which did not significantly affect growth. The growth of *P. aeruginosa* PAK, under a range of different concentrations of aminoglycosides (tobramycin, kanamycin and gentamycin) is shown in Figure 3.1. The aminoglycosides used are closely related, and share structural similarity. The main core of these molecules is the same, with variation in the side groups.

The growth of *P. aeruginosa* PAK under a range of different concentrations of kanamycin is shown in Figure 3.1A. The growth curves show that *P. aeruginosa* PAK, in the absence of kanamycin, has a typical bacterial growth pattern, reaching a stationary phase optical density of around 10 OD after 7 h of growth. Upon addition of kanamycin to the growth medium the growth of *P. aeruginosa* PAK responds in a dose dependent manner. At lower kanamycin concentrations (10 to 50 μgml^{-1}) the growth of *P. aeruginosa* is equivalent to growth without kanamycin. At concentrations of 70 μgml^{-1} and above the rate of exponential growth *P. aeruginosa* PAK is slowed, and concentrations of 100 and 110 μgml^{-1} significantly impact bacterial growth. At a concentration of kanamycin up to 70 μgml^{-}

[1] the final cell density of stationary phase growth is equivalent to cells grown without kanamycin, indicating that concentrations below this do not significantly impact the growth of *P. aeruginosa* PAK. Dr Luke Allsopp is acknowledged for his assistance in generating the data used showing *P. aeruginosa* PAK growth with increasing concentrations of kanamycin.

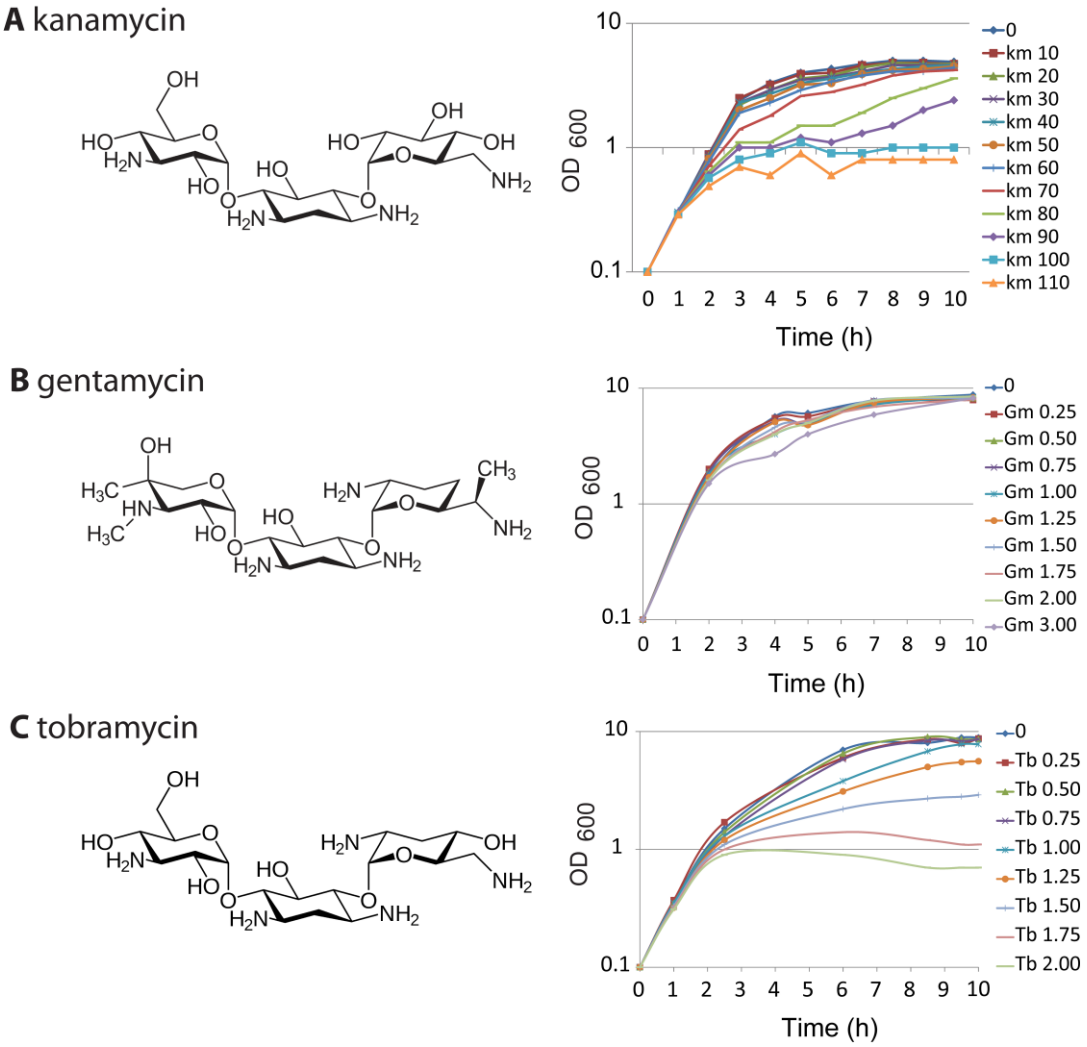


Figure 3.1 Growth of *P. aeruginosa* PAK in response to aminoglycosides
Planktonic growth (OD_{600}) of *P. aeruginosa* PAK incubated with increasing concentrations of kanamycin (A), gentamycin (B) and tobramycin (C) are shown over a ten-hour period. The chemical structure of each aminoglycoside is included for comparison. The concentration of antibiotic used is shown to the right of each growth curve.

Figure 3.1B shows that the concentrations of gentamycin tested are all sub inhibitory towards *P. aeruginosa* PAK. The highest concentration tested, 3 $\mu g ml^{-1}$, has a small impact on the growth of

PAK after 2 hours, but the resulting final OD is equivalent to cells grown without gentamycin. Figure 3.1C shows the growth of PAK with increasing concentrations of tobramycin. At a concentration of 1.75 μgml^{-1} and above PAK growth stops after 2 hours. The lower concentrations of tobramycin, at around 0.25 to 1.0 μgml^{-1} represent concentrations sub inhibitory to the growth of *P. aeruginosa* PAK.

In conclusion, concentrations of kanamycin at 60 μgml^{-1}, gentamycin at 2 μgml^{-1} and tobramycin at 2 μgml^{-1} can be considered the maximum subinhibitory concentrations of these antibiotics that do not impact the growth of *P. aeruginosa* PAK.

3.3.2 Subinhibitory aminoglycosides and biofilm formation

Using the information gathered on the subinhibitory concentrations of kanamycin, gentamycin and tobramycin, their effect on biofilm *P. aeruginosa* PAK biofilm formation was investigated. Figure 3.2A shows the formation of biofilm by PAK incubated with a range of concentrations of each of these aminoglycosides. Visualisation of crystal violet stained biofilm biomass in glass test tubes indicates that PAK without kanamycin forms very low levels of biofilm, and that there is a dose dependent induction of biofilm formation between 10 and 60 μgml^{-1} of kanamycin. This result is confirmed by quantifying the absorbance of the dissolved crystal violet stain (Figure 3.2A). At 60 and 70 μgml^{-1} kanamycin there is a statistically significant increase in biofilm formation (students T-test, $P<0.01$), with biofilm levels 2.58 and 2.49 times higher respectively than PAK grown without kanamycin. Dr Luke Allsopp is acknowledged for his assistance in generating the data used showing biofilm formation by kanamycin.

Analysis of biofilm formation by *P. aeruginosa* PAK grown with increasing concentrations of gentamycin and tobramycin are shown in Figure 3.2B and 3.2D respectively, which show that

subinhibitory concentrations of gentamycin and tobramycin induce higher levels of biofilm formation compared to kanamycin. The maximum effect on biofilm induction by gentamycin occurs at 0.6 μgml^{-1} where an induction of 3.25 fold is observed (Students T Test, $P<0.05$). A maximum induction of 4.05 fold (Students T Test, $P<0.05$) occurs at 0.5 μgml^{-1} of tobramycin. Biofilm assays using gentamycin and tobramycin were performed by Mr John Horlick (Undergraduate project student).

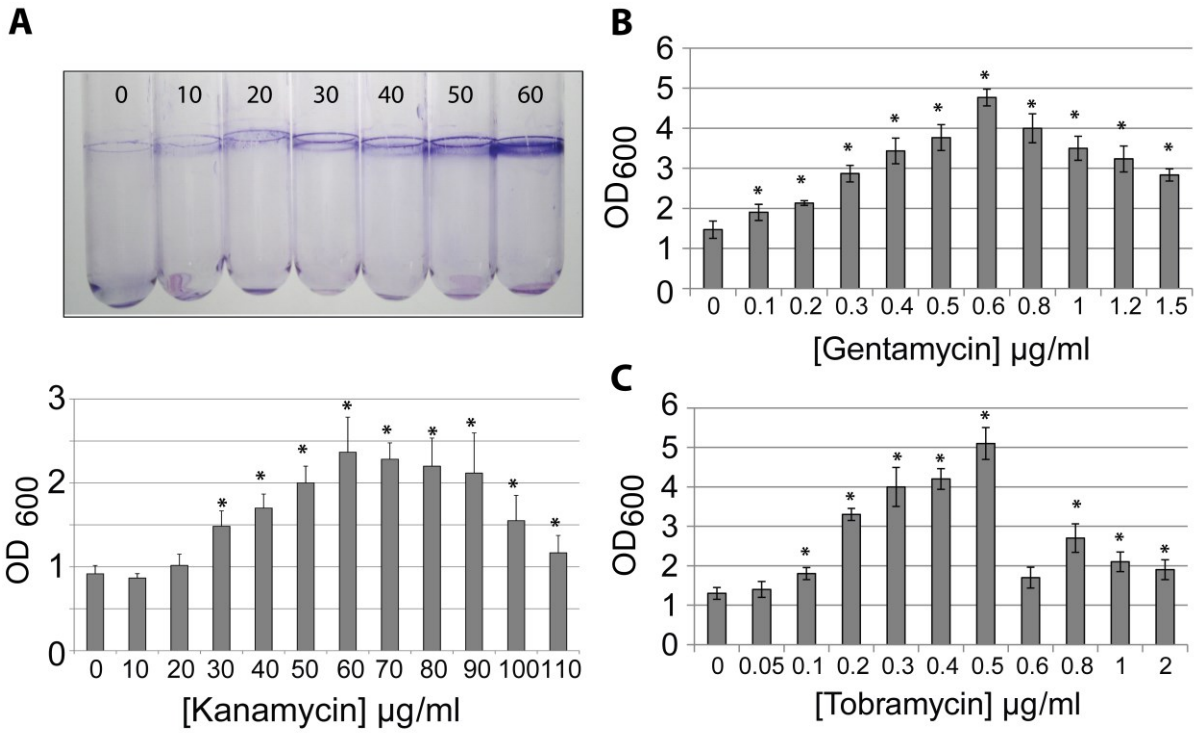


Figure 3.2 Induction of PAK biofilm formation by subinhibitory aminoglycoside concentrations
Biofilm formation upon addition of kanamycin (A), gentamycin (B) and tobramycin (C). A. top panel shows staining of biofilm biomass with crystal violet in glass test tubes. The concentration of kanamycin used (in µg/ml) is shown above each tube. Biofilm production is quantified (lower panel, B and C) by measuring the optical density of the dissolved crystal violet stain. Error bars show standard deviation. Antibiotic concentration is indicated below each bar. * indicates a statistically significant difference compared to biofilm formation without antibiotic (student's T Test, $P<0.05$).

These results indicate that the induction of biofilm formation by aminoglycosides reported for *P. aeruginosa* PAO1 also occurs in *P. aeruginosa* PAK. Kanamycin, gentamycin, and tobramycin induce biofilm formation in this strain.

3.3.3 Dependence on *arr* and ci-di-GMP

In *P. aeruginosa* PAO1 the induction of biofilm formation by aminoglycosides and quinolone antibiotics has been shown to depend on a gene encoding Arr, a predicted inner-membrane phosphodiesterase (Gotoh et al., 2008; Hoffman et al., 2005). When *arr* is disrupted, *P. aeruginosa* PAO1 no longer forms biofilms in response to aminoglycosides. Arr, is shown to cause a decrease in the c-di-GMP pool upon addition of aminoglycosides, since membranes from strains where *arr* is disrupted have reduced phosphordiesterase activity (Hoffman et al., 2005).

It has already been confirmed in the previous section that aminoglycosides induce biofilm formation in *P. aeruginosa* PAK, similar to published observations in *P. aeruginosa* PAO1 (Hoffman et al., 2005). However, since the genome sequence of the PAK strain is not currently available, the conservation of the *arr* gene in this strain is not known. Genomic DNA from PAK was probed by PCR for the presence of a fragment of *arr* 686 bp in length, as was genomic DNA from PAO1, known to encode *arr*, and PA14, which lacks *arr*. The result of this reaction can be seen in Figure 3.3A. The positive product is clearly amplified from PAO1 genomic DNA, but not from PAK and PA14 gDNA. This indicates that the *arr* gene is absent from PAK, and therefore the biofilm formation observed in this strain occurs in an *arr* independent mechanism. Interestingly, the gene encoding *arr* is only conserved in three of the thirteen *P. aeruginosa* genomes available for comparison at pseudomonas.com, which are *P. aeruginosa* PAO1, M18, and PA7. The lack of *arr* in PAK is therefore not surprising since it is not conserved in the majority of *P. aeruginosa* strains.

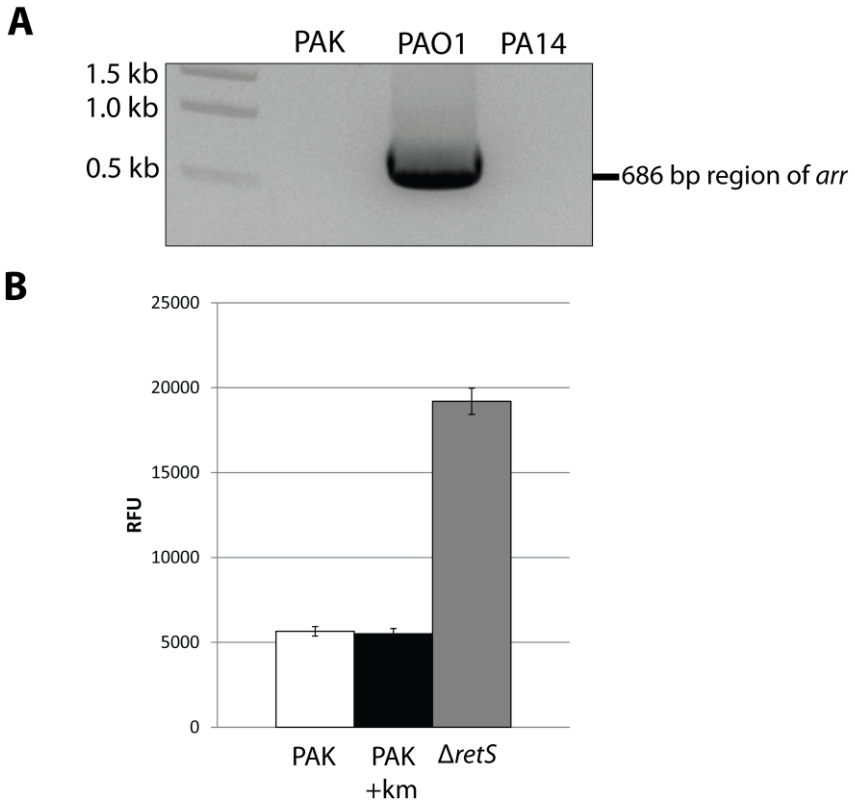


Figure 3.3 Analysis of c-di-GMP levels in *P. aeruginosa* PAK strains
A. Result of PCR screening for a 686 base pair region of arr in PAK, PAO1 and PA14. B. Relative fluorescence values shown for strains carrying the c-di-GMP reporter plasmid (*cdrA-gfp* fusion). Levels shown for wild type PAK, PAK incubated with kanamycin at 30 µg/ml kanamycin, and a control strain (PAK Δ*retS*), known to have elevated levels of c-di-GMP. RFU indicates arbitrary relative fluorescence units corrected for optical density. Error bars show standard deviation.

The biofilm phenotype induced by subinhibitory concentrations of aminoglycosides in *P. aeruginosa* PAK may still be dependent on c-di-GMP, despite the absence of *arr*, involving instead other c-di-GMP modifying enzymes. This was investigated using a *cdrA-gfp* promoter fusion reporter which is responsive to c-di-GMP levels (Rybtke et al., 2012). Measurements of relative c-di-GMP were made to see if they correlated with biofilm formation upon addition of aminoglycosides. *P. aeruginosa* PAK carrying the reporter plasmid was incubated with or without kanamycin, and a PAK Δ*retS* control also carrying the reporter plasmid was included since it is known to have elevated c-di-GMP levels (Moscoso et al., 2011). Figure 3.3B shows the relative fluorescence units, corrected for optical density, for each condition tested. A clear increase is observed in the PAK Δ*retS* strain, indicating

that the assay is capable of detecting increases in ci-di-GMP levels. A comparison of PAK grown with or without kanamycin shows no detectible change in c-di-GMP levels upon addition of this antibiotic.

The fact that *arr* is absent from PAK, and that there is no change in the c-di-GMP levels in this strain upon addition of kanamycin, suggests that the increased biofilm formation in this strain is *arr* and c-di-GMP independent, and occurs via an uncharacterised mechanism. These observations indicate that PAO1 and PAK both respond to subinhibitory aminoglycosides by forming biofilms, but the precise mechanism of this response is not the same in each strain.

3.4 Sub inhibitory aminoglycosides and the H1-T6SS

In addition to being induced by subinhibitory concentrations of aminoglycoside antibiotics, *P. aeruginosa* biofilm formation is also regulated by the RetS/Gac/Rsm cascade (Goodman et al., 2004). The RetS/Gac/Rsm cascade co-regulates biofilm formation and the H1-T6SS (Mougous et al., 2006). Due to this co-regulation by the regulatory cascade it was hypothesised that these two phenotypes may also be co-regulated by subinhibitory concentrations of aminoglycosides, and that the H1-T6SS may also be activated under these conditions.

3.4.1 Subinhibitory aminoglycosides and production of H1-T6SS components

The production of a key structural component of the H1-T6SS, Hcp1, was tested upon addition of subinhibitory concentrations of various aminoglycosides. As described in section 1.7, Hcp forms hexameric rings, which stack on top of each other to form a structure similar to the tail tube of bacteriophage, strictly required for the activity of the T6SS (Mougous et al., 2006). Antibodies directed towards specific Hcp1 peptides have previously been generated and were available for use

(Hachani et al., 2011). Western blots were performed on cell extracts from *P. aeruginosa* cultures grown with subinhibitory concentrations of kanamycin, gentamycin, and tobramycin. The resulting western blots can be seen in Figure 3.4. Figure 3.4A shows that increasing concentrations of kanamycin result in a dose dependent increase in the intensity of a band corresponding to a protein of around 17 kDa, proposed to be Hcp1 (with an expected molecular weight of 17.5 kDa). Analysis of Figure 3.4B and 3.4C shows that increasing the concentration of gentamycin and tobramycin do not result in a comparable band. This suggests that under the conditions tested, kanamycin, but not gentamycin or tobramycin, is able to induce production of a core component of the H1-T6SS.

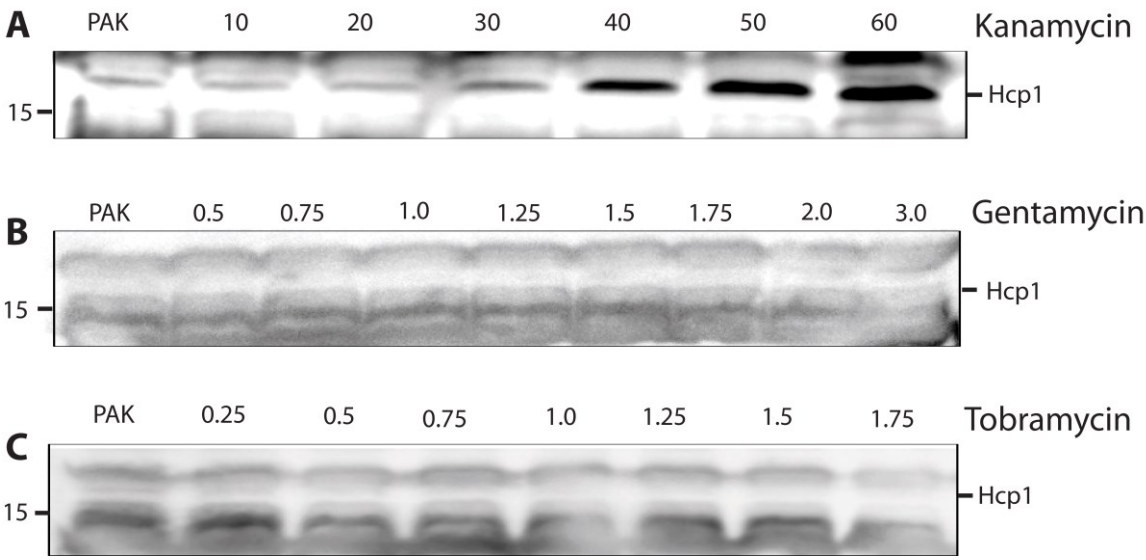


Figure 3.4 Effect of subinhibitory concentrations of aminoglycosides on Hcp1 production
Western blots probing whole cell lysates with anti-Hcp1 antibody. Cells were previously incubated with kanamycin (A), gentamycin (B) or tobramycin (C) at concentrations indicated in µg/ml above each blot. The expected location of Hcp1 is indicated on the right of each blot, and molecular weight standards (in kDa) on the left.

The positive effect of kanamycin on the H1-T6SS was tested further by investigating the production of Hcp1, and another core component of the H1-T6SS, VgrG1a. VgrG, as described in section 1.7, forms the trimeric puncturing device of the T6SS system, essential for T6SS function (Leiman et al., 2009). An antibody raised against full length VgrG1a, also able to recognise other closely related VgrG associated with the H1-T6SS cluster (VgrG1b and VgrG1c), was used in this experiment

(Hachani et al., 2011). The locations of the genes encoding these H1-T6SS components are indicated in Figure 3.5A.

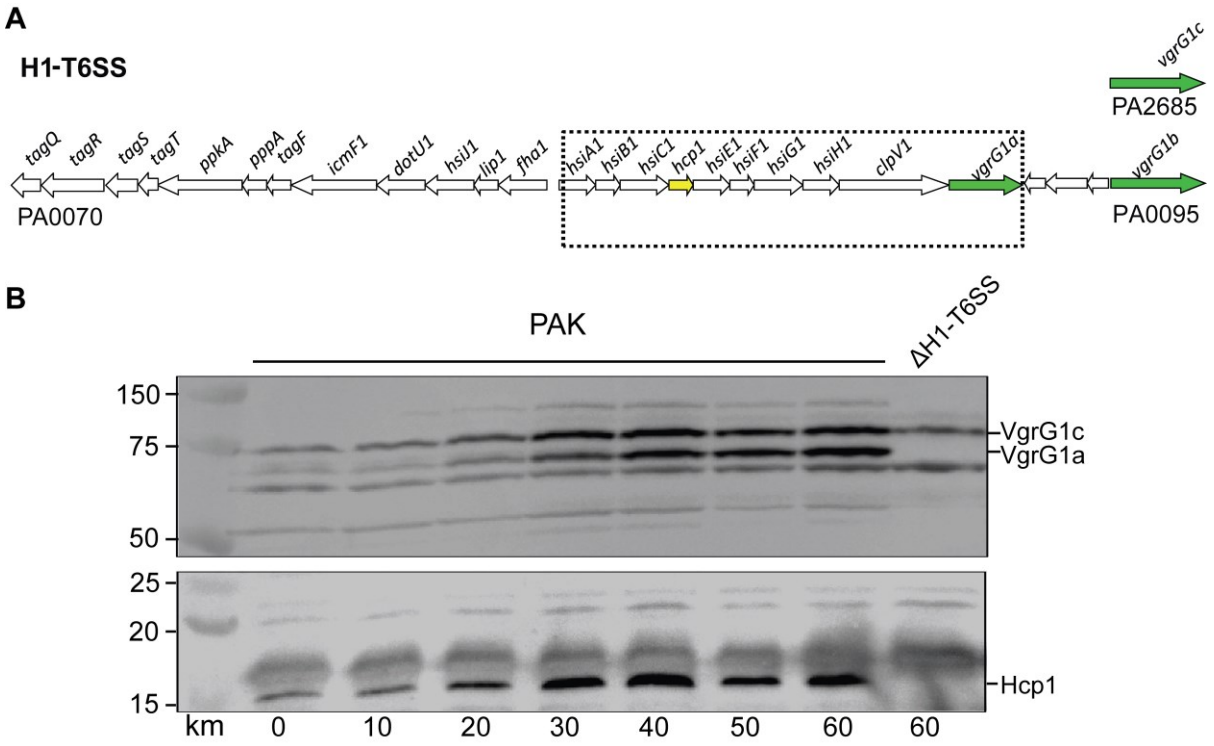


Figure 3.5 Kanamycin induces components of the H1-T6SS in *P. aeruginosa* PAK
A. Genetic organisation of the H1-T6SS cluster. The region deleted in the PAK ΔH1-T6SS strain is indicated by the hatched box. B. western blot of PAK whole cell extracts from cultures incubated with increasing concentrations of kanamycin, indicated in µg/ml below the blots. Cell extracts were probed with anti-VgrG1a antibody (upper blot) and anti-Hcp1 antibody (lower blot). The expected position of the proteins of interest are indicated on the right of the blot, and molecular weight standards (in kDa) are indicated on the left. Cell extract from a PAK ΔH1-T6SS strain is included as a control to confirm the identity of VgrG1a and Hcp.

Cell extracts from cultures of *P. aeruginosa* PAK grown with increasing concentrations of kanamycin were analysed by western blot. The resulting blots can be seen in Figure 3.5B. The upper panel shows blots probed with anti-VgrG1a antibody, and the lower with anti-Hcp1. *P. aeruginosa* PAK cells grown without kanamycin have very low levels of Hcp1 and VgrG1a production. Upon addition of kanamycin, the intensity of bands corresponding to VgrG1a and Hcp1 increases, with maximum

induction occurring at 60 μgml^{-1} of kanamycin, confirming that subinhibitory concentrations of kanamycin induce the production of two core H1-T6SS components.

The identity of bands corresponding to Hcp1 and VgrG1a are confirmed using an isogenic PAK strain in which part of the H1-T6SS cluster has been deleted (PAK ΔH1-T6SS). This strain carries a clean deletion of genes from *hsiA1* to *vgrG1a,* as indicated by the hatched box in Figure 3.5A. When PAK ΔH1-T6SS is incubated with 60 μgml^{-1} kanamycin, the bands observed in the wild type strain under the same conditions are absent, confirming the identity of these proteins as Hcp1 and VgrG1a (Figure 3.5B).

A second band recognised by the VgrG1a antibody shows a dose dependent increase in intensity with increasing kanamycin concentration, which migrates with a molecular weight higher than that of VgrG1a. Comparisons with previously published observations indicate that this band likely corresponds to VgrG1c, which is also recognised by this antibody (Hachani et al., 2011). However, the exact identity of this protein cannot be confirmed since the gene encoding VgrG1c is not disrupted by the mutation used.

This data shows that in addition to inducing biofilm formation, sub inhibitory concentrations of kanamycin also induce the production of core components of the H1-T6SS. This observation suggests that biofilm formation and the H1-T6SS are co-regulated by subinhibitory concentrations of kanamycin, which is of interest, since they have previously been shown to be co-regulated by the RetS/Gac/Rsm regulatory cascade (Goodman et al., 2004). However, the same cannot be said for gentamycin and tobramycin, which induce biofilm formation but do not appear to have an effect on the production of H1-T6SS components under the conditions tested.

3.4.2 Dependency on the Ret/Gac/Rsm pathway

The results previously discussed in this chapter show that kanamycin is responsible for the induction of two phenotypes; biofilm formation and production of core H1-T6SS components. These two phenotypes are also co-regulated by the RetS/GacS/Rsm cascade, as described in section 1.9. However, it is not known if the regulatory cascade and kanamycin converge in a single regulatory pathway, or if both act independently to control these phenotypes. To address this question the dependency of the kanamycin induced phenotypes on the components of the cascade was investigated.

The components and hierarchy of the Ret/Gac/Rsm cascade is shown in Figure 3.6A. *P. aeruginosa* PAK strains carrying clean deletions in components of the cascade have previously been generated and were available for testing (Bordi et al., 2010; Ventre et al., 2006). The parental wild type PAK strain and isogenic deletion mutants in components of the regulatory cascade were incubated with and without kanamycin at 30 $\mu g ml^{-1}$, and cell extracts were analysed by western blot. Kanamycin 30 $\mu g ml^{-1}$ was used, since at this concentration the growth of all strains was not significantly affected by addition of kanamycin, as shown in Figure 3.6B.

The western blot obtained can be seen in Figure 3.6B. Cell extracts of the wild type PAK strain shows that production of VgrG1a, VgrG1c, and Hcp1 are induced upon addition of kanamycin, as expected. Cell extracts from mutants in two membrane bound sensor kinases (*ladS* and *gacS*), the response regulator *gacA*, and a double mutant in the regulatory RNAs *rsmY* and *rsmZ* incubated with or without kanamycin, are shown. Addition of kanamycin to the growth media of each deletion mutant tested does not result in increased production of VgrG1a, VgrG1c or Hcp. This observation suggests that in the absence of a functional RetS/GacS/Rsm regulatory cascade the induction of the H1-T6SS by subinhibitory concentrations of kanamycin is unable to occur.

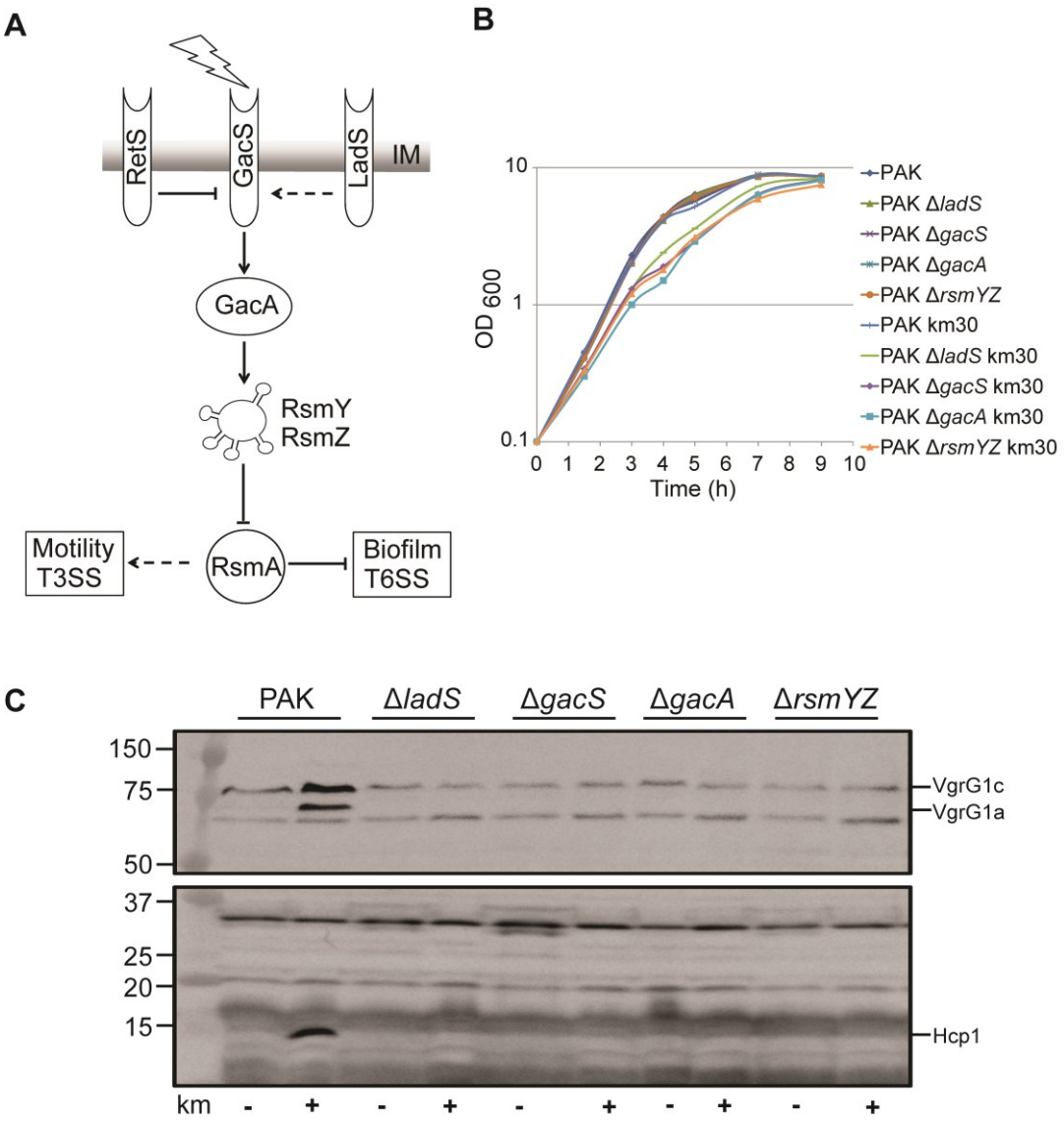


Figure 3.6 Kanamycin induction of the H1-T6SS requires a functional LadS/Gac/Rsm pathway
A. Schematic representation of the Gac/Rsm signalling cascade, as previously introduced. B. Planktonic growth of PAK and isogenic mutants in components of the LadS/Gac/Rsm cascade with and without kanamycin at 30 µg/ml. Western blot of whole cell extracts from PAK and isogenic deletion mutants in components of the regulatory cascade as indicated above the blot. Strains were incubated with or without kanamycin at 30 µg/ml as indicated below the blot. Cell extracts were probed with anti-VgrG1a (upper blot) or anti-Hcp1 (lower blot). The expected positions of the proteins of interest are indicated on the right of the blot, with molecular weight markers (in kDa) on the left.

To place induction of H1-T6SS components by subinhibitory kanamycin concentrations into context, the levels observed under these conditions were compared to the levels seen by deletion of *retS*. Deletion of the membrane sensor *retS* is known to strongly activate the H1-T6SS (Goodman et al., 2004; Mougous et al., 2006). PAK and PAK Δ*retS* were incubated with or without kanamycin, and cell extracts probed with anti-Hcp1 antibody. The resulting blot can be seen in Figure 3. 7. The levels of Hcp1 produced in response to kanamycin are much lower than observed in the *retS* mutant, which is considered to be a constitutively active T6SS. Addition of kanamycin to a *retS* mutant does not affect the level of Hcp1 produced. From this observation it is clear that although kanamycin results in increased Hcp1 production, the induction is at quite a low level compared to PAK Δ*retS*.

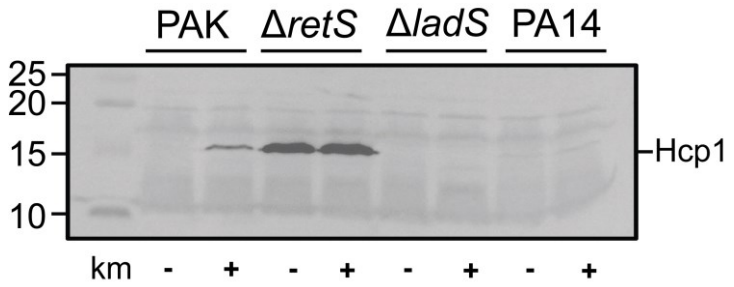


Figure 3.7 Impact of kanamycin on Hcp1 production in various *P. aeruginosa* backgrounds
Western blot showing Hcp1 production in whole cell lysates of *P. aeruginosa* PAK, PAKΔ*retS*, PAKΔ*ladS* and *P. aeruginosa* PA14. Each strain was grown with or without kanamycin at 30 µg/ml, as indicated below the blots. The position of Hcp1 is indicated on the right, with molecular weight markers indicated on the left (in kDa).

In addition, Figure 3.7 shows the effect of subinhibitory concentrations of kanamycin on the production of Hcp1 in PAK Δ*ladS* and *P. aeruginosa* PA14. The lack of induction of Hcp1 production in a *ladS* mutant confirms previous observations. Hcp1 production is not observed in PA14 with or without kanamycin, indicating that kanamycin induction of the H1-T6SS is not conserved in all *P. aeruginosa* strains. The PA14 strain has a similar phenotype to a PAK Δ*ladS* mutant, and PA14 carries a defective *ladS* gene encoding a truncated LadS protein (Mikkelsen et al., 2011a). Given that

PA14 is a *ladS* mutant, this confirms the observation that in the absence of a functional RetS/Gac/Rsm cascade, the induction of the H1-T6SS by subinhibitory concentrations of kanamycin is unable to occur. The PAO1 strain already has a high basal level of H1-T6SS activity in the absence of kanamycin, and the addition of kanamycin does not affect Hcp levels (data not shown). The genetic basis for the increased H1-T6SS production in PAO1 compared to PAK is unknown.

3.4.3 Effect of kanamycin the transcription and translation of the H1-T6SS

As previously described in section 1.8, the Ret/Gac/Rsm pathway influences the H1-T6SS at the translational level by reducing the levels of free RsmA, a translational repressor of H1-T6SS mRNA. Transcriptional and translational fusions of *lacZ* to *hsiA1*, the first gene of the H1-T6SS cluster, at neutral chromosomal locations, were obtained from Dr Stephen Lory (Harvard University) and used to investigate the impact of kanamycin at the transcriptional and translational levels of H1-T6SS expression (Brencic and Lory, 2009). The activity of these fusions was tested under varying concentrations of kanamycin. A strain carrying the transcriptional or translational fusion in an *rsmA* mutant background, in which the H1-T6SS is de-repressed and constitutively active, was included as a positive control.

The result of β-galactosidase assays performed on cell lysates is shown in Figure 3.8. Figure 3.8A shows the activity of the transcriptional *lacZ-hsiA1* fusion. Addition of kanamycin to strains carrying this construct does not result in a detectable increase in the transcription of *hsiA1*. At 15 μgml^{-1} kanamycin the activity of the fusion is equivalent to that with no kanamycin. At 30 and 50 μgml^{-1} there seems to be a reduction in the activity of this transcriptional fusion, which is not consistent with increased production of H1-T6SS components. Therefore, it is not likely that the effect of kanamycin on the production of the H1-T6SS occurs at the transcriptional level.

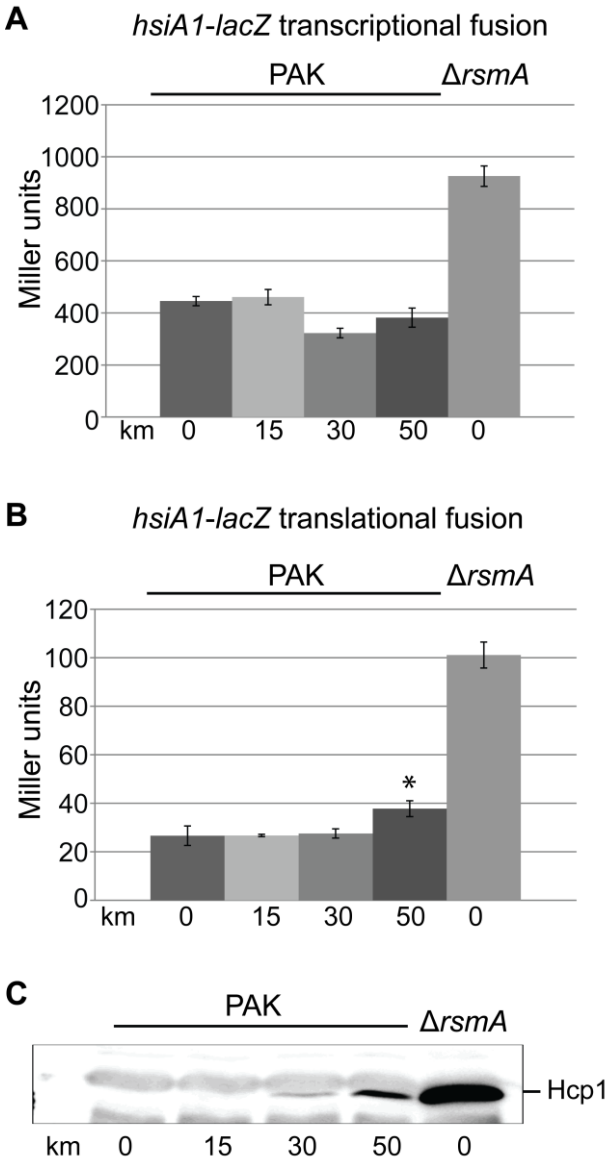


Figure 3.8 Effect of kanamycin on expression of PA0082 (*hsiA1*)

Levels of β–galactosidase activity in PAK carrying chromosomal transcriptional (A) or translational (B) fusions of the *hsiA1* gene (PA0082) to *lacZ* with increasing concentrations of kanamycin as indicated on the x-axis. Both fusions introduced into PAK Δ*rsmA* background were included as a positive control showing increased transcription and translation. * indicates a reproducible statistically significant difference compared to incubation without kanamycin (Student's T Test, $P < 0.05$). C. Western blot showing levels of Hcp1 in the translational fusion strain under corresponding kanamycin concentrations.

Figure 3.8B shows the activity of a translational *hsiA1-lacZ* fusion. At lower concentrations of kanamycin (15 and 30 $\mu g ml^{-1}$) there is no detectible increase in the activity of the *hsiA1-lacZ* fusion. However, at 50 $\mu g ml^{-1}$ there is a small, but statistically significant and reproducible, increase in the activity of this fusion. This suggests that the induction of H1-T6SS by kanamycin may be due to

increased translation of H1-T6SS mRNA. This correlates with the levels of Hcp1 produced under these conditions, as shown in Figure 3.8C, with the small increase in *hsiA1* translation corresponding to a small increase in the amount of Hcp1 produced under the same conditions, in the strain carrying the translational reporter construct.

The kanamycin induced production of H1-T6SS components seem to occur, at least in part, at the translational level. This corresponds to the regulation of the H1-T6SS at the translational level by the Gac/Rsm pathway. However, it cannot be concluded that the induction by these two mechanisms occurs via the same regulatory pathway.

3.5 Sub inhibitory aminoglycosides and the T3SS

Two RetS/Gac/Rsm cascade regulated phenotypes (biofilm formation and the H1-T6SS) are induced by subinhibitory concentrations of kanamycin. Because of this, the effect of kanamycin on a third cascade dependent phenotype, the T3SS, was investigated. The T3SS is inversely regulated with biofilm formation by this cascade as discussed in section 1.9. The *P. aeruginosa* T3SS is responsible for cytotoxicity during acute infections, and secretes potent effectors, as described in Section 1.6.

To monitor T3SS activity cell extracts and purified supernatants were probed with an antibody targeted towards a core structural T3SS component, PcrV. PcrV, is the tip of the T3SS needle, and is found in the supernatant of cells with an active T3SS. *P. aeruginosa* PAK cells were incubated with increasing concentrations of kanamycin, and the resulting western blot can be seen in Figure 3.9.

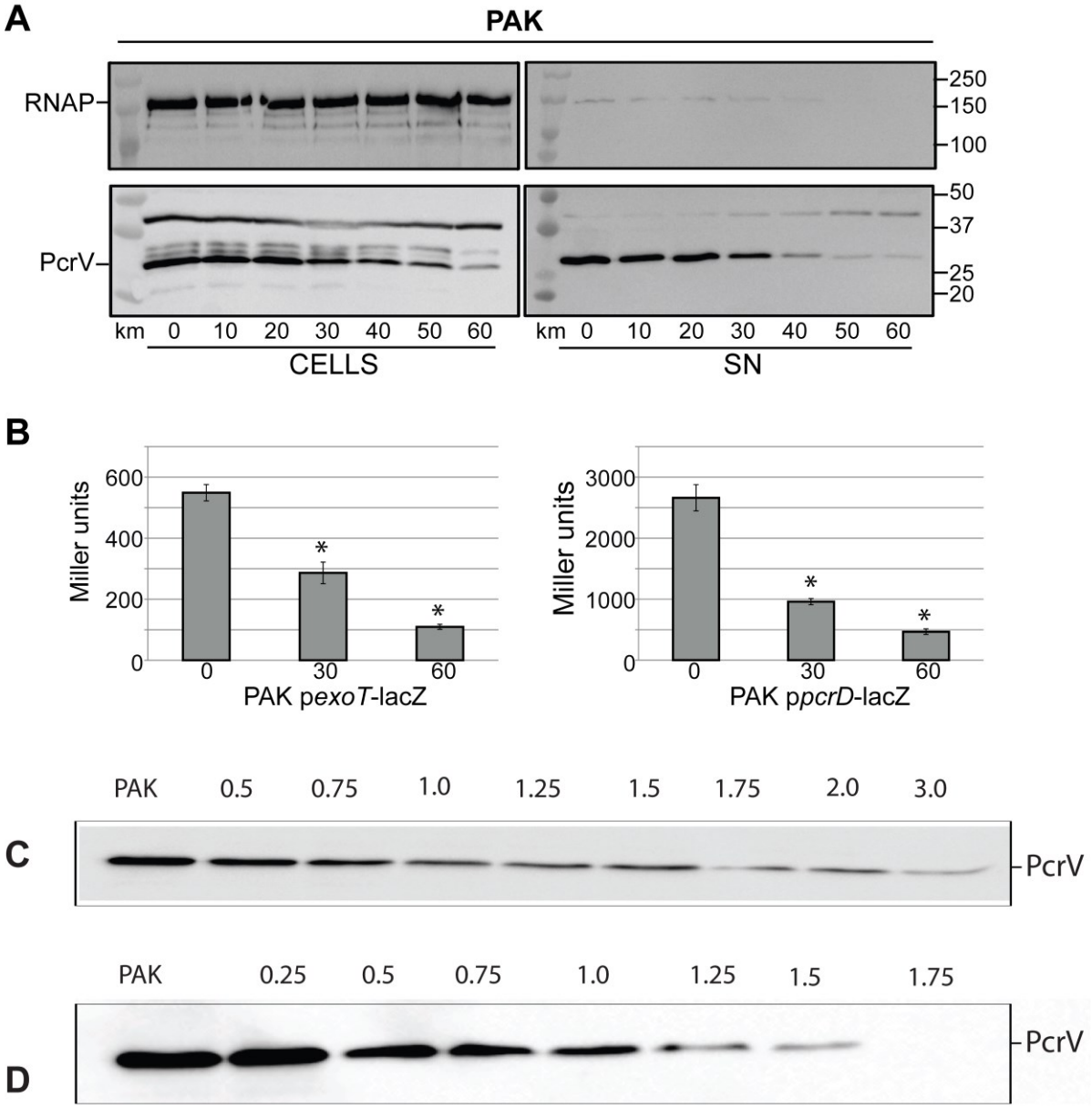


Figure 3.9 Kanamycin represses the T3SS

A. Western blots of whole cell extracts (left) and supernatant fractions (right) from PAK cells grown under increasing levels of kanamycin, as indicated below the blots. Upper blots are probed with anti-RNA polymerase, and lower blots are probed with anti-PcrV. The expected position of these proteins is indicated on the left of the blots, while molecular weight markers in kDa are shown on the right. B. Effect of kanamycin on the activity of *lacZ* transcriptional fusions with *exoT* or *pcrD*. β–galactosidase activity (Miller units) is shown for PAK strains carrying these plasmid borne fusions, incubated with increasing kanamycin concentration, as indicated on the x axis. * indicates a statistically significant difference in activity compared to the strain incubated without kanamycin. Error bars show standard deviation. C. and D. show western blots probing whole cell extracts from cell incubated with gentamycin (C) and tobramycin (D) with anti-PcrV antibody.

Analysis of whole cell fractions in Figure 3.9A reveal a background level of PcrV production in *P. aeruginosa* PAK incubated without kanamycin, observed as a band at 32 kDa. With increasing kanamycin concentration the intensity of this band decreases, indicating that production of PcrV is reduced with increasing kanamycin. This effect can also be observed in the supernatant fractions, where the level of secreted PcrV decreases as the concentration of kanamycin is increased. RNA polymerase, a cytoplasmic protein, is observed at a constant level in all cell extracts, but is not see in the supernatant samples, indicating that the localisation of PcrV in the supernatant is due to secretion and not an artefact of cell lysis.

In addition to western blot analysis, the effect of kanamycin on the transcription of T3SS genes was analysed, with fusions of T3SS promoters to the *lacZ* gene as previously described (Bleves et al., 2005). Two fusions were tested, a fusion to the promoter region of *exoT*, a secreted effector of the T3SS, and a fusion to the promoter region of *pcrD*, a structural component of the T3SS. Cells containing the indicated plasmid borne gene fusion were incubated with increasing kanamycin concentration, and the resulting beta-galactosidase activity measured, as shown in Figure 3.9B. It is clear that as kanamycin concentration increases, the activity of both fusions is reduced. The activity of the *exoT* fusion is reduced 1.9 fold upon addition of 30 μgml^{-1} and 5.0 fold at 60 μgml^{-1} (Students T Test, $P < 0.05$ in both cases). Similarly, the activity of the *pcrD* fusion is reduced 2.8 fold at 30 μgml^{-1} and 5.7 fold at 60 μgml^{-1} (Students T test, $P<0.05$ in both cases).

Two other aminoglycosides, gentamycin and tobramycin, were also tested for their effect on the T3SS by analysing the amount of PcrV in the supernatant with increasing concentrations of these antibiotics, as seen in Figure 3.9C and 3.9D. The amount of secreted PcrV decreases with increasing concentrations of both tobramycin and gentamycin, similar to the effect seen with kanamycin. Unlike that seen with induction of the H1-T6SS, which was only seen with kanamycin, the repression of the T3SS seems to be common to all of the aminoglycosides tested.

These results show that the addition of aminoglycosides has a negative effect on the T3SS and leads to a reduction in the production and secretion of PcrV, and in the expression of *exoT* and *pcrD*. This observation is similar to the downregulation of the T3SS by the RetS/Gac/Rsm cascade. All of the aminoglycosides tested are able to induce biofilm formation and repress the T3SS, but only kanamycin induces the H1-T6SS under the conditions tested. It is clear that the response to subinhibitory aminoglycosides, especially kanamycin, shows striking similarity to regulation by the RetS/Gac/Rsm cascade. It still remains to be shown whether these two mechanisms converge into a single pathway or are distinct regulatory mechanisms.

3.6 Effect of tetracycline, a non-aminoglycoside

To further understand the response of *P. aeruginosa* PAK to subinhibitory aminoglycosides, the response of *P. aeruginosa* PAK to tetracycline was investigated in order to observe the broader effects of antibiotic challenge. Tetracycline has a different chemical structure to the antibiotics described thus far, as shown in Figure 3.10A. Tetracycline targets protein synthesis, binding to the 30S subunit of the microbial ribosome, but unlike aminoglycosides, prevents the introduction of new amino acids into the growing polypeptide chain at the A-site, rather than causing misreading of the mRNA. Tetracycline is bacteriostatic, and inhibits bacterial growth.

The effect of a range of tetracycline concentrations was tested on the growth of *P. aeruginosa* PAK, and the resulting growth curves are shown in Figure 3.10B. At concentrations of tetracycline below 1.5 μgml^{-1} *P. aeruginosa* PAK growth is not affected. At concentrations between 3.0 μgml^{-1} and 10.0 μgml^{-1} tetracycline has a small impact on the growth of this strain; however, even at the highest concentration tested the final cell density is equivalent to cells grown in the absence of tetracycline.

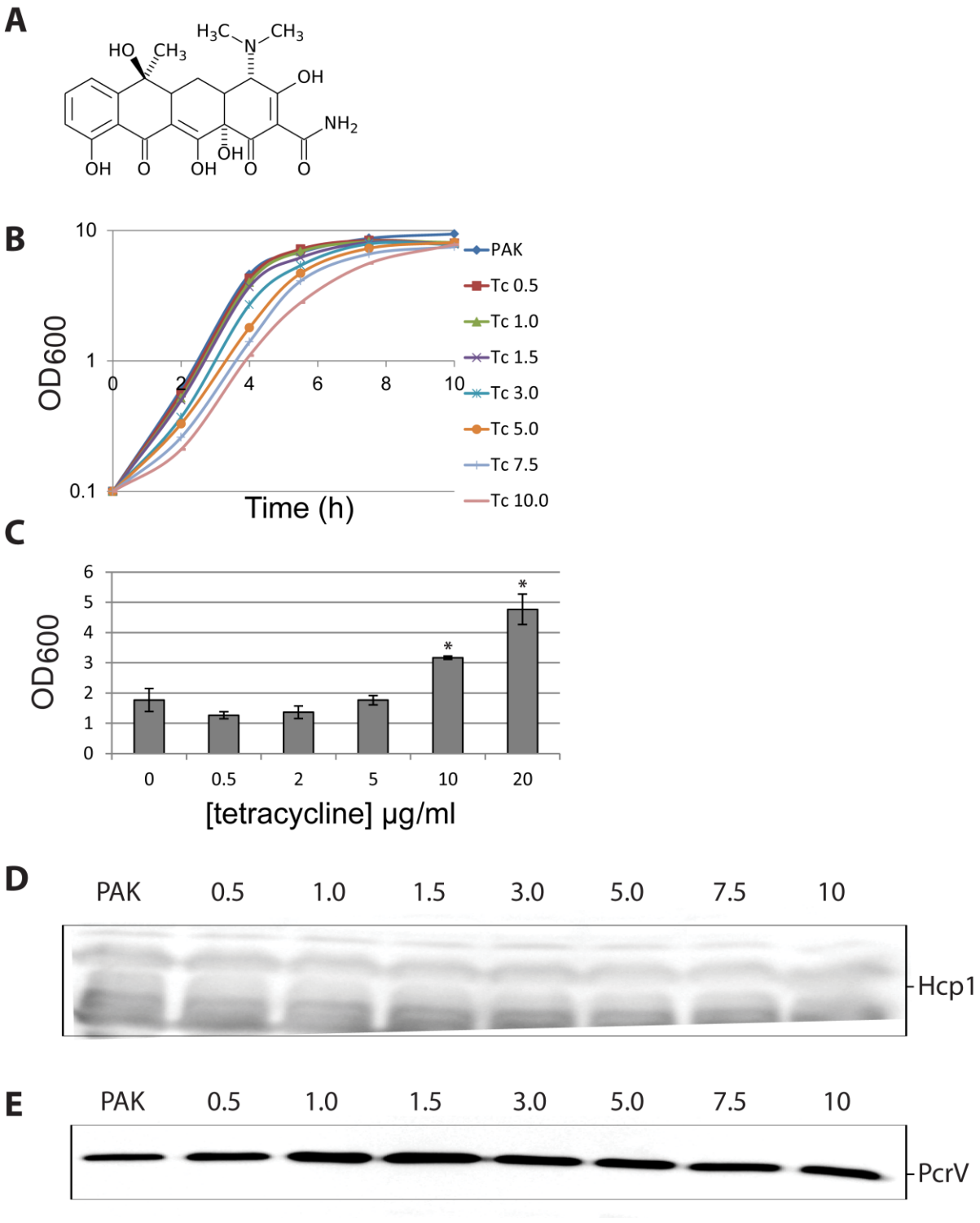


Figure 3.10 Response of PAK to subinhibitory concentrations of tetracycline

A. Molecular structure of tetracycline. B. Planktonic growth of PAK grown with increasing concentrations of tetracycline over a ten-hour period. C. Biofilm formation by PAK incubated with increasing concentrations of tetracycline. D. and E. Hcp1 and PcrV production, respectively, in PAK cells incubated with increasing concentrations of tetracycline.

A biofilm production assay was performed with *P. aeruginosa* PAK, using a suitable range of tetracycline concentrations. The result of this assay is shown in Figure 3.10C. As observed with aminoglycosides, tetracycline also induces biofilm formation in *P. aeruginosa* PAK. At tetracycline concentrations of 10 μgml^{-1} and 20 μgml^{-1} the level of biofilm formation is 1.7 fold higher ($P<0.05$, students T test), and 2.6 fold higher ($P<0.05$, students T test) respectively. This suggests that the impact on biofilm formation may be a general response by *P. aeruginosa* PAK to antibiotic induced stress or protein synthesis inhibition, and the specific chemical structure of the antibiotic may not be important.

The effect of tetracycline on the H1-T6SS phenotypes of *P. aeruginosa* PAK is shown in Figure 3.10D. There is no production of Hcp1 in cells incubated with tetracycline, even at the highest concentration used. The effect on production of H1-T6SS components appears specific to kanamycin, and not due to a reduction in growth rate or protein synthesis inhibition, since other aminoglycosides and tetracycline do not show a similar effect on Hcp production, despite similarly impacting the growth of PAK.

Secreted levels of PcrV are constant with increasing tetracycline concentration, as shown in Figure 3.10E, suggesting that the T3SS is not repressed by subinhibitory concentrations of this antibiotic. This is a striking observation, since the T3SS was seen to be downregulated with kanamycin, gentamycin, and tobramycin (Figure 3.9). However, even though the range of tetracycline concentrations used produces a similar effect on the growth rate as other antibiotics tested, there is no similar reduction in the secretion of PcrV, indicating that the reduction of T3SS activity is a specific feature of aminoglycosides. The reduction of T3SS is not caused by a slowed growth rate or protein synthesis inhibition, since it is not seen when tetracycline induces similar effects.

In summary, the results outlined show a complex effect of subinhibitory antibiotics on various phenotypes of *P. aeruginosa* PAK. Biofilm formation is induced by all aminoglycosides tested and tetracycline and is likely a non-specific effect of reducing growth rate or protein synthesis inhibition. The effect on secretion phenotypes is not as clear. Induction of the H1-T6SS components appears to be specific to kanamycin, since other aminoglycosides and tetracycline do not induce a similar response. Repression of the T3SS system appears to be aminoglycoside specific, as kanamycin, gentamycin, and tobramycin cause decreased PcrV secretion, whereas tetracycline does not.

3.7 Kanamycin as an activator of H1-T6SS

The H1-T6SS is known to be regulated by the Ret/Gac/Rsm cascade, but the precise signals required for its activation are unknown. The observation that kanamycin causes an increase in production of core H1-T6SS components, Hcp1 and VgrG1a, led to the hypothesis that kanamycin may be a natural signal activating this secretion system. Additional H1-T6SS mediated phenotypes were tested to investigate the role of kanamycin in the secretion and activity of the H1-T6SS.

3.7.1 Effect of kanamycin on Hcp1 and VgrG1a Secretion

Production of Hcp1 and VgrG1a has been shown to increase in response to the addition of subinhibitory concentrations of the aminoglycoside antibiotic kanamycin. Despite being structural parts of the secretion machinery, these components are routinely found in the culture supernatant in a T6SS dependent manner, and Hcp secretion is a hallmark of an active T6SS (Hachani et al., 2011; Mougous et al., 2006). The secretion of Hcp1 and VgrG1a was investigated upon addition of kanamycin to test if these components are also secreted.

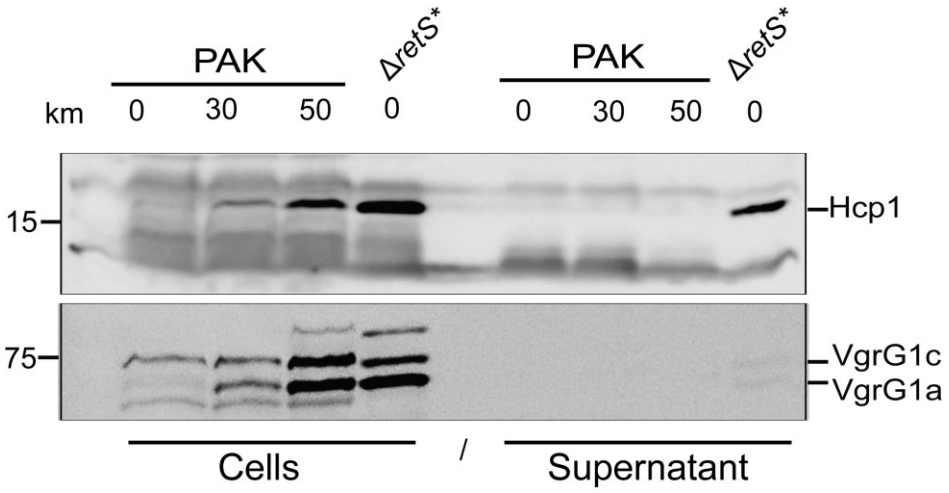


Figure 3.11 Kanamycin does not induce H1-T6SS-dependent secretion
A secretion assay performed on PAK cells incubated with increasing concentrations of kanamycin as indicated above the blots. Whole cell extracts are shown on the left, while supernatants are shown on the right. Blots are probed with anti-Hcp1 (upper blot) or anti-VgrG1a (lower blot). The expected location of each protein is indicated on the right, and molecular weight standards shown on the left. The PAKΔ*retS* strain is included as a positive control for secretion. * denotes that the PAKΔ*retS* sample has been diluted 1 in 10 with loading buffer to reduce Hcp1 signal in this strain.

Analysis of the western blot shown in Figure 3.11 shows that production of this Hcp1 is increased as kanamycin concentration increases, as expected. However, Hcp1 is not detectible in the supernatant fractions of PAK strains incubated with kanamycin. A *retS* mutant is included as a positive control, as it is known to produce and secrete Hcp1 abundantly. Detection of Hcp1 in the supernatant of the *retS* control indicates that the secretion assay was successfully performed, and confirms that Hcp1 is not secreted in response to subinhibitory concentrations of kanamycin.

Production of VgrG1a and VgrG1c are also increased upon arabinose induction, but as with Hcp1, their presence in the supernatant fraction is not detectible. VgrG1a and VgrG1c are detectible in the cell fraction of the *retS* mutant, however, only traces of these proteins are detected in the supernatant. This is possibly due to the lower abundance of these proteins compared to the multicopy Hcp tubes. Although components of the H1-T6SS are produced in response to incubation

with subinhibitory concentrations of kanamycin, their secretion into the supernatant cannot be detected. This suggests that the system is not functional under these conditions, when compared to the activated H1-T6SS activated by the deletion of *retS*.

3.7.2 Effect of kanamycin on H1-T6SS toxin production

Three toxins, Tse1-3, are known to be bacterial targeted secreted substrates of the H1-T6SS, described in details in Section 1.7 (Hood et al., 2010). Tse1-3 are encoded distantly to the core H1-T6SS cluster, but are known to be coregulated with it via *retS* (Hood et al., 2010). To further investigate the role of kanamycin as a signal for the activation of the H1-T6SS, the production of Tse3, a H1-T6SS peptidoglycan targeting substrates was tested in response to kanamycin (Russell et al., 2011). If the H1-T6SS is activated in response to subinhibitory kanamycin concentrations, the levels of Tse3 will be expected to be higher under the same conditions.

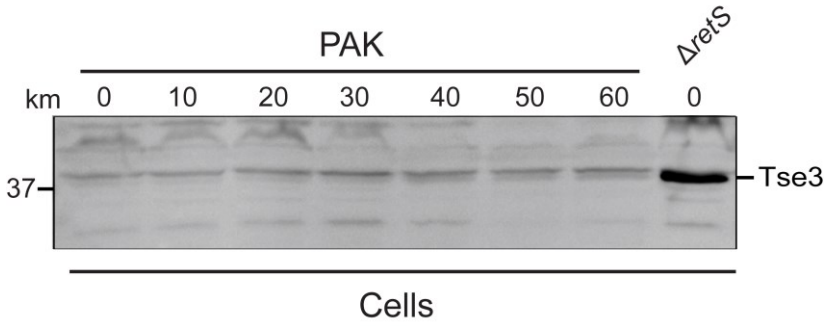


Figure 3.12 Kanamycin does not induce Tse3 production
Western blot performed with anti-Tse3 antibody on whole cell extracts prepared from PAK cells grown with increasing concentrations of kanamycin. PAKΔ*retS*, is included as a control as it is known that Tse3 is induced in this background.

Antibodies were previously generated against specific peptides within Tse3 (Hachani et al., 2011). Cell extracts from cultures incubated with increasing kanamycin concentration were probed by

western blotting for production of Tse3, as shown in Figure 3.12. A *retS* deletion mutant shows a band of high intensity corresponding to Tse3, confirming the specificity of this antibody.

Comparison of cell extracts from wild type PAK with this *retS* control shows that a corresponding Tse3 band is absent, even at the highest concentration of kanamycin. This suggests that even though Hcp1 and VgrG production is increased, Tse3 production is not induced by subinhibitory concentrations of kanamycin, again suggesting these conditions may not induce a functionally active H1-T6SS.

3.7.3 Effect of kanamycin on H1-T6SS mediated bacterial killing

The killing of competing bacteria through T6SS mediated puncturing and toxin delivery has been shown to be the role of the *P. aeruginosa* H1-T6SS (Basler et al., 2012). Investigating bacterial H1-T6SS mediated bacterial killing is therefore a useful alternative phenotype to observe the effect of kanamycin on the activity of the H1-T6SS. A visual assay has been developed in our laboratory to observe T6SS mediated *E. coli* killing by *P. aeruginosa* (Hachani et al., 2013). This assay was used to investigate if incorporation of subinhibitory concentrations of kanamycin to the growth medium improved the ability of *P. aeruginosa* PAK to kill competing *E. coli* cells in a H1-T6SS dependent manner.

In this experiment *P. aeruginosa* PAK or an isogenic PAK Δ*retS* was co-incubated with *E. coli* carrying a plasmid allowing alpha complementation of β-galactosidase, causing colonies to appear blue on X-gal plates. The individual strains used in the assay can be seen in Figure 3.13A. The *E. coli* prey strain shows a clear blue colour, while the *P. aeruginosa* strains do not. Each of these strains also grew to comparable levels on plates containing 50 $\mu g ml^{-1}$ of kanamycin (data not shown).

The co-incubation steps were performed on agar plates either without kanamycin or with 50 µgml^{-1} kanamycin for five hours, to allow bacterial competition to take place. Following co-incubation, cells were harvested and serially diluted onto LB plates supplemented with X-gal, but without kanamycin.

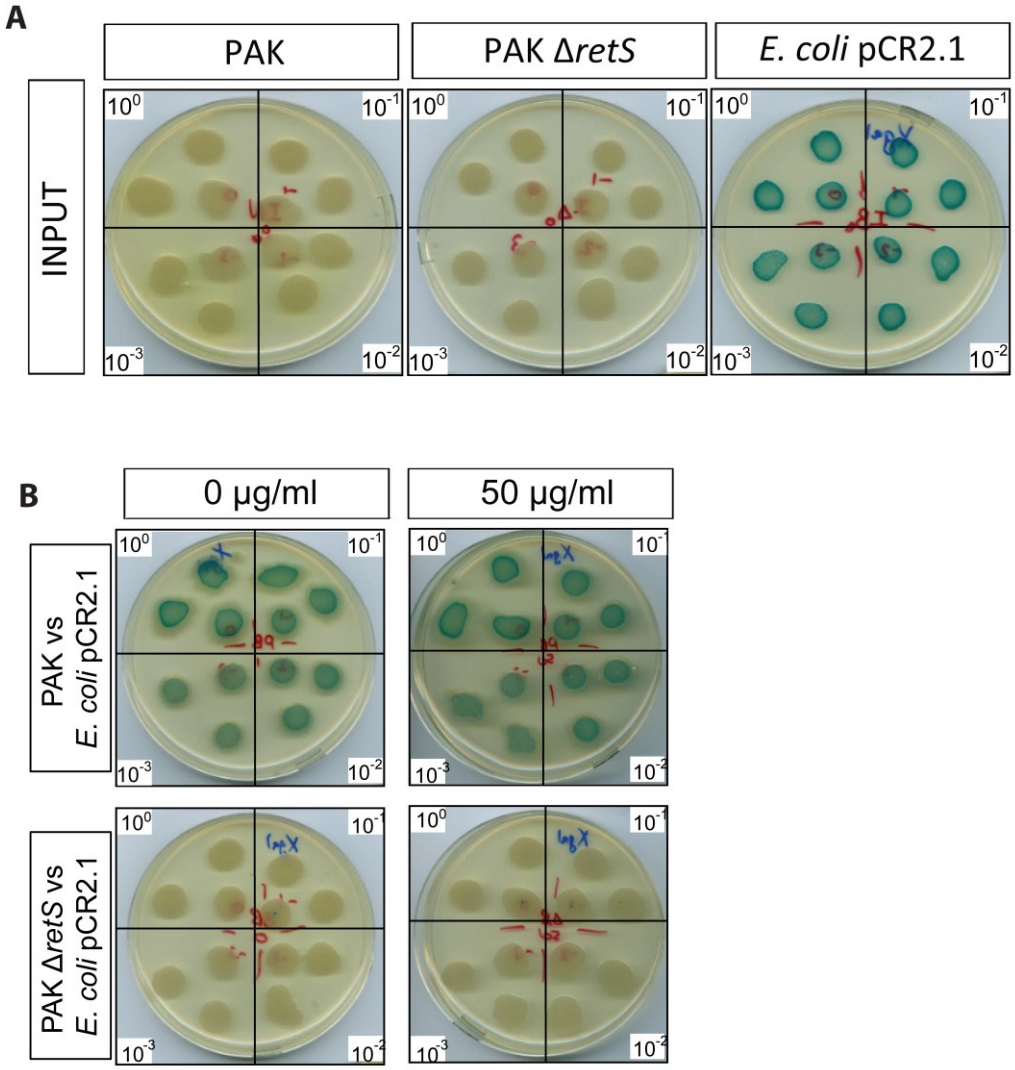


Figure 3.13 Kanamycin does not improve *P. aeruginosa* H1-T6SS mediated bacterial competition

E. coli cells carrying to pCR2.1 vector were co-incubated with either PAK wild type or PAKΔ*retS*. Panel A. shows the strains input into the assay in monoculture. Panel B shows the result of mixed culture competition assays on LB plates without antibiotics or in the presence of kanamycin at a concentration of 50 µg/ml for five hours. Following competition, cells were harvested and resuspended in LB broth, and a dilution series ranging from 10^0 to 10^{-3} was plated in triplicate onto LB plates supplemented with X-gal, as indicated by the white box in each corner. Visualisation of a blue colour gives an indication of *E. coli* survival.

Figure 3.13B shows the output of this bacterial competition assay. A blue colour, indicating recovery of viable *E. coli*, is observed when PAK is co-incubated with *E. coli*, indicating that this strain does not kill *E. coli*, as previously observed, due to low T6SS activity in the PAK strain (Hachani et al., 2013). *E. coli* is not recoverable when co-incubated with PAK Δ*retS* due to the constitutively active H1-T6SS in this strain. When PAK is co-incubated with *E. coli* in the presence of 50 $\mu g ml^{-1}$ kanamycin, there is no change in the levels of *E. coli* recovered, indicating that kanamycin does not enhance the *E. coli* killing activity of the *P. aeruginosa* PAK H1-T6SS under the conditions tested. It is clear from this assay that addition of kanamycin does not result in activation of the H1-T6SS.

In conclusion, despite activating the production of core components of the H1-T6SS, Hcp and VgrG, subinhibitory concentrations of kanamycin do not result in activation of other H1-T6SS dependent phenotypes. Although produced, Hcp and VgrG are not localised to the supernatant, indicating that the system is either not correctly assembled or not active. Production of a toxin associated with the system is not increased upon incubation with kanamycin, and the killing of *E. coli* by the *P. aeruginosa* PAK H1-T6SS is not improved in the presence of this antibiotic.

3.8 Discussion

The results presented in this chapter describe the response of *P. aeruginosa* to subinhibitory concentration of antibiotics. It has previously been reported that subinhibitory concentrations of aminoglycosides induce biofilm formation in *P. aeruginosa* PAO1 (Hoffman et al., 2005), and this response was tested here in *P. aeruginosa* PAK. The effect of subinhibitory aminoglycosides on additional phenotypes, namely the activity of the T3SS and T6SS, known to be coregulated with biofilm formation, was also tested. The data collected shows that *P. aeruginosa* PAK responds in different ways to subinhibitory concentrations of antibiotics.

All antibiotics tested caused increased levels of PAK biofilm formation at subinhibitory concentrations, however, an increase in the production of the T6SS was only observed with kanamycin, and not other very similar aminoglycosides. The T3SS system was downregulated in response to the aminoglycosides tested, but not when exposed to subinhibitory concentrations of tetracycline. These observations support the conclusions by Linares and colleagues that antibiotics are able to act as signalling agents as well as antibacterial weapons (Linares et al., 2006). The molecular mechanism of the response was also tested, and our data suggest that it is independent of c-di-GMP, while requiring a functional Gac/Rsm cascade.

3.8.1 PAK biofilm response to subinhibitory antibiotic concentrations

The results presented here show that subinhibitory concentrations of three aminoglycosides, namely kanamycin, gentamycin, and tobramycin, induce biofilm formation in *P. aeruginosa* PAK. This supports the observation by Hoffman and colleagues that aminoglycosides, namely tobramycin, amikacin, streptomycin, and gentamycin, induced biofilm formation in *P. aeruginosa* PAO1 (Hoffman et al., 2005). The fact that non-aminoglycosides (polymyxin B, carbenicillin and chloramphenicol) did not induce biofilm formation led to the conclusion that the response was specific to aminoglycosides (Hoffman et al., 2005). Additional studies, and data presented in this chapter show that non-aminoglycosides such as tetracycline and members of the quinolone family also induce bacterial biofilm formation in *P. aeruginosa* PAO1 and PAK (Gotoh et al., 2008; Linares et al., 2006), indicating that the biofilm response is likely not specific to one class of antibiotics.

A limited microarray probing the expression of 555 PAO1 genes in response to subinhibitory concentrations of tobramycin, ciprofloxacin, and tetracycline has been previously performed (Linares et al., 2006). A distinct expression profile was obtained for each of these antibiotics, which are from distinct classes and have different modes of action. It is likely that the response by *P.*

aeruginosa is a general response to antibiotic induced stress, and not due to specific recognition of such a diverse group of antibiotic molecules with varying cellular targets. Given the current evidence it is not clear why some antibiotics can and others cannot induce biofilm formation, and the effect of any untested antibiotics is unpredictable.

The biofilm experiments performed in this chapter are focused on the early stages of biofilm formation (attachment), since the crystal violet assays measure the ability of *P. aeruginosa* to attach to surfaces. To test the influence of aminoglycosides on mature biofilm formation, flow cell assays could be employed, which would allow direct microscopic visualisation of biofilm development (Sternberg and Tolker-Nielsen, 2006)

3.8.2 The molecular determinants of the biofilm response

A striking observation in this work is that *P. aeruginosa* PAK lacks the *arr* gene, which was shown to be involved in the *P. aeruginosa* PAO1 biofilm response to subinhibitory concentrations of tobramycin. The induction of biofilm formation induced by tobramycin does not occur when *arr* is disrupted by a transposon insertion, and can be restored by the expression of *arr* from a plasmid (Hoffman et al., 2005). Membranes from cells in which the *arr* is disrupted show reduced phosphodiesterase activity, confirming a role for Arr in the degradation of the second messenger c-di-GMP in the biofilm response (Hoffman et al., 2005). This remains the only example of low levels of c-di-GMP increasing biofilm formation, since low levels of c-di-GMP usually reduce biofilm formation.

It is proposed that in PAK the biofilm response is independent of c-di-GMP, since a change in the levels of this second messenger could not be detected upon addition of kanamycin by the methods employed, despite this antibiotic having a significant effect on biofilm formation. The levels of c-di-

GMP in wild type PAK were low, it is reported that the fusion is not sensitive enough to detect small changes in c-di-GMP levels (Rybtke et al., 2012). To confirm that biofilm formation in PAK in response to subinhibitory antibiotics is independent of c-di-GMP a more sensitive assay should be employed, such as measuring the levels using mass spectroscopy techniques such as LC-MS/MS (Simm et al., 2009), which would also represent a direct approach compared to using the Gfp reporter assay.

In the publication of Hoffman et al, *arr* is shown to be required for biofilm formation in response to subinhibitory concentrations of tobramycin, and to have phosphodiesterase activity, but it was not directly shown that subinhibitory tobramycin concentrations resulted in a measurable change in cellular c-di-GMP levels in PAO1. To confirm that using the *cdrA-gfp* fusion reporter is a suitable approach, it should be shown, using this fusion, that c-di-GMP levels are measurably affected by tobramycin in *P. aeruginosa* PAO1. Even though Arr has phosphodiesterase activity, the changes induced by antibiotics may not be detectible.

The fact that *arr* is absent from PAK indicates that other regulatory components mediate the response to subinhibitory antibiotics in this strain. The role of *arr* in the biofilm response of PAO1 was identified in a screen of transposon mutants in each of the 38 PAO1 GGDEF/EAL domain proteins, which were unable to form biofilms in response to subinhibitory tobramycin concentrations. A similar screen in the PAK strain would reveal if GGDEF or EAL proteins other than Arr are required for the response in this strain, especially if there is such a protein encoded by PAK which is absent in PAO1. If all GGDEF/EAL proteins are dispensable in the PAK biofilm response to aminoglycosides then this confirm a c-di-GMP independent response. If the screen reveals a GGDEF/EAL is required for the aminoglycoside biofilm response, then this would confirm c-di-GMP dependence, and suggest the Arr function is conserved by a different EAL protein. An alternative approach would be to perform a random transposon mutagenesis screen to identify genes required

for the response of *P. aeruginosa* PAK to aminoglycosides. This approach would not be focused on the GGDEF/EAL proteins alone, and should provide information on other regulatory proteins involved, which would be particularly interesting if the PAK response is c-di-GMP independent.

3.8.3 The T3SS response to subinhibitory antibiotics

Kanamycin, gentamycin, and tobramycin were shown to reduce the expression of the T3SS. Strikingly, the levels of PcrV, a core T3SS component, did not decrease upon increasing concentrations of tetracycline, despite a similar impact on bacterial growth. This may suggest that the aminoglycosides specifically repress the T3SS. However, Linares et al (2006) have shown using semi-quantitative RT-PCR, that a subinhibitory concentration of tetracycline induces the expression of *exsA*, the master regulator of the T3SS, and *exoS*, a secreted effector, in *P. aeruginosa* PAO1. The expression of these genes is not affected by subinhibitory levels of tobramycin or ciprofloxacin. The cytotoxicity of bacteria exposed to subinhibitory concentrations of tetracycline is increased, correlating with the increased expression of the T3SS (Linares et al., 2006). Considering these observations, it is likely that the reduction of the T3SS by aminoglycosides may be a non-specific effect on the growth of *P. aeruginosa* PAK, which is not observed with tetracycline due to a specific induction of the T3SS which compensates for the growth effect.

3.8.4 The H1-T6SS response to subinhibitory antibiotics

In contrast to that observed for biofilm formation and expression of the T3SS, only kanamycin had an effect on the production of components of the H1-T6SS. Despite having a similar chemical structure, neither gentamycin nor tobramycin showed an effect on the H1-T6SS. The subinhibitory concentration of kanamycin (around 50 μgml^{-1}) is around 100 fold higher than those of either tobramycin or gentamycin (around 0.5 μgml^{-1}). *P. aeruginosa* is much more resistant to kanamycin

than to the other aminoglycosides tested, which may enhance the ability of kanamycin to induce the H1-T6SS.

The effect observed in response to the addition of subinhibitory concentrations of kanamycin mirrors that observed following the addition of subinhibitory concentrations of bile (Reen et al., 2012). Subinhibitory concentrations of bile salts induce biofilm formation and the H1-T6SS in *P. aeruginosa* PAO1, while negatively influencing the expression of the T3SS, which the authors concluded represents a switch towards a chronic *P. aeruginosa* lifestyle. However, the effect of bile on the H1-T6SS was tested at the transcriptional level and it remains to be shown if this results in a fully assembled and active H1-T6SS. Subinhibitory concentrations of both bile and kanamycin have a strikingly similar effect on the lifestyle of *P. aeruginosa,* but it is not known if this response is mediated via the same regulatory pathway.

Exact elucidation of the molecular mechanism in both of these cases is essential to understand the responses observed. This could be investigated by comparing the microarray data from cells incubated with either kanamycin or bile. Since the regulatory mechanism responsible for the response of *P. aeruginosa* to bile is not characterised, it would be interesting to investigate if this involves either c-di-GMP or the Gac/Rsm pathway. It was reported that quorum sensing was activated in response to bile, while swarming motility was reduced, and effect on these phenotypes by subinhibitory concentrations of kanamycin should be tested to see if the response is conserved.

3.8.5 Dependency of the H1-T6SS response on the RetS/Gac/Rsm cascade

A striking observation of this work is that the effect of subinhibitory concentrations of kanamycin is very similar to those observed upon activation of the RetS/Gac/Rsm cascade. Either subinhibitory concentrations of kanamycin or deletion of *retS* activates biofilm formation and the H1-T6SS, while

simultaneously repressing the T3SS, phenotypes which promote a chronic *P. aeruginosa* lifestyle. Due to this similar effect it was proposed that kanamycin may exert its effects by directly signalling into the regulatory cascade, or intersecting at some point within the network.

The dependency of kanamycin on the cascade was tested by observing the induction of the H1-T6SS in a panel of clean deletion mutants, in which components of the cascade were absent. A functional cascade is required for the effects of kanamycin on the H1-T6SS to be observed. The only significant effect of kanamycin upon transcriptional and translational fusions to *hsiA1*, the first gene of the H1-T6SS cluster, was a small increase in the translation of *hsiA1* at 50 µg/ml of kanamycin, which correlated with the levels of Hcp1 in this strain. The effect of the cascade and kanamycin on the H1-T6SS is thus likely exerted at the translational level. These observations alone do not confirm that the effect of kanamycin is directly mediated through the RetS/Gac/Rsm cascade, only that the functional cascade is required for the kanamycin response. The possibility remains while the T6SS is repressed by RsmA, addition of kanamycin has no effect, and is unable to relieve this dominant repression.

To further understand the link between the kanamycin response and the cascade, the impact of antibiotics on the expression of cascade components could be investigated. Addition of kanamycin may result in increased expression of the regulatory RNAs, RsmY and RsmZ, if the mechanism of T6SS activation by kanamycin is the same as the activation of the cascade achieved by deleting *retS*. Similarly, kanamycin may exert its effect through reduced expression of the translational regulator *rsmA*. These effects could be tested using β-lactamase reporter fusions to the promoter regions of these genes.

It is interesting to note that in the limited microarray performed by Linares et al (2006), the expression of *rsmA* is affected by each of the antibiotics tested. *rsmA* expression is upregulated

3.12-fold by tobramycin, but modestly downregulated 1.26-fold by ciprofloxacin, and 1.42-fold by tetracycline (Linares et al., 2006). Despite indicating a possible role for the Gac/Rsm cascade in the response to different antibiotics, the data does not correlate with what is observed, since although *rsmA* is inversely regulated by tobramycin and tetracycline, we observe biofilm formation in both cases, and only observe T3SS activation with tetracycline. Consistent with what is known about the cascade, increased *rsmA* expression by tobramycin should lead to suppression of biofilm formation, while the downregulation observed in response to tetracycline should result in downregulation of the T3SS. These inconsistencies suggest complex regulation of responses to antibiotics, involving numerous pathways depending on the antibiotic and strain used.

It was also observed that the tolerance of RetS/Gac/Rsm cascade mutants to antibiotics is lower than that of the wild type strain, suggesting that a response mediated via this cascade is protective to *P. aeruginosa* upon antibiotic stress. It has been reported that a *gacS* mutant has a minimum inhibitory concentration for gentamycin four fold lower than the wild type strain, and is more sensitive to amikacin, chloramphenicol, and cefpirome (Brinkman 2001). This observation provides indirect evidence that the RetS/Gac/Rsm cascade is important in the survival of *P. aeruginosa* exposed to antibiotics, however it is still unclear whether this effect is due to the inability to respond by adopting a protective biofilm lifestyle with associated secretion phenotypes.

The recent reporting of the crystal structure of the RetS periplasmic sensing domain revealed homology to a carbohydrate sensing domain (Vincent et al., 2010). It could be suggested that kanamycin directly binds to the sensing domain of RetS, since kanamycin is essentially a trisaccharide carbohydrate molecule. Once bound, kanamycin could potentially influence the oligomerisation and subsequent activity of RetS, acting as a direct signal for activation of the cascade and inducing the associated phenotypes. However, due to the diverse chemical nature of the antibiotics influencing the biofilm phenotype is it unlikely that this is the correct model.

3.8.6 Kanamycin as a signal for H1-T6SS activation

The observation that incubation with subinhibitory concentrations of kanamycin leads to increased production of H1-T6SS components is noteworthy, since the exact signals required for H1-T6SS activation remain elusive. The initial observation of the effect on production of both VgrG1a and Hcp1, core H1-T6SS components, led to further investigation of the role of kanamycin as a signal for H1-T6SS activation.

Despite our promising observation, it became clear that the H1-T6SS was not fully activated by subinhibitory concentrations of kanamycin. VgrG1a and Hcp1 could not be detected in the supernatant, indicating that the system was unlikely to be assembled and functional. In support of this, the production of a toxin transported by the H1-T6SS and coregulated by the RetS/Gac/Rsm cascade, was not induced by kanamycin in contrast to VgrG1a and Hcp1. Another test to probe the activity of the system showed that the H1-T6SS mediated killing of *E. coli* by *P. aeruginosa* was not enhanced when kanamycin was included in the growth media.

Comparison of the levels of Hcp1 produced following incubation with kanamycin with those of a *retS* mutant, in which the H1-T6SS is constitutively active, indicates that the kanamycin-induced levels are far lower. It is therefore unclear whether this low level of induction is sufficient to observe a biologically relevant phenotype. However, deletion of *retS* represents an artificially over-active H1-T6SS, and the native levels of H1-T6SS are likely much lower.

The observation that components of the system are up regulated in response to kanamycin is still an important observation. Despite not resulting in an active H1-T6SS, production of the components may result in an intermediate state where the T6SS is produced in readiness for an H1-T6SS attack. *P. aeruginosa* has co-existed with natural antibiotic producers in the soil for much longer than

antibiotics have been used clinically. Therefore, the response of *P. aeruginosa* to subinhibitory concentrations of antibiotics may represent a mechanism that has evolved to allow *P. aeruginosa* to sense bacterial competitors in the soil environment. The recognition of diffusible antibiotic molecules would indicate to *P. aeruginosa* that an antibiotic producing organism is in the vicinity. Up-regulation of H1-T6SS components would therefore be advantageous in allowing a rapid response should *P. aeruginosa* subsequently come into direct contact with antibiotic producing competitors. *Streptomyces* spp. are responsible for the production of many antibiotics, but the activity of the T6SS on Gram-positive organisms is yet to be observed. Preliminary experiments were performed using co-cultures of *P. aeruginosa* with *Streptomyces kanamyceticus*, the natural producer of kanamycin (data not shown). It was observed that *P. aeruginosa* was able to outcompete *S. kanamyceticus*, but this ability was independent of a functional H1-T6SS.

Observations by the Mekalanos laboratory have shown that the *P. aeruginosa* H1-T6SS is activated as a defensive response to a T6SS attack from a competing organism (Basler et al., 2013). The lack of competing organisms in the secretion assays performed with kanamycin may explain why H1-T6SS components are not secreted, since this would be a wasteful use of resources by *P. aeruginosa*. It is likely that additional regulatory mechanisms are in place to prevent H1-T6SS activity in the absence of an aggressive competitor. The direct contact with competing bacteria may represent one such regulatory mechanism, involving activation of the PpkA/PppA post-translational regulation of the H1-T6SS (Silverman et al., 2011), which is likely not affected by kanamycin. It would therefore be interesting to observe the effect of kanamycin on H1-T6SS secretion in a strain in which the PpkA/PppA system is de-regulated.

Experiments investigating the effect of kanamycin on the H1-T6SS mediated killing of *E. coli* failed to show an effect upon the addition of kanamycin. This evidence does not support the hypothesis that a kanamycin induced H1-T6SS is primed for attack and capable of a fast response targeted against

competing bacteria. However, observations at the cellular level of H1-T6SS dynamics indicate that *P. aeruginosa* does not attack *E. coli* cells routinely, and launches T6SS attacks only following T6SS attacks from competing bacteria (Basler et al., 2013). *E. coli* may not be the most suitable prey cell for testing the effect of kanamycin, since it is not an aggressive competitor. A prey that provokes a *P. aeruginosa* T6SS attack, e.g. *Vibrio cholerae*, could be used and the effect of kanamycin in increasing the speed or efficiency of the *P. aeruginosa* counterattack could be tested.

Chapter 4 Characterisation of the H2-T6SS

4.1 Introduction

The genome of *P. aeruginosa* encodes three Type Six Secretion systems, known as the H1-, H2- and H3-T6SS (Hcp-secretion Island 1-3). The most widely studied of these is the H1-T6SS, which is responsible for the secretion of three toxins (Tse1-3), and has a role in bacterial killing, as described in section 1.7. Study of the H1-T6SS has been possible since this cluster is known to be regulated by the Ret/Gac/Rsm pathway, and deletion of the *retS* sensor kinase results in overproduction of the H1-T6SS components and hyper-secretion of its substrates, as described in section 1.8. A *P. aeruginosa retS* deletion mutant is sufficient to allow for the study of the H1-T6SS under normal laboratory culture conditions. Analysis of microarray data shows that the genes of the H2- and H3-T6SS are not affected by *retS* deletion, indicating that the three *P. aeruginosa* T6SS clusters are not co-regulated (Goodman et al., 2004; Mougous et al., 2006). The *P. aeruginosa* H2- and H3-T6SS remain poorly characterised in comparison to the H1-T6SS. The H2- and H3-T6SS are not active under normal laboratory conditions, and the conditions required for their activation are not fully understood. Despite this, a number of studies have investigated the regulation and role of these systems.

Regulation of the H2-T6SS

The publication by Lesic and colleagues (2009) was the first to report on the regulation and function of the H2-T6SS, which is the focus of this chapter. Their work shows that quorum sensing is involved in the regulation of the H2-T6SS in *P. aeruginosa* PA14 (Lesic et al., 2009). The authors reviewed earlier microarray data from a PA14 *mvfR* mutant and find that the expression of genes

encoded within the H2-T6SS cluster is repressed between 2- and 12-fold in an *mvfR* mutant compared to the wild type strain (Deziel et al., 2005). This indicates that MvfR, also known as PqsR, and consequently the PQS quorum sensing system which it regulates, both positively influence H2-T6SS expression. They confirmed by RT-qPCR analysis, showing that *hcp2* expression was reduced in an *mvfR* mutant. Expression of *hcp2* was also reduced in a *lasR* mutant, implicating the Las quorum sensing system in the regulation of H2-T6SS. The discovery of LasR binding sites in the H2-T6SS promoter provides further evidence for quorum sensing regulation of this system (Lesic et al., 2009). Despite this information, how strong and efficient the effect of quorum sensing is on the production, assembly and activity of the H2-T6SS system remains to be investigated, especially since no phenotypic assays were readily available for this system.

The regulation of the H2-T6SS was also studied in another *P. aeruginosa* isolate, PAO1 (Sana et al., 2012). In this case, a transcriptional fusion was generated between the promoter region of *hsiA2*, the first gene in the H2-T6SS cluster, and *lacZ*. The activity of this fusion was increased around 10-fold at the transition to stationary phase, and this effect was significantly delayed in quorum sensing mutants, with maximal levels reduced 22-fold in a *lasR* mutant and 8.5-fold in a *rhlI* mutant (Sana et al., 2012). However, the impact of MvfR/PqsR, which was shown to impact the H2-T6SS in PA14, has not been investigated in this study using the PAO1 strain.

In their study, Sana and colleagues also emphasise another regulatory pathways impacting H2-T6SS expression. They observed that the activity of the *hsiA2* transcriptional fusion is modestly increased, around 2-fold, under iron limiting conditions (Sana et al., 2012). The discovery of Fur boxes in the promoter region of the H2-T6SS provides further evidence that iron limitation may be an environmental signal important in the activation of the H2-T6SS. As with the previous study by Lesic et al, this study clearly identified regulatory pathways which modulate expression of the H2-T6SS,

but whether, and to what extent, they influence the production, assembly and activity of the H2-T6SS was not clearly addressed.

The role of the H2-T6SS

A phenotype associated with the H2-T6SS is the involvement in virulence, which was observed using several infection models (Lesic et al., 2009). For example, virulence in plants was tested using an *Arabidopsis thaliana* model, and leaves were infiltrated with wild type *P. aeruginosa* PA14, or an isogenic mutant in the H2-T6SS. Colony forming units recovered from leaves showed that the H2-T6SS mutant exhibited significantly decreased growth at 2 and 3 days post infection compared to the wild type strain (Lesic et al., 2009). Furthermore, virulence in a mammalian model was tested using an acute mouse lung infection model. Mortality was delayed when mice were infected with a H2-T6SS mutant, compared to mice infected with wild type PA14, however survival of mice was not affected. Finally, in a model of mouse burn wound infection, deletion of H2-T6SS did not affect mortality (Lesic et al., 2009), suggesting it is not a central player in all infections. Nevertheless, the authors concluded that the H2-T6SS plays an important role in *P. aeruginosa* virulence. The molecular mechanism of the H2-T6SS contribution to virulence remains unclear, since firstly, the effects observed could be due to direct interaction with eukaryotic cells, or indirect effects due to the fitness of *P. aeruginosa* strains, and secondly, the specific effectors delivered by the H2-T6SS responsible for these phenotypes were not described in this work.

Sana and colleagues also present evidence for the involvement of the H2-T6SS in interaction with the host, and more specifically with eukaryotic cells (Sana et al., 2012). The internalisation of *P. aeruginosa* PAO1 into HeLa cells was investigated using gentamycin protection assays. Deletion of *clpV2*, a core gene of the H2-T6SS cluster, results in an invasion defect, since the internalisation of this mutant is 75% lower than the wild type strain, although only 1.7% of wild type cells were

internalised. This internalisation defect was shown to depend on the Akt phosphorylation pathway, also known as protein kinase B, a component of a host cell pathway involved in *P. aeruginosa* internalisation from the apical surface (Kierbel et al., 2005). Infection with a *clpV2* mutant resulted in a 50% decrease in Akt phosphorylation, compared with the wild type strain (Sana et al., 2012).

H2-T6SS effectors

Despite the extensive work presented in the papers by Lesic et al (2009) and Sana et al (2012), neither of these studies described the effector proteins responsible for the H2-T6SS associated phenotypes in host cell interactions. Bioinformatic analyses have previously uncovered putative lipase effectors associated with the H2-T6SS, due to the presence of lipase encoding genes in genetic clusters with *vgrG* genes (Barret et al., 2011). One of these lipases, PldA, was shown to have phospholipase activity, but was not shown to be T6SS dependent at the time (Wilderman et al., 2001).

The first experimental evidence of a H2-T6SS dependent effector, known as Tle5^{PA}, a type VI lipase effector, was recently reported (Russell et al., 2013). The observation that *vgrG* genes are often linked with genes encoding putative lipases was expanded and a bioinformatic approach uncovered 377 putative lipases from five families. It was also noticeable that each lipase gene is adjacent to a gene encoding a putative periplasmic localised immunity protein, required to protect *P. aeruginosa* from self-toxicity, indicating that the activity of the H2-T6SS may not be restricted to eukaryotic cells, but may also be involved in bacterial competition.

Tle5^{PA} was shown to catalyse the in vitro release of choline from phosphatidylcholine, a component of the bacterial membrane. *P. aeruginosa tle5*/*tli5* mutants, lacking both the Tle5 effector and cognate immunity protein, were susceptible to killing by a wild type *P. aeruginosa* competitor.

However, they survived incubation with *P. aeruginosa* Δ*tle5*, indicating that Tle5 is an antibacterial toxin against *P. aeruginosa* strains lacking the cognate immunity (Russell et al., 2013). Inactivation of the H2-T6SS by deleting *clpV2* abrogated the $Tle5^{PA}$ dependent toxicity, indicating that the H2-T6SS was responsible for the delivery of $Tle5^{PA}$.

Previous studies on the H2-T6SS provide clues to conditions activating the transcription of this gene cluster, but defined conditions resulting in a fully assembled and active system are yet to be shown. However, regulatory controls involving quorum sensing or iron limitation have a very global effect on bacterial cell physiology, and might not be best appropriate to specifically study the role and function of the H2-T6SS. Engineering strains in which the H2-T6SS is tightly controllable would allow the system to be activated on demand and studied in more detail.

4.2 Specific aims of the study

The work presented in this chapter aims to further characterise the H2-T6SS, to control the production of H2-T6SS components, investigate their assembly and study the role of the system. An important feature that has driven the rational of the work is a major difference between the H2-T6SS clusters from PAO1 and PA14. Indeed, we observed that in PA14, additional genes are present which may provide a clue on the nature of the H2-T6SS effectors and thus on the role and function of the system. The chapter is split into four main sections covering genetic characterisation of the H2-T6SS cluster, artificial activation of the H2-T6SS, analysis of the production and secretion of putative substrates, and investigation of the role of the H2-T6SS. The aim of each section is described below.

- To characterise the *P. aeruginosa* H2-T6SS genetic cluster and highlight similarities and differences to the well characterised H1-T6SS.

- To engineer a strain in which the H2-T6SS can be artificially activated under standard laboratory culture conditions, generating a tool to allow in vitro study of this system.

- To define and characterise putative secreted substrates or effectors of the H2-T6SS, and follow their production and secretion under conditions where the H2-T6SS is artificially activated.

- Perform experiments to investigate the role of the *P. aeruginosa* PA14 H2-T6SS in bacterial and eukaryotic targeting.

4.3 Characterisation of the H2-T6SS gene cluster

The *P. aeruginosa* PAO1 H2-T6SS cluster

The genetic arrangement of the H1- and H2-T6SS clusters from PAO1 are shown in Figure 4.1. Comparison of the two clusters reveals a set of components common to both systems (white arrows) which have previously been reported as core T6SS components (Zheng et al., 2011), but one may see that the arrangement of these genes varies greatly between the two clusters. Only one gene, *sfa2* is encoded within the H2-T6SS and absent from the H1-T6SS (blue arrow) (Figure 4.1). *sfa2* encodes a sigma factor activating protein. These proteins have been shown to interact with sigma factors and RNAP, driving the expression of specific genes as described in section 1.7.5. This suggests an alternative regulation of the H2-T6SS and H1-T6SS.

Figure 4.1 Comparison of the *P. aeruginosa* PAO1 H1-T6SS and H2-T6SS gene clusters
Arrows represent the direction of open reading frames at the H1-T6SS (top) and H2-T6SS (bottom) gene clusters of *P. aeruginosa* PAO1. The gene identifiers (PA numbers) are given for the first and final genes in each cluster, and each gene is annotated with a short identifier. Grey, yellow and green shaded arrows represent genes that are present in the H1-T6SS but absent in the H2-T6SS, while blue shaded arrows represent the gene present specifically in the H2-T6SS.

13 genes present in the H1-T6SS are absent in the H2-T6SS cluster (grey, yellow and green arrows) (Figure 4.1). Five of these genes are type six associated genes (*tag*) and are not core T6SS components. The *tagQRST* genes encode proteins involved in the post-translational regulation of the H1-T6SS, described in section 1.7.5. The absence of these *tag* genes in the H2-T6SS again further highlights the different regulation of these two systems. H1-T6SS also encodes a gene annotated as *hsiE1* (*tagJ*) which is not essential for H1-T6SS function, although its product interacts with the tail sheath component *hsiB1* (*tssB1*) (Lossi et al., 2012). Recent data observed in our laboratory suggest that HsiE1 may be responsible for actively targeting ClpV1 to the HsiBC sheath, and therefore catalyse recycling of the sheath (Filloux personal communication). The lack of *hsiE* in the H2-T6SS shows that this function is not conserved (Lossi et al., 2012).

More strikingly, some of the remaining H1-T6SS genes not found in the PAO1 H2-T6SS encode core components, essential for T6SS activity, namely *hcp* (yellow arrow) and *vgrG* (green arrows) (Figure 4.1). The Hcp and VgrG proteins form the tail tube and puncturing device respectively, and are required for the secretion of T6SS toxins (section 1.7.1). An H2-T6SS cluster lacking these proteins

would be non-functional, unless genes encoded elsewhere on the chromosome must provide these essential functions. Several orphan *vgrG* and *hcp* genes are found on the PAO1 genome, as described further in Section 4.6, but their involvement in the H2-T6SS remains to be demonstrated experimentally.

The H2-T6SS in other *P. aeruginosa* strains

The H2-T6SS cluster is conserved in the thirteen sequenced *P. aeruginosa* strains currently available for comparison at pseudomonas.com (Winsor et al., 2011). The PAO1 H2-T6SS genes, from *hsiA2* to *stk2*, and their genetic organisation, are conserved in all of the available strains, while differences are apparent in the region upstream of *hsiA2* (Figure 4.2). The genetic arrangement of the H2-T6SS shown on the top in Figure 4.2 is the most common, found in PAO1 and eight other strains (PA2191, C3719, DK2, LESB58, M18, PA7, PACS2 and RP73). The genetic arrangement of the 39016, B136-33, NCGM2.S1 and PA14 strains show some striking differences. In PA14 and B136-33 there is an insertion of four genes into the genome, orientated in the opposite direction to the core cluster, and absent from the majority of the other strains analysed. The organisation in *P. aeruginosa* 39016 and NCGM2.S1 represent a hybrid between PAO1 and PA14, since it involves some but not all of the additional genes. Such organisation may represent an evolutionary step in the acquisition or loss of this four gene cluster.

Two of the additional genes present in PA14 and B136-33 encode core T6SS components (Barret et al., 2011; Lesic et al., 2009). These components are a *hcp* gene and a *vgrG* gene, essential for T6SS. In contrast to PAO1, orphan Hcp and VgrG may not be required to build a functional H2-T6SS in these strains. The *P. aeruginosa* PA14 strain thus presents a H2-T6SS cluster which encodes a complete set of the core T6SS components, and for this reason is our chosen model for the present study.

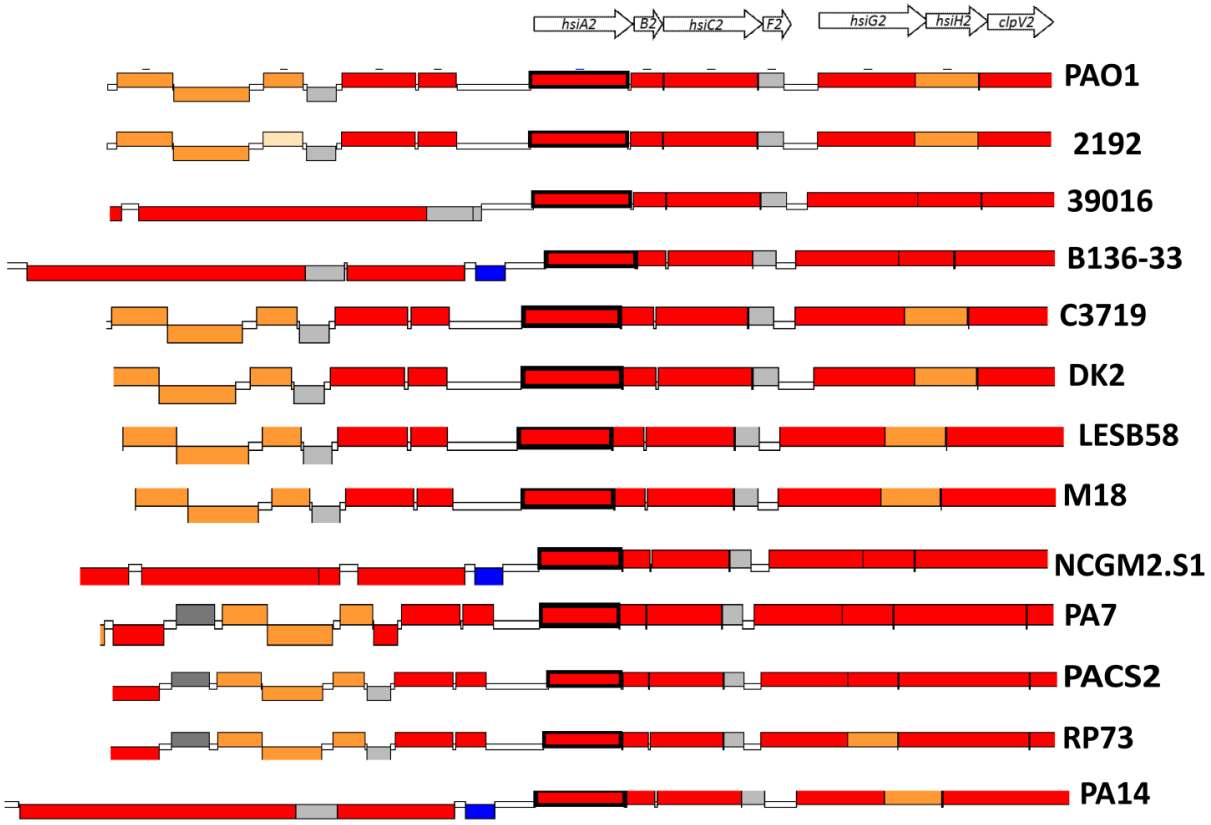


Figure 4.2 Comparison of the H2-T6SS across 13 *P. aeruginosa* strains

The genetic arrangement of the H2-T6SS cluster in the 13 *P. aeruginosa* genomes currently available for comparison, the strain name is indicated to the right. Gene clusters are taken from pseudomonas.com. A diagrammatic representation of the core H2-T6SS cluster is shown above (white arrows). While the core cluster (right) is conserved in all of the strains analysed, the genes encoded adjacent to the cluster (left) show some variability. The gene boxed with thick black lines is *hsiA1* in each case.

Because of its location adjacent to the H2-T6SS cluster, this Hcp is referred to here as Hcp2, and the VgrG is referred to as VgrG14 as it was originally found in the PA14 strain. The other two genes found in PA14 are not similar to any characterised T6SS components, and their potential role is discussed later in this chapter.

A

P. aeruginosa PAO1			*P. aeruginosa* PA14		
Hcp1	H1-T6SS	PA0085	Hcp1	H1-T6SS	PA14_01030
Hcp3	H3-T6SS	PA2367	Hcp3	H3-T6SS	PA14_34030
HcpA	Orphan	PA1512	HcpA	Orphan	PA14_44890
HcpB	Orphan	PA5266	HcpB	Orphan	PA14_03240
HcpC	Orphan	PA0263	HcpC	Orphan	PA14_69560
			Hcp2	H2-T6SS	PA14_43070

B

	Hcp1	Hcp3	HcpA	HcpB	HcpC	Hcp2
Hcp1		25.93	8.64	8.64	8.64	8.7
Hcp3	25.93		17.58	17.58	17.58	17.58
HcpA	8.64	17.58		100	100	100
HcpB	8.64	17.58	100		100	100
HcpC	8.64	17.58	100	100		100
Hcp2	8.7	17.58	100	100	100	

C

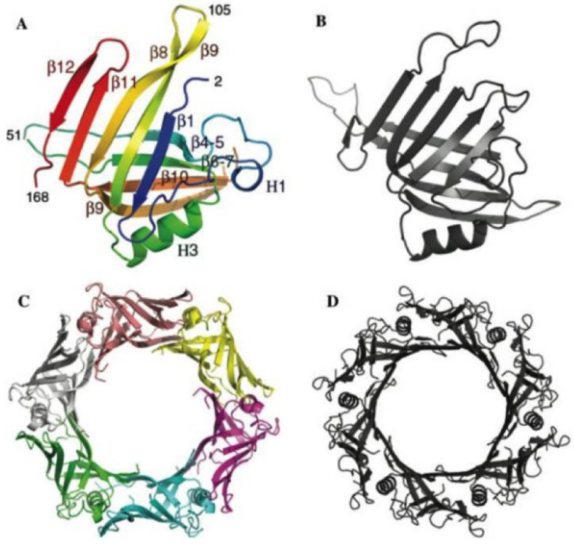


Figure 4.3 Characterisation of the PA14 specific Hcp

A. The names of each Hcp protein from PAO1 and the corresponding protein from PA14 are shown, along with their associated T6SS cluster, or orphan if not, and the gene identifier of the corresponding *hcp* gene. B. Pairwise comparison of the percentage amino acid similarity of the six Hcp proteins from PA14. C. Structural comparison of the Hcp2 structure (left, coloured) with Hcp2 (right, black and white (Mougous et al., 2006). Hcp monomers (above) and the ring complex formed from Hcp hexamers (below). Reproduced from (Osipiuk et al., 2011) with permission.

Characterisation of Hcp2

Whereas *hcp1* is located within the H1-T6SS, and *hcp3* within the H3-T6SS, no *hcps* are encoded in the vicinity of the H2-T6SS in PAO1. The presence of the *hcp2* gene adjacent to the H2-T6SS cluster is an important feature of *P. aeruginosa* PA14. The PA14 strain is equipped with six Hcps, as it also contains a homolog of each of the five PAO1 Hcps (Figure 4.3A), including three orphan *hcp* genes are encoded distantly to the T6SS clusters.

The protein sequence of Hcp2 was compared to the sequences of the other five PA14 Hcps, and protein identity scores are shown in Figure 4.3B. Hcp2 shows 100% identity to the three orphan Hcps (ABC), while only being 8.7% and 17.58% identical to Hcp1 and Hcp3 respectively. A publication reporting the structure of Hcp3 from *P. aeruginosa* in fact shows the solved structure of the protein encoded by PA0263, which is annotated as *hcpC* on the *P. aeruginosa* genome, and is not *hcp3* (encoded by PA2367) (Osipiuk et al., 2011). The protein sequence of the protein analysed in this publication is 100% identical to Hcp2 from PA14, and the structure for this protein is shown in Figure 4.3C. Comparison with the solved structure of Hcp1 shows that these proteins have strong structural homology, despite having low sequence similarity, indicating that the tail tube function is likely conserved by Hcp2, in the PA14 H2-T6SS, but also for each of the identical Hcp proteins (Mougous et al., 2006; Osipiuk et al., 2011).

4.4 Activation of H2-T6SS

4.4.1 Engineering H2-T6SS promoters

A system in which the H2-T6SS in *P. aeruginosa* PA14 could be artificially controlled was proposed, allowing the H2-T6SS to be studied under standard laboratory culture conditions. Such a system has

already been successfully used to activate a T6SS cluster from *Citrobacter rodentium* (Gueguen and Cascales, 2013). A promoter engineering approach was adopted to control artificial expression of the H2-T6SS. A pBAD promoter under the control of the AraC transcriptional regulator was the preferred choice and allowed the system to be expressed upon addition of arabinose to the growth medium. This promoter is found in *E. coli*, controlling the expression of arabinose uptake and metabolism genes in the presence of arabinose (Gallegos et al., 1997). It has subsequently been used in *Pseudomonas* species for which broad host range expression vectors, e.g. pJN105, allowing tightly controlled protein production, have been engineered (Newman and Fuqua, 1999).

A strategy was designed to introduce the pBAD promoter and *araC* gene onto the PA14 chromosome, immediately upstream of the H2-T6SS cluster. Due to the divergent arrangement of the H2-T6SS clusters in *P. aeruginosa* PA14 two copies of the pBAD promoter were introduced, acting in opposite orientations, interspaced by one copy of the *araC* gene, represented diagrammatically in Figure 4.4A.

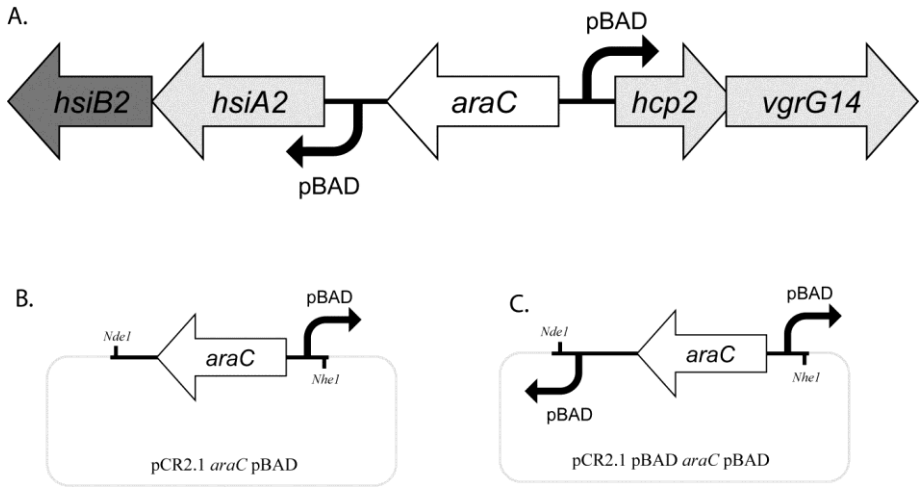


Figure 4.4 Design of the divergent promoter strategy

A. Final organisation of the PA14 strain carrying two copies of the pBAD promoter (curved black arrows) and a single copy of the *araC* gene (white arrow) in the promoter region of the H2-T6SS cluster. B. pCR2.1 strain carrying the *araC* gene and a single copy of the pBAD promoter, flanked by *Nde1* and *Nhe1* restriction sites. C. The pCR2.1 vector manipulated to contain two copies of the pBAD promoter, acting divergently, either side of the *araC* gene, flanked by *Nde1* and *Nhe1* restriction sites.

The cloning strategy for generation of the divergent promoter is shown in Figure 4.4. Briefly, a pCR2.1 vector containing the *araC* gene with a single pBAD promoter was obtained from Dr Helga Mikkelsen (Figure 4.4B) (Mikkelsen et al., 2013). The pBAD promoter and operator regions were amplified by PCR and duplicated into this construct upstream of the *araC* gene, generating divergent copies of the pBAD promoter, generating pCR2.1-DP, as shown in Figure 4.4C.

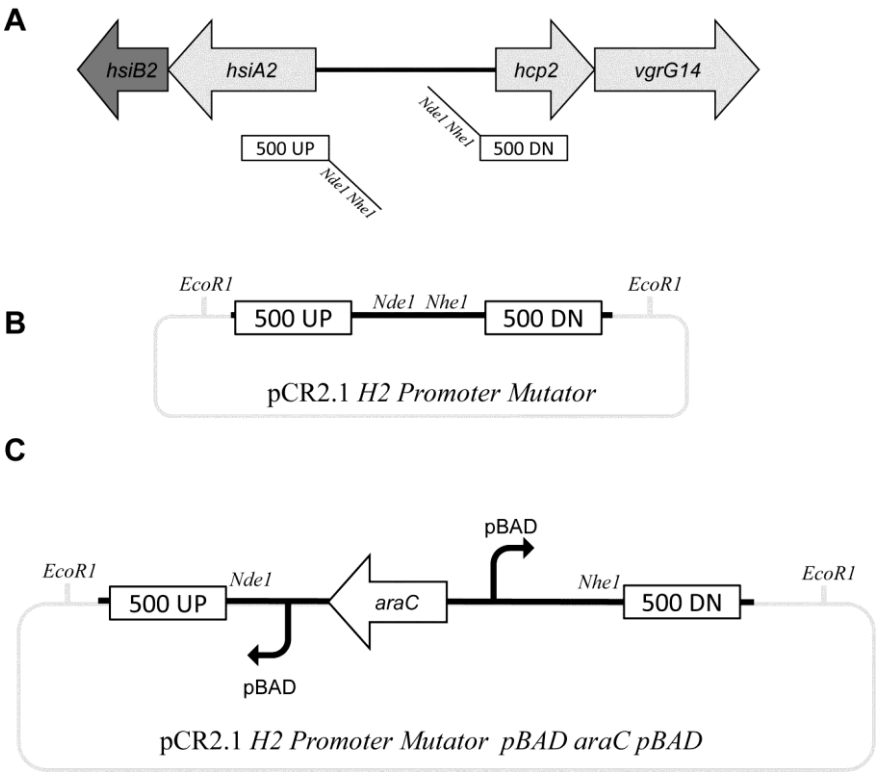


Figure 4.5 Introduction of the divergent promoter into the H2-T6SS promoter region
A. 500 base pair regions defining the H2-T6SS promoter region were amplified by PCR, with *Nde1* and *Nhe1* restriction sites incorporated into the primers. B. The two 500 bp regions were fused by overlap extension PCR and cloned into the pCR2.1 vector, with *Nhe1* and *Nde1* sites separating the two regions. C. The divergent pBAD promoter araC construct was subcloned into the Nde1 and Nhe1 sites, generating the mutator fragment required for mutagenesis of PA14.

In order to insert the divergent pBAD promoter region into the required chromosomal location, the strategy outlined in Figure 4.5 was used. 500 base pair regions upstream and downstream of the native H2-T6SS promoter region were amplified as indicated in Figure 4.5A. 50 base pairs upstream of the start codons of *hsiA2* and *hcp2* were maintained in order to retain any cis-acting elements. The two fragments were fused by overlap extension PCR, and ligated into pCR2.1 (Figure 4.5B). The

overlapping region contained appropriate restriction sites (Nde1/Nhe1) to allow insertion of the divergent pBAD promoter sequence (Figure 4.5C).

The resulting DNA fragment was subcloned into the broad host range suicide vector pKNG101 (Sarker and Cornelis, 1997). Standard mutagenesis protocol using pKNG101 derivatives was followed, which allowed insertion of the divergent promoter region into the H2-T6SS promoter region of the *P. aeruginosa* PA14 chromosome, and, following two events of homologous recombination, a strain referred to as *P. aeruginosa* PA14-DP (divergent promoter) was generated.

4.4.2 Testing of the activity of the Divergent Promoter

Quantitative Reverse transcriptase PCR analysis (q-RT-PCR) was performed on the newly constructed *P. aeruginosa* PA14-DP strain to test the activity of the pBAD promoters, and to observe if arabinose induction resulted in significantly increased expression of H2-T6SS encoded genes. Transcript levels of H2-T6SS genes from the PA14-DP were compared in the absence and presence of arabinose (2%), and in the parental PA14 strain (Figure 4.6a). Primers specific to 100 base pair amplicons of several H2-T6SS encoded genes were designed, as detailed in Table 9. Expression of these genes was measured relative to the expression of the reference gene *rpoD*, encoding the housekeeping sigma factor of RNA polymerase.

Expression of the first gene of the H2-T6SS cluster, *hsiA2*, is increased 8.5-fold upon induction with arabinose (Figure 4.6a), compared to the un-induced sample, and similarly induced when compared to the parental strain lacking the inducible promoter. The *hsiF2* gene transcription is increased about 19-fold, and *hsiG2* around 8-fold upon arabinose induction. Finally, expression of s*tk2*, the distal gene of this cluster, is increased around 5-fold upon arabinose addition.

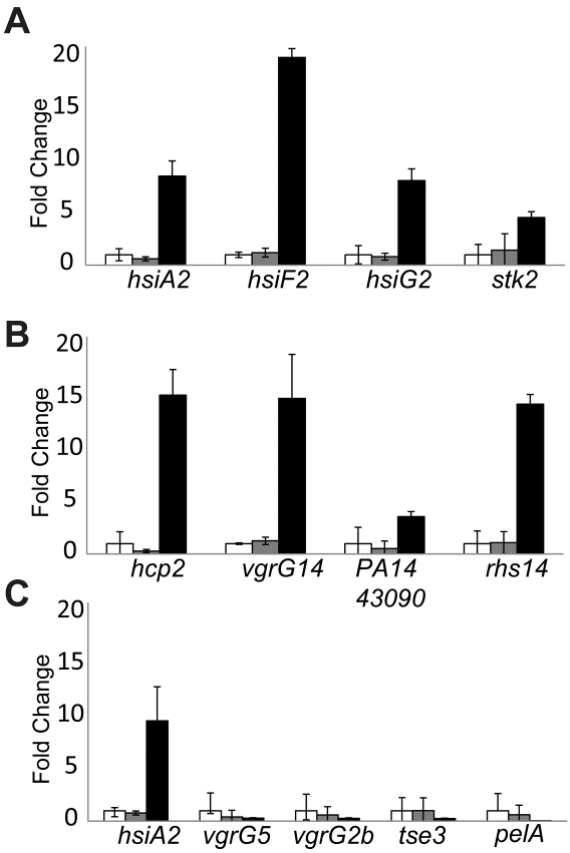


Figure 4.6 Expression of H2-T6SS genes upon arabinose induction
The expression of H2-T6SS genes were tested in wild type PA14 (white bars) uninduced PA14 DP and PA14 DP induced with 2% arabinose. The fold change in expression of genes encoded in the core H2-T6SS is shown in A, expression of the additional genes encoded adjacent to the H2-T6SS cluster in PA14 is shown in B, and a control showing genes encoded outside the H2-T6SS cluster are shown in C, where *hsiA2* expression was included as a positive control. Error bars show the standard deviation of three replicates. Primers used in this experiment are defined in Table 1.

The expression levels of genes encoded within the PA14 specific cluster adjacent and divergent to the H2-T6SS, were also tested and compared in PA14 and PA14-DP (Figure 4.6b). Expression of all four of these genes is increased in response to arabinose induction. The first gene in the cluster, *hcp2*, is upregulated around 15-fold, as is *vgrG14*. The PA14_43090 open reading frame, currently un-named, is upregulated 3.6-fold, and the last gene in this cluster, named *rhs14*, characterised further in the upcoming sections, is upregulated 14-fold.

As a control, the expression of genes located outside the H2-T6SS was tested (Figure 4.6c). None of these, including *vgrG5*, *vgrG2b*, *tse3* and *pelA*, were upregulated upon arabinose addition, in contrast to the *hsiA2* gene.

These results show that the control of the divergent arabinose inducible promoter on H2-T6SS gene expression was effective and specific, and the PA14-DP strain can be used for experiments requiring activation of the H2-T6SS cluster.

4.4.3 Production of H2-T6SS components

Although qPCR analysis using the PA14-DP strain has shown significant expression of the H2-T6SS gene cluster upon arabinose addition, further evidence is required to confirm that the H2-T6SS multi protein complex is produced, assembled and active under these conditions.

Antibodies were generated against two H2-T6SS proteins, namely Hcp2 and HsiB2, encoded on opposite sides of the divergent promoter construct and within the two divergent transcriptional units. The gene encoding a his tagged Hcp2 homolog (PA1512), cloned into pDEST42 vector was provided by Dr A. Hachani from our laboratory. The *hsiB2* gene was amplified and cloned into a pET28a vector using primers defined in Table 8, generating a his-tagged version of HsiB2. Both genes were over-expressed in *E. coli* grown in terrific broth, and corresponding proteins purified using nickel affinity and size exclusion chromatography. Protein purification was performed in the lab by Miss E. Manoli, and purified proteins were injected into rabbits for production of a polyclonal antibody (Eurogentec). Both antibodies were shown to specifically detect Hcp2 or HsiB2 when tested on whole cell extracts of *E. coli* overexpressing the corresponding genes and on purified protein (data not shown).

The production of Hcp2 and HsiB2 was then tested in PA14-DP upon arabinose induction. The western blot presented in Figure 4.7 shows that Hcp2 and HsiB2 are not produced in significant amounts in cell extracts from a wild type PA14 culture, and this is not influenced by addition of arabinose or glucose to the medium. However, analysis of cell extracts from PA14-DP cultures shows that upon addition of arabinose at concentrations above 0.1% significant production of Hcp2 and HsiB2 is observed, which is not the case in the absence of arabinose or the presence of glucose. This confirms the q-PCR data, and also confirms that arabinose induction of the divergent promoters results in tightly controlled production of H2-T6SS proteins.

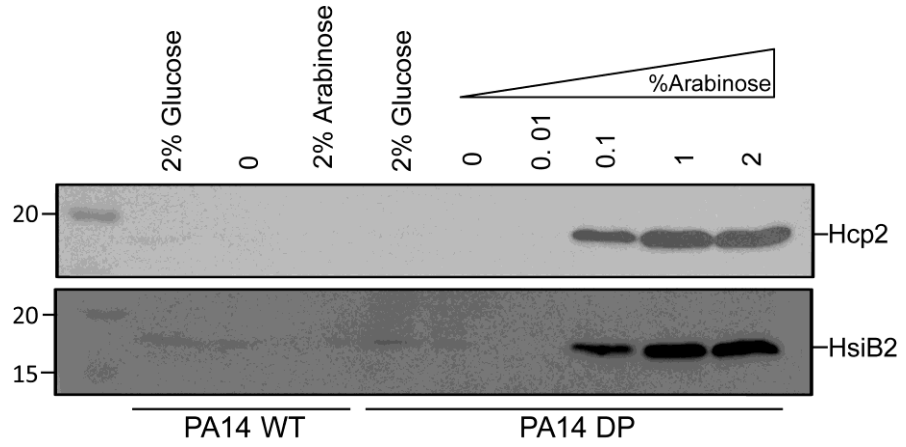


Figure 4.7 Production of HsiB2 and Hcp2 proteins upon arabinose induction
Western blot analysis of Hcp2 (top) and HsiB2 (bottom) production from whole cell extracts from PA14 and PA14-DP derivative. The strain used is indicated below the blot, while glucose or arabinose concentrations are indicated above the blot. Molecular weight markers are indicated to the left of the blot, and expected sizes of Hcp2 and HsiB2 are indicated on the right.

4.4.4 Secretion of Hcp2

An assay often used to demonstrate that the T6SS is active and functional is to follow the appearance of Hcp in culture supernatant (Mougous et al., 2006). Although Hcp is no longer considered to be a true secreted effector, but rather a structural component of the T6SS machinery, its localisation in the supernatant is a hallmark for a correctly assembled T6SS.

To investigate the fate of Hcp2, a secretion assay was performed. Cells were separated from the extracellular medium and subjected to Western blot analysis, the result of which is shown in Figure 4.8A. As previously shown, induction of the PA14-DP strain with arabinose results in the production of Hcp2. Analysis of the supernatant fraction indicates that Hcp2 is also located in the extracellular medium of PA14-DP cells. The absence of RNA polymerase in the supernatant indicates that the Hcp2 protein in the supernatant is not the result of cell lysis, but likely due to specific secretion.

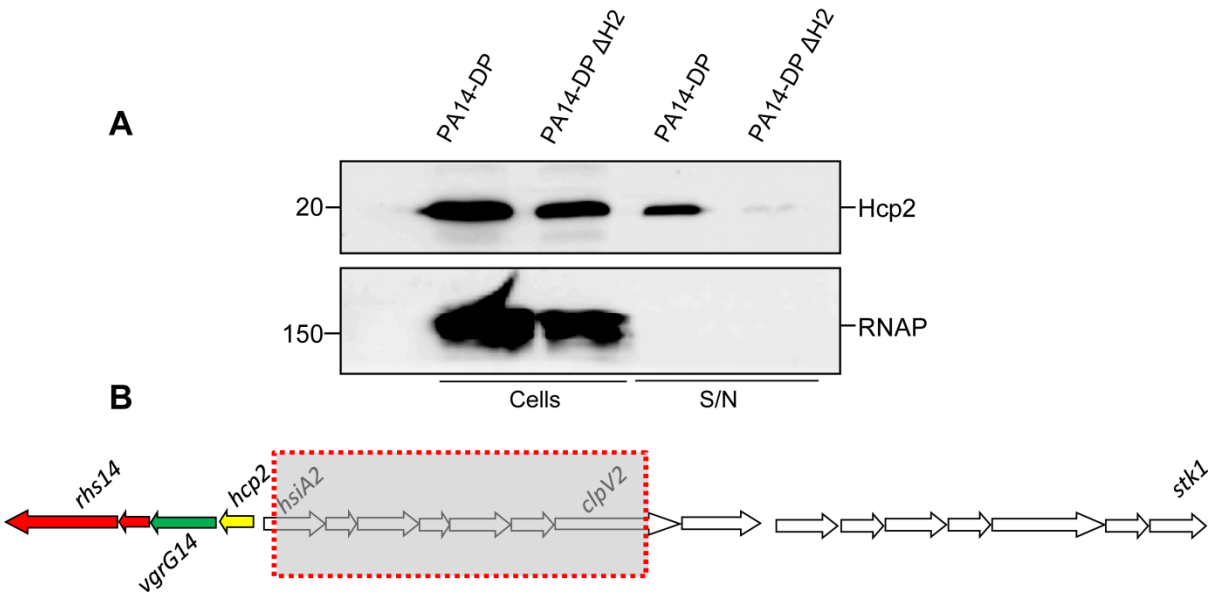


Figure 4.8 Hcp2 is secreted in a H2-T6SS dependent manner
A. Western blot analysis of whole cell extracts and supernatant from PA14 DP and PA14 DP strain carrying a deletion in the H2-T6SS cluster as indicated by the hatched box in the H2-T6SS cluster diagram. Cells were probed with either Hcp2 (top) or RNA polymerase (bottom) antibody. The strain used is indicated above, and the sample type is indicated below the blot. Molecular weight standards are indicated to the left of the blot, and the expected sizes of Hcp2 and RNAP are indicated to the right of the blot. B. The location of the region deleted to generated PA14ΔH2-T6SS is shown in the hatched red box.

To further show that the Hcp2 secretion is specific and dependent on the H2-T6SS a strain lacking the core H2-T6SS genes was generated. The DNA region spanning from *hsiA2* to mid-*clpV2* was deleted in the PA14-DP background, yielding PA14-DP ΔH2-T6SS (PA14-DP ΔH2). However, in this strain, the operon encoding Hcp2, VgrG14 and the two downstream gene products was left intact, as

shown in Figure 4.8B. The arabinose-induced production of Hcp2 is unaffected in the PA14-DP ΔH2 strain, but its secretion is drastically reduced when compared to PA14-DP. Overall, this suggests that arabinose induction of the divergent promoters in PA14-DP leads to the correct assembly of a functioning H2-T6SS.

4.5 Quorum Sensing and Iron Limitation

Now that we have tools to monitor production of H2-T6SS components, we revisited the impact of quorum sensing and iron limitation, which were shown in previous studies to control the expression of H2-T6SS genes (Lesic et al., 2009; Sana et al., 2012). PA14 wild type cells were grown in tryptone soya broth (TSB) in the presence or absence of 2,2'-bipyridyl (250 μM) to chelate iron, and samples were taken at various time points during growth. Cell lysates were prepared and analysed by western blot for the production of Hcp2.

As shown in Figure 4.9, the presence of absence of iron chelators makes no difference to the amount of Hcp2 produced. Bands of weak intensity appear at the expected size of Hcp2 at the stationary phase of growth (6h and 7.5h), and the intensity of these bands is not affected by the availability of iron. In contrast, the production of Hcp2 by arabinose induction in PA14-DP is much higher.

In conclusion, iron limitation does not result in significant production of Hcp2 in *P. aeruginosa* PA14. Interestingly, the traces of Hcp2 production from cultures at high cell density suggest that quorum sensing may have a visible impact on production of H2-T6SS components such as Hcp2, yet the levels of Hcp2 production obtained by the divergent promoter construct in PA14-DP are significantly higher.

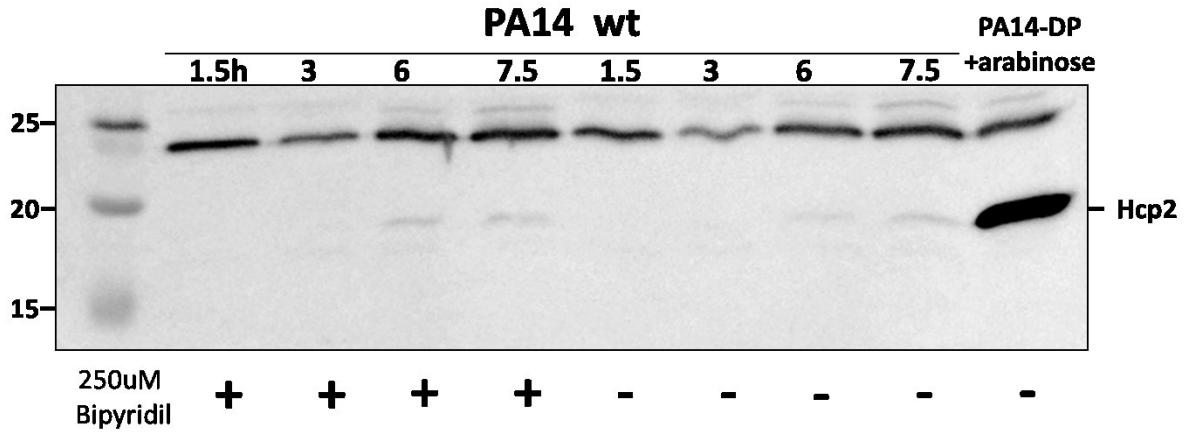


Figure 4.9 Effect of population density and iron limitation on Hcp2 production
Western blot probing for Hcp2 production in wild type PA14 cultures at various times of growth (shown above the blot in hours), with or without bipyridil (shown below the blot). Molecular weight markers are shown (left), and the expected size of Hcp2 is indicated (right). The PA14-DP strain, incubated in the presence of arabinose, is included as a positive control for Hcp2 production.

4.6 VgrG14: A Putative H2-T6SS Secreted Substrate

4.6.1 *P. aeruginosa* VgrG proteins

A gene encoding a VgrG protein is encoded directly downstream of the *hcp2* gene in *P. aeruginosa* PA14, and is named VgrG14 to recognise its presence in this strain, and absence in PAO1 (Figure 4.10). VgrG proteins are usually described as a puncturing device, and are essential for T6SS activity (Hachani et al., 2011). *P. aeruginosa* encodes multiple copies of *vgrG* genes which are often found scattered on the chromosome in so-called *vgrG* islands containing few other genes (Barret et al., 2011). The distribution of *vgrG* in the PAO1 genome is shown in Figure 4.11.

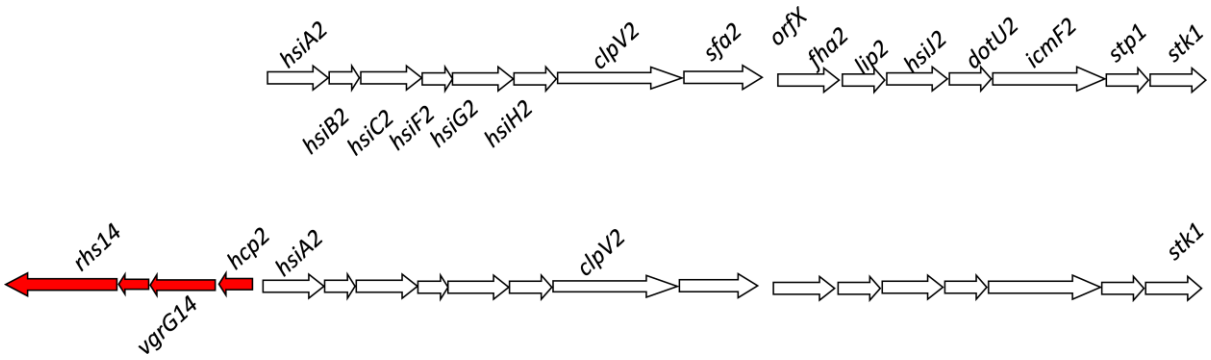


Figure 4.10 Comparison of the PAO1 and PA14 H2-T6SS

The H2-T6SS cluster is shown from *P. aeruginosa* PAO1 (upper) and PA14 (lower). Genes present in the PA14 strain but absent in PAO1 are highlighted in red.

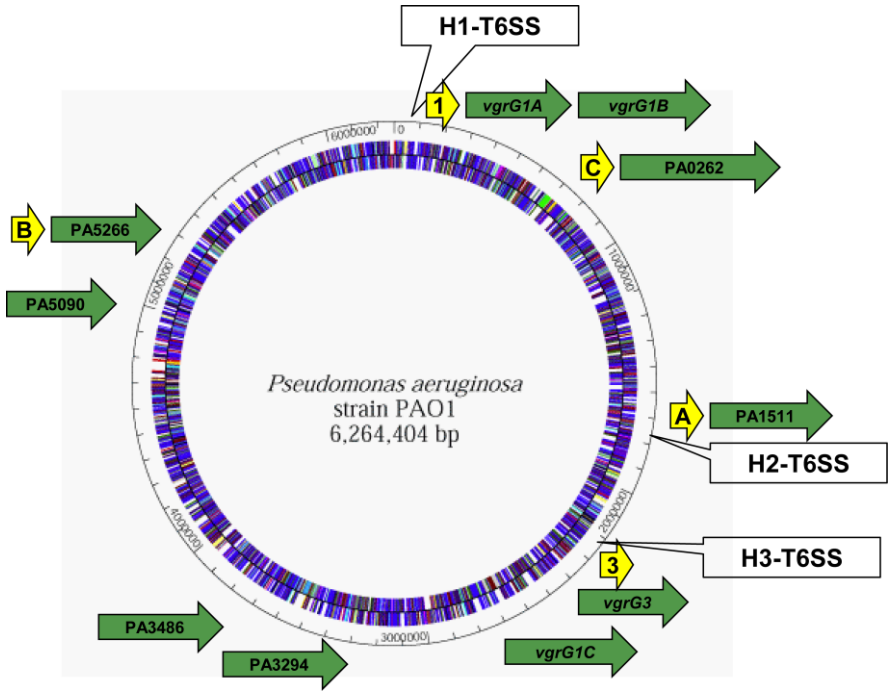


Figure 4.11 Distribution of *vgrG* in PAO1

Graphical representation of the genomic distribution of the three T6SS genetic clusters (white arrows), five *hcp* genes (yellow arrows) and ten *vgrG* genes (green arrows) on the genome of *P. aeruginosa* PAO1.

The additional *vgrG14* gene equips *P. aeruginosa* PA14 with 11 *vgrG* genes, while 10 are found in PAO1, as listed in Figure 4.12. These genes encode proteins ranging from 72.0 kDa to 113 kDa (Table 11). VgrG proteins are composed of two conserved domains, structurally similar to the bacteriophage gp27 and gp5 gene products, fused into a single peptide (Leiman et al., 2009). In addition, variable C termini are often found following the gp5 domain. The domain organisation of the 21 VgrGs from PA14 and PAO1 are shown in Table 11, defined using structural homology predicted by the Phyre server (Kelley and Sternberg, 2009). Approximately 450 residues of the VgrG N terminus comprise the gp27-like domain, and the next 200 amino acids comprise the gp5-like domain (table 11).

P. aeruginosa PAO1			*P. aeruginosa* PA14		
VgrG1a	H1-T6SS	PA0091	VgrG1a	H1-T6SS	PA14_01110
VgrG1b	H1-T6SS	PA0095	VgrG1b	H1-T6SS	PA14_01160
Vgr1c	H1-T6SS	PA2685	Vgr1c	H1-T6SS	PA14_29390
Vgr2a	Orphan	PA1511	Vgr2a	Orphan	PA14_44900
Vgr2b	Orphan	PA0262	Vgr2b	Orphan	PA14_03220
Vgr3	H3-T6SS	PA2373	Vgr3	H3-T6SS	PA14_33960
VgrG4a	Orphan	PA3294	VgrG4a	Orphan	PA14_21450
Vgr4b	Orphan	PA3486	Vgr4b	Orphan	PA14_18985
VgrG5	Orphan	PA5266	VgrG5	Orphan	PA14_69550
VgrG6	Orphan	PA5090	VgrG6	Orphan	PA14_67230
			VgrG14	H2-T6SS	PA14_43080

Figure 4.12 Comparison of VgrG from PAO1 and PA14
The names of each VgrG protein from PAO1 and the corresponding proteins from PA14 are shown, along with their association with a T6SS cluster, or 'orphan' if not, and the gene identifier of the corresponding *hcp* gene.

VgrG Name	MWt (kDa)	N° Amino Acids	gp27 region	gp5 region	ct length
			PAO1		
VgrG1a	72.0	643	8-447	439-639	4
VgrG1b	82.7	741	11-451	442-649	92
VgrG1c	81.7	726	48-488	479-725	1
VgrG2a	93.6	842	9-451	442-668	174
VgrG2b	113.0	1019	9-451	442-757	262
VgrG3	71.9	668	10-433	424-668	0
VgrG4a	77.0	688	10-457	449-641	47
VgrG4b	90.6	808	10-457	449-666	142
VgrG5	76.7	680	10-453	444-648	32
VgrG6	87.8	790	10-457	449-653	137
			PA14		
VgrG14	75.7	680	10-452	444-651	29

Table 11 Characteristics of *P. aeruginosa* VgrG

The size of each protein in kilodaltons and number of amino acids is indicated. The start and end of regions showing structural homology to the bacteriophage gp27 and gp5 proteins are shown, as is the number of amino acids remaining at the c terminus of the protein, following the region of gp5 homology (ct length).

In this analysis the C terminal extensions are defined by the end of structural homology to the gp5 domain, and vary in length. VgrG1a, VgrG1b and VgrG3 terminate at, or soon after, the end of the gp5 domain, and have no C terminal extension. The other VgrG contain extension ranging from 47 amino acids (VgrG4a) to 262 amino acids for VgrG2b. As previously described in section 1.7, VgrG C terminal extensions may carry a domain with catalytic or toxic activities, exemplified by the actin cross linking domain of VgrG from *V. cholerae* and *A. hydrophila* (Pukatzki et al., 2007; Suarez et al., 2010), and the peptidoglycan targeting domain of VgrG3 from *V. cholerae* (Brooks et al., 2013; Dong et al., 2013). To date, no experimentally characterised examples of VgrG with catalytic activity from *P. aeruginosa* has been reported. VgrG2b has been predicted to have zinc dependent metallopeptidase activity due to the presence of a HExxH motif, which is a conserved feature of this protein family (Jongeneel et al., 1989; Pukatzki et al., 2007). No other obvious activity can be predicted for the remaining VgrG carrying C terminal extensions. These shorter extensions may alternatively act as adaptor domains allowing interaction of VgrG with other proteins, including the recently characterised PAAR domain proteins, allowing VgrG to act as transporter proteins (Shneider et al., 2013).

Given that the subset of VgrGs found in PAO1 and PA14 differ, the VgrGs found in 13 sequenced genomes currently available for comparison at www.pseudomonas.com were analysed. VgrG proteins belong to a cluster of orthologous group (COG) numbered COG3501. All COG3501 proteins from the 13 genomes were identified, yielding 133 proteins. Each protein was classified by length, and compared to the named VgrG from PAO1, and the presence or absence of genes encoding each VgrG protein is indicated in Table 12. If comparison of protein length was not sufficient for classification, the genetic arrangement of *vgrG* genes was analysed and compared to PAO1. This search yielded no additional VgrG proteins, and the 11 VgrG found in PA14 is the maximum found in any single strain. Many strains are lacking several VgrGs, for example, *P. aeruginosa* PA7 has only 7 VgrGs, lacking VgrG4a, VgrG4b, and VgrG6 compared to PAO1. Analysis of the genome of *P. aeruginosa* 39016 yielded only 3 VgrG, but due to the draft nature of this genome additional VgrG may be uncovered. The number of VgrG vary in different strains of *P. aeruginosa*, but since not all VgrGs have been characterised it is difficult to understand the significance of this variation. The VgrG proteins which have been characterised are described in some depth below

	PAO1	B136-33	DK2	LESB58	M18	NCGM 2.S1	PA7	RP73	PA14	PACS2*	C3719*	2192*	39016*	TOTAL
vgrG1a	+	+	+	+	+	+	+	+	+	+	+	+	-	12
vgG1b	+	+	+	+	+	+	+	+	+	+	+	-	-	11
vgrG1c	+	+	+	+	+	+	+	+	+	+	+	-	-	11
vgrG2a	+	+	+	+	+	+	+	+	+	+	+	+	-	12
vgrG2b	+	-	+	+	+	+	+	+	+	-	+	+	-	10
vgrG3	+	+	+	+	+	+	+	+	+	+	+	+	+	13
vgrG4a	+	+	+	+	+	+	-	+	+	+	+	+	-	11
vgrG4b	+	+	-	-	-	+	-	-	+	+	-	-	-	5
vgrG5	+	+	+	+	+	+	+	+	+	+	+	+	+	13
vgrG6	+	+	+	+	+	+	-	+	+	+	+	+	+	12
vgrG14	-	+	-	-	-	+	-	-	+	-	-	-	-	3
TOTAL	10	10	9	9	9	11	7	9	11	9	9	7	3	113

Table 12 Distribution of *vgrG* genes across sequenced *P. aeruginosa* strains.
Presence (+) or absence (-) of each *vgrG* gene within *P. aeruginosa* genomes. Draft genomes are indicated by * and alternative shading. All proteins belonging to COG3501 group were obtained and the gene size and chromosomal locations were compared to classify each VgrG corresponding to the PAO1 notation.

4.6.2 VgrG subgroups

VgrG1a/b/c

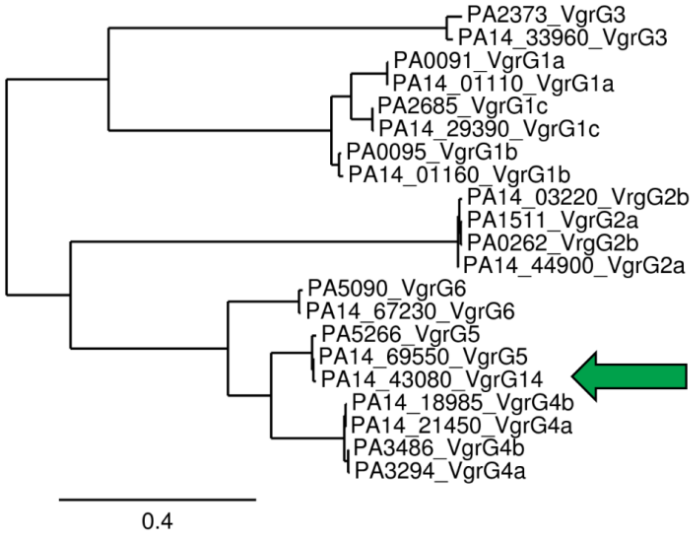
The 11 VgrGs found in *P. aeruginosa* PA14 are compared in Figure 4.13. Phylogenetic analysis shows that VgrG1a/b/c are closely related to each other (Figure 4.13A), and share around 75% sequence identity (Figure 4.13B). These VgrG have been shown experimentally to be associated with the H1-T6SS (Hachani et al., 2011). Hcp1 secretion requires VgrG1a or VgrG1c, but not VgrG1b. The secretion of VgrG1a and VgrG1c is dependent on the H1-T6SS cluster, while VgrG1b is not. However, *vgrG1b* is co-regulated with *vgrG1a*, *vgrG1c* and the whole H1-T6SS gene cluster by the previously described RetS cascade. The *vgrG1b* gene is located at the same chromosomal location as *vgrG1a*, separated only by three genes (Hachani et al., 2011). Therefore, VrgG1a/b/c are a closely related, and may define a functional subgroup of VgrGs. The function of the other *P. aeruginosa* VgrGs have not been experimentally studied, and remain unknown.

VgrG14

VgrG14 is phylogenetically distinct from VgrG1a, VgrG1b and VgrG1c (Figure 4.13A) suggesting VgrG14 is not associated with the H1-T6SS cluster. The chromosomal location of the *vgrG14* gene, adjacent to the H2-T6SS cluster in PA14, strongly suggests its role is connected to this system. VgrG14 is most similar to VgrG5 from PAO1, and is closely related to VgrG6, VgrG4a and VgrG4b. VgrG14 is 96.62% identical to VgrG5 at the amino acid level, the highest similarity observed between any pair of VgrGs (Figure 4.13B). VgrG14 is also ~70% identical to VgrG4a, VgrG4b and VgrG6, while it is only ~30% identical to the remaining VgrGs. This high similarity could suggest that these five VgrGs (4a, 4b, 5, 6 and 14) also represent a functional subgroup, and may all be associated with the

H2-T6SS. This hypothesis is supported by global phylogenetic analysis performed by Barret *et al.* who showed that this subset of VgrG are distributed within a single phylogenetic clade that corresponds to a phylogenetic cluster containing the H2-T6SS, and that VgrG4a and VgrG4b are co-regulated with the H2-T6SS cluster (Barret et al., 2011). Additionally, the recent publication describing an H2-T6SS dependent substrate, Tle5, encoded downstream of VgrG4b, provides experimental evidence for such a link (Russell et al., 2013).

A



B

PA14	VgrG1a	VgrG1b	VgrG1c	VgrG2a	VgrG2b	VgrG3	VgrG4a	VgrG4b	VgrG5	VgrG6	VgrG14
VgrG1a		74.03	74.81	29.24	29.24	32.97	32.19	33.28	32.19	31.57	31.88
VgrG1b	74.03		75.19	25.51	25.64	35.03	30.67	28.34	31.32	28.21	31.03
VgrG1c	74.81	75.19		27.72	27.72	32.49	31.4	31.06	31.47	30.33	31.18
VgrG2a	29.24	25.51	27.72		90.74	26.65	29.36	26.24	31.91	28.61	31.76
VgrG2b	29.24	25.64	27.72	90.74		28.29	29.36	27.23	33.24	29.37	32.65
VgrG3	32.97	35.03	32.49	26.65	28.29		29.79	29.97	30.39	29.94	29.94
VgrG4a	32.19	30.67	31.4	29.36	29.36	29.79		94.33	72.94	64.53	72.94
VgrG4b	33.28	28.34	31.06	26.24	27.23	29.97	94.33		72.21	58.48	72.06
VgrG5	32.19	31.32	31.47	31.91	33.24	30.39	72.94	72.21		69.71	96.62
VgrG6	31.57	28.21	30.33	28.61	29.37	29.94	64.53	58.48	69.71		69.26
VgrG14	31.88	31.03	31.18	31.76	32.65	29.94	72.94	72.06	96.62	69.26	

Figure 4.13 Phylogenetic and amino acid sequence comparisons of *P. aeruginosa* VgrG
A. Phylogenetic tree of VgrG protein sequences from *P. aeruginosa* PA14 and PAO1 using phylogeny.fr. A graphical representation of the inferred tree is shown. The position of the PA14 specific VgrG14 is indicated by the grey arrow. B. Pairwise comparison of the percentage amino acid similarity of the six Hcp proteins from PA14. Subsets of VgrG showing high sequence similarity to each other are boxed in red and highlighted in green.

VgrG2a and VgrG2b

It is currently unclear as to which system VgrG2a and VgrG2b associate; although their similarity indicates that they too should belong to a functional-subgroup, since they are over 90% similar to each other (figure 4.13B). VgrG2a was initially thought to be functionally associated to the H2-T6SS, since *vgrG2a* is encoded close to the H2-T6SS cluster. Due to its high similarity at the protein sequence level, vgrG2b was also proposed to be associated with the H2-T6SS cluster.

Preliminary experiments were performed to examine the secretion of VgrG2a and VgrG2b, dependent on the H2-T6SS cluster in *P. aeruginosa* PAO1. Antibodies were generated against the full length VgrG2a protein, and directed against a mix of two specific peptides within VgrG2b (QRRFVLKQLDGQTA and YRLEPGKTLKDYNIEQ), and deletion mutants of *vgrG2a* and *vgrG2b* were generated. However, production of neither VgrG2a nor VgrG2b could be detected by western blot performed on whole cell extracts, while antibodies successfully recognised these proteins when overexpressed in *E. coli.* VgrG2a and VgrG2b were clearly not produced under the conditions tested, and their functional association with the H2-T6SS cluster remains to be confirmed.

4.6.3 Chromosomal tagging of *vgrG14*

The *vgrG14* gene is encoded adjacent to the H2-T6SS cluster, and is likely to have a functional link with this system, providing one of the missing core T6SS components. VgrG14 might still be seen as a secreted substrate for the H2-T6SS, similar to VgrG1a/c for the H1-T6SS (Hachani et al., 2011). Much like Hcp, the localisation of VgrG proteins in the supernatant in a T6SS dependent manner is a characteristic of a functional T6SS (Hachani et al., 2011). In order to monitor the production and secretion of VgrG14, the *vgrG14* locus in the PA14-DP strain was genetically manipulated so that the VgrG14 protein contained a C terminal V5 epitope and a poly-histidine tag. The V5 tag is an epitope

tag found in the P and V proteins of the paramyxovirus SV5 and is composed of a fourteen amino acid sequence GKPIPNPLLGLDST (Southern et al., 1991). Combined with arabinose induction, this strategy will allow detection of VgrG14 with commercial antibodies against the V5 or His tag.

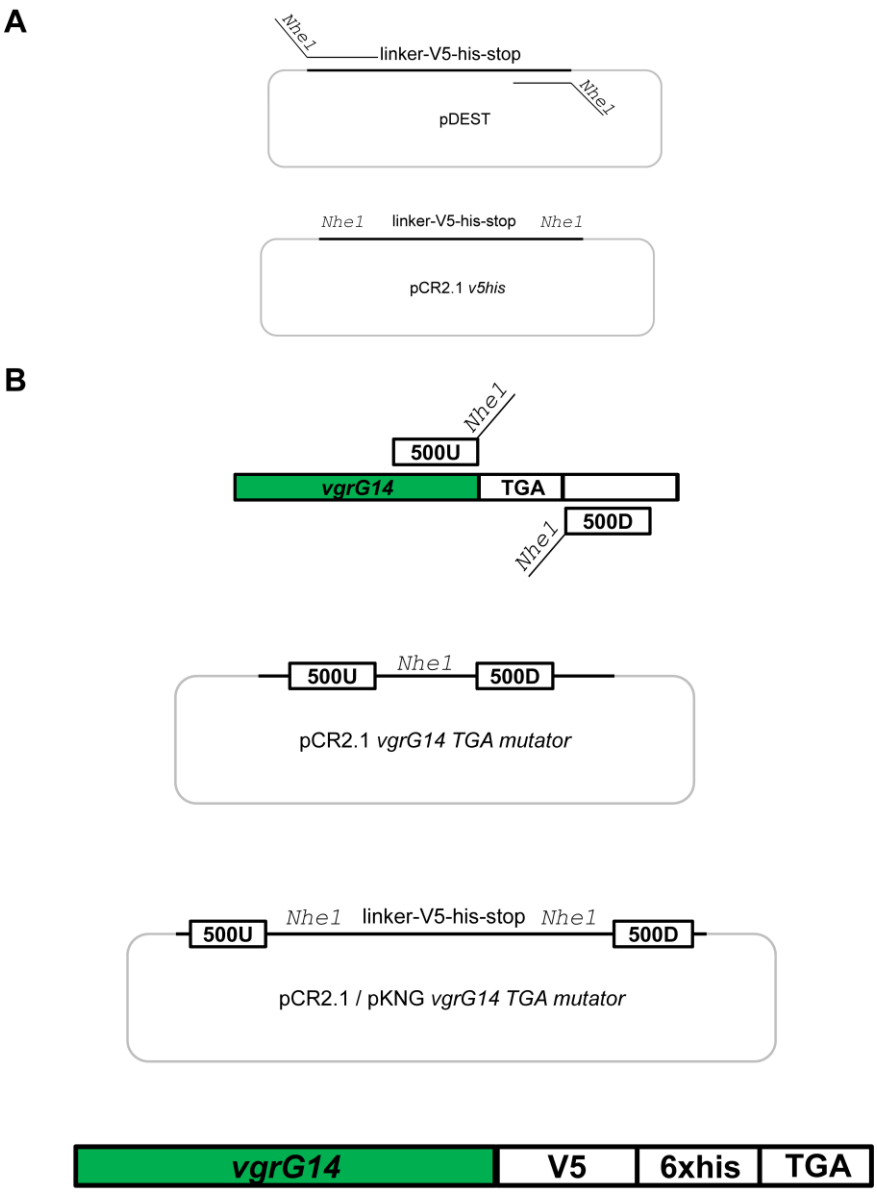


Figure 4.14 Cloning strategy used for chromosomal tagging of *rhs14*

A. The genetic sequence encoding the V5 sequence was amplified from pDEST42, flanked with *Nhe1* restriction sites, and cloned into the pCR2.1 cloning vector. B. 500 base pair regions either side of the vgrG14 stop codon were amplified and fused by overlap extension, with a *Nhe1* site incorporated into the overlapping region. The previously cloned genetic sequence encoding the V5 tag was subcloned into the overlapping region, generating the mutator plasmid required for mutagenesis. Subcloning into the pKNG101 suicide vector and subsequence homologous recombination resulted in a strain producing a V5 tagged version of the VgrG14 protein.

The cloning strategy to introduce the genetic sequence encoding the V5-His tag at the 3'end of the *vgrG14* gene is outlined in Figure 4.14. Briefly, a mutator plasmid was generated by amplifying 500 base pair regions either side of the TGA stop codon of *vgrG14,* which were fused by overlap extension incorporating a suitable restriction site into the overlap region. The V5-His epitope was amplified from the pDEST vector (Invitrogen) and inserted between two regions of homology, in frame with *vgrG14*. This construct was transferred to the suicide vector pKNG101 and the V5-His tag introduced to the required chromosomal location following two events of homologous recombination, generating PA14-DP *vgrG14-V5*.

4.6.4 Production and secretion of VgrG14-V5

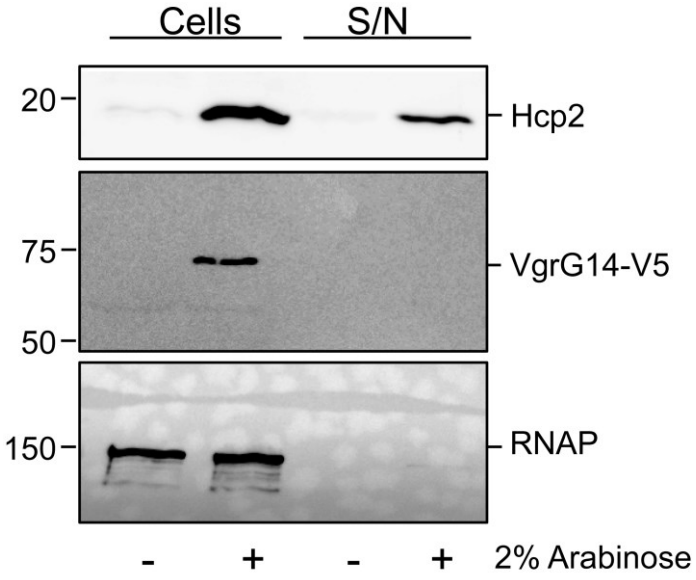


Figure 4.15 V5-tagged VgrG14 is produced but not secreted
Western blot analysis of whole cell extracts and supernatant from PA14-DP strain carrying a chromosomal tagged version of *vgrG14* (PA14 DP *vgrG14-v5*). The upper blot is probed with anti-Hcp2, the central blot is probed with anti-V5, and the lower blot is probed with anti-RNAP antibody. The molecular weights standards are indicated on the left of each blot, and the expected location of each protein indicated on the right. The type of sample (cells or supernatant) is indicated above each set of blots, and the addition of arabinose to the growth medium is indicated below.

A secretion assay was performed to check for production and secretion of VgrG14-V5. The PA14-DP *vgrG14-v5* strain was grown in the presence or absence of arabinose. Cell extracts and supernatants were prepared and analysed by western blot, the result of which is shown in Figure 4.15.

Analysis of the cell fractions show that upon arabinose induction, Hcp2 is produced and its secretion has not been affected by the genetic manipulation at the *vgrG14* locus. Analysis of cell extracts with an anti-V5 antibody reveals a band, at around 75 kDa, specifically produced upon arabinose induction. The band has the expected size of the VgrG14-V5 protein (75.7 kDa native, 77 kDa with tag), which indicates that the chromosomal tagging strategy was successful. Despite Hcp2 being secreted, VgrG14-V5 is not detectable in the supernatant fraction, suggesting it is not secreted. Other possibilities are that it is secreted in very low abundance, or that the tag has interfered with VgrG14 transport.

4.6.5 Localisation of VgrG14

Despite not being located in the supernatant, VgrG14-V5 could potentially be targeted to an extracytoplasmic subcellular location as part of T6SS assembly, including the inner or outer membranes, or the periplasm. To investigate this, proteins from whole cell extracts were separated into a soluble fraction (containing the cytoplasm and periplasm), and an insoluble fraction (containing the total membranes). Spheroplasts were prepared to recover the periplasmic content. Differential solubilisation of the membrane fraction in detergents was performed to isolate inner and outer membrane proteins. The protein content of these various fractions was analysed by SDS-PAGE and western blotting, and the resultant blots are shown in Figure 4.14.

A series of controls was used to confirm that the fractionation experiments were successful, including detection of RNA polymerase in the cytoplasm, LasB, an enzyme secreted by the T2SS and XcpY, an inner membrane protein of the T2SS. Antibodies directed against an outer membrane located protein were not available at the time of testing.

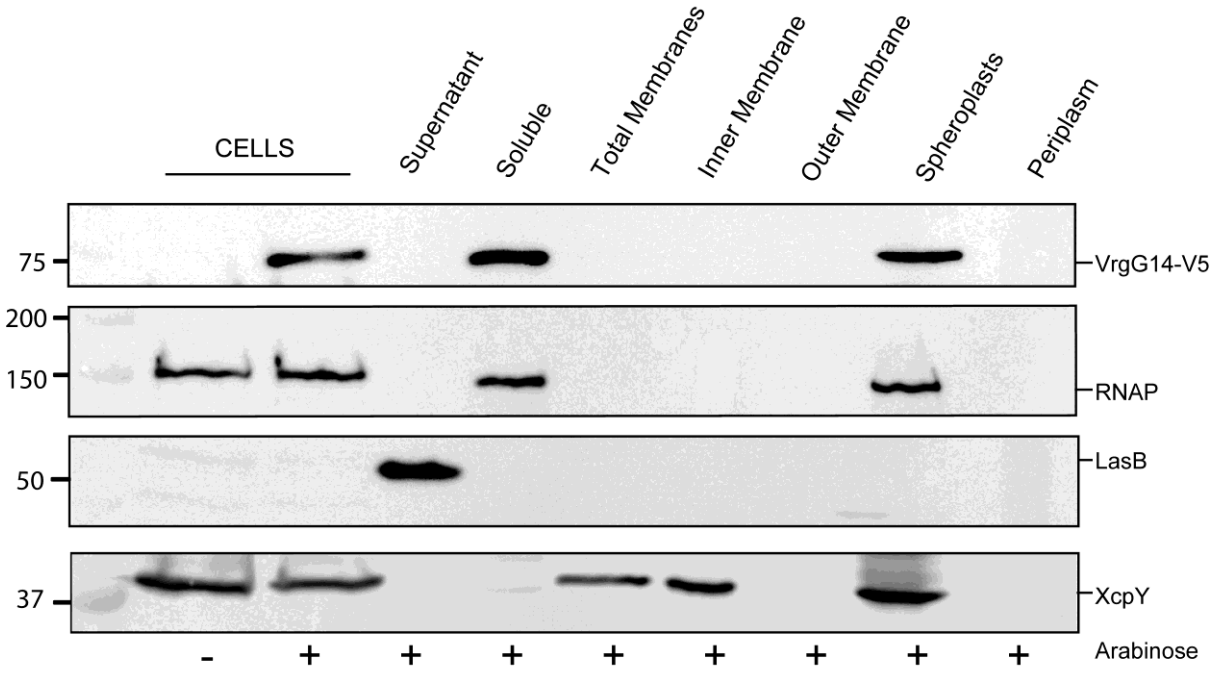


Figure 4.16 Sub cellular fractionation of VgrG14-V5
Fractionation experiments from PA14 DP cells producing V5 tagged versions of VgrG14. Cell fraction indicated above the blots, and the presence or absence of arabinose is indicated below the blots. Samples are probed with (from top to bottom) anti-V5, anti-RNAP, anti-LasB and anti-XcpY, the expected locations of which are indicated to the right of the blots.

VgrG14-V5 is observed in the soluble fraction and in the spheroplasts. The data further confirmed that VgrG14-V5 is not detected in the supernatant fraction. It is associated with the spheroplast fraction, but not detectible in the periplasmic fraction. VgrG14-V5 is absent from the membrane fractions, and thus mostly is a soluble cytoplasmic protein under the conditions tested.

The fact that Hcp2 is secreted suggests that VgrG-dependent membrane puncturing occurs under these conditions, since previous work suggests that Hcp secretion is dependent of the puncturing activity of the VgrG protein (Hachani et al., 2011), though the membrane puncturing role of VgrG14 remains to be shown.

4.7 Rhs14: A Putative H2-T6SS Secreted Effector

One of the four genes found in PA14, adjacent to the H2-T6SS gene cluster, is predicted to encode a 182 kDa protein of the Rhs family. The r*hs* genes were first described in *E. coli* K12, originally classified as 'recombination hot spots' due to the observation of chromosomal rearrangements between *rhs* elements (Lin et al., 1984). However, it is now clear that recombination is not a biologically relevant function of *rhs* genes, and that they encode a family of structurally related proteins with diverse activities (Jackson et al., 2009).

4.7.1 Characterisation of Rhs proteins

A comprehensive analysis of enterobacterial *rhs* genes provides a detailed overview of the structure and function of these genes and the proteins they encode (Jackson et al., 2009). RHS proteins are composed of distinct domains as shown in Figure 4.17. Rhs proteins from enterobacteriaceae contain an N terminal domain, a core YD-peptide repeats domain, a highly conserved 61 amino acid motif ending in PxxxxDPxGL, and a C-terminal tip of 21 to 168 amino acids (Jackson et al., 2009). These C-terminal 'core extensions' are highly variable, with GC content and codon bias that differs from the sequences encoding the N terminal region, suggesting the insertion of horizontally transferred core extension sequences, described further below (Jackson et al., 2009). A key feature of *rhs* genes is that they are often, but not exclusively, found in the vicinity of *vgrG* and *hcp* genes,

and thus are potentially linked to the T6SS. The *rhs* genes are distributed throughout the β- γ- and δ-proteobacteria, and more distantly related YD repeat proteins are found in Gram-positive bacteria, some fungi and higher metazoans (Jackson et al., 2009).

Figure 4.17 Model of Rhs protein domains
A model of a characteristic Rhs protein, as described by (Jackson et al., 2009). The central core domain contains a 61 amino acid hyper-conserved region (red line), and terminates in a conserved consensus sequence as shown. Models were generated using ExPaSy MyDomains image creator.

4.7.2 RhsT from *P. aeruginosa*

The role of an Rhs protein from *P. aeruginosa* PSE9, has been described (Kung et al., 2012). *P. aeruginosa* PSE9 is a highly virulent clinical strain isolated from a patient with ventilator associated pneumonia (Battle et al., 2009). PSE9 contains genomic islands absent from PAO1, and one such island (*P. aeruginosa* genomic island PAGI-9) encodes for an Rhs protein, named RhsT (Battle et al., 2009). RhsT production was not detected in wild type PSE9, but production and secretion was confirmed when RhsT was expressed from an arabinose inducible plasmid (Kung et al., 2012). Indirect immunofluorescence experiments using fixed, but not permeabilised, cells revealed that RhsT is exposed on the bacterial surface, as shown in Figure 4.18A (Kung et al., 2012).

The CCF2 reporter system was used to investigate the translocation of RhsT into eukaryotic cells. J774 macrophages were loaded with CCF2, a green fluorogenic substrate, consisting of two fluorophores separated by a β-lactam ring. In the presence of β-lactamase the substrate is cleaved, and loss of fluorescence resonance energy transfer results in a change to blue fluorescence (Charpentier and Oswald, 2004). A PSE9 strain was generated that expressed RhsT fused to β-

lactamase, and was used to show that RhsT is translocated into macrophages as previously described (Kung et al., 2012).

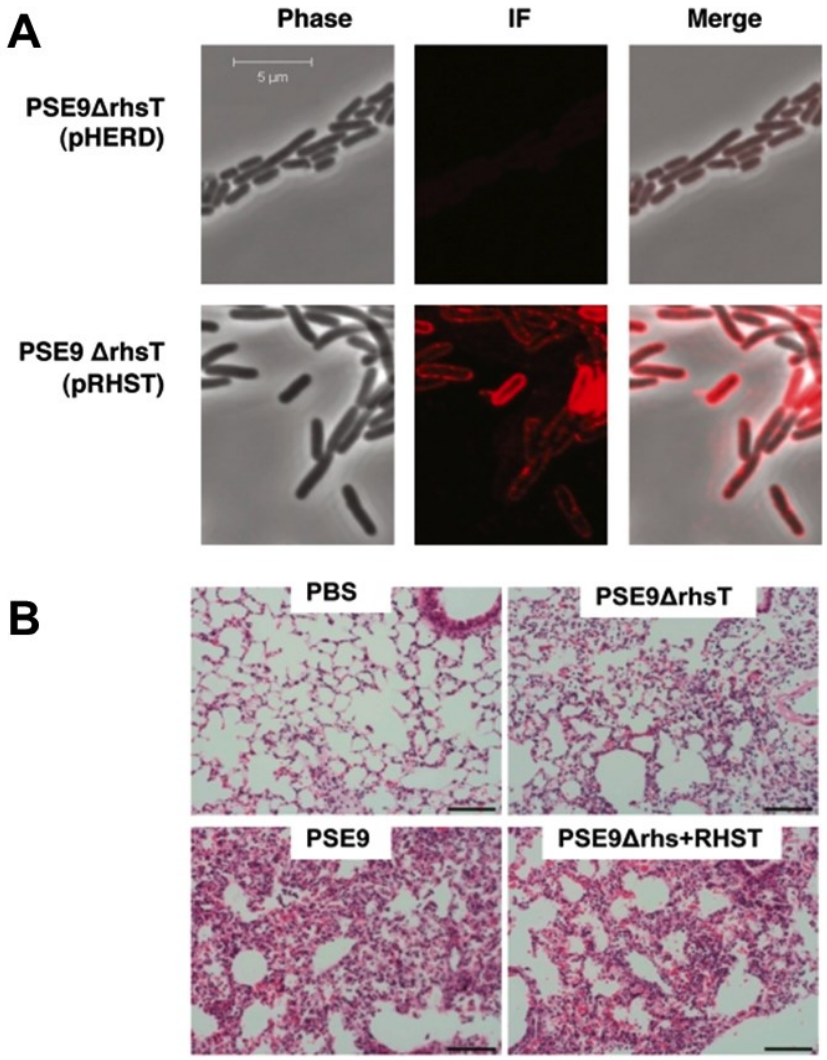


Figure 4.18 *P. aeruginosa* PSE9 RhsT is surface localised and causes pulmonary inflammation
A. Visualisation by indirect immunofluorescence microscopy of RhsT in PSE9Δ*rhsT* containing the empty pHERD vector (upper) or overexpressing *rhsT* form pRHST (lower). Bacteria were fixed but not permeabilised. B. Pulmonary inflammation associated with RhsT in a mouse model of acute pneumonia. Hematoxylin and eosin stained mouse lung sections taken at 26h post-infection with PSE9, PSE9Δ*rhsT*, PSE9Δ*rhsT +* RHST or PBS.

Histological examination of mouse lung tissue shows marked inflammatory cell infiltrates in mice infected with PSE9, while *rhsT* mutant strains show modest levels of inflammatory infiltrates in comparison, as shown in Figure 4.18B. The impact of RhsT on macrophages is clearly demonstrated

by the observation that disruption of *rhsT* results in a 50% reduction in lactate dehydrogenase release compared to infection with wild type PSE9. Pre-treatment with a caspase-1 inhibitor abrogated RhsT-dependent cytotoxicity, indicating that killing involved inflammasome signalling (Kung et al., 2012). Mice infected with PSE9 strains lacking *rhsT* showed increased survival from infection, confirming a role for RhsT in pathogenicity.

The mechanism involved in the transport or secretion of RhsT to the cell surface and translocation into eukaryotic cells is not known. RhsT does not possess an N-terminal Tat or Sec signal sequence, and secretion by a one-step mechanism is likely (Kung et al., 2012). One possibility is that RhsT is directly injected into host cells by the T6SS, but there is no evidence to support this (Kung et al., 2012). Alternatively, RhsT may not be secreted but directly inserted into host cells similar to that seen for contact-dependent inhibition proteins, described in the next section (Aoki et al., 2005).

4.7.3 Contact-dependent inhibition

Bacteria are able to successfully outcompete neighbouring bacteria through a mechanism known as contact dependent growth inhibition (CDI). The first example was described in *E. coli* EC93 which inhibits the growth of *E. coli* K12 strains, a common laboratory strain. This growth inhibition was mediated by CdiAB proteins (Aoki et al., 2005). *cdiA* and *cdiB* encode a two partner secretion system, with CdiB forming a β-barrel pore in the outer membrane, allowing CdiA transport. CdiA is predicted to extend from the cell surface and bind to receptors on target bacteria, as shown in Figure 4.19. Upon contact the C terminal domain appears to be cleaved and translocated into target cells, but the precise mechanism is not known. Expression of the CdiA-Ct inside *E. coli* K-12 cells results in dissipation of the proton motive force, indicative of pore formation in the inner membrane (Aoki et al., 2009). It has been shown that CdiA recognises and binds to the BamA outer membrane protein on target cells, since *E. coli* K12 strains lacking *bamA* are resistant to CdiA mediated killing

(Aoki et al., 2008). Self-toxicity is avoided due to the presence of a third protein, CdiI, which provides specific immunity by binding to CdiA (Aoki et al., 2005). CdiI contains two predicted transmembrane domains, and its localisation to the inner membrane may inhibit the toxic activity of CdiA. The presence of the *cdiABI* locus thus confers a competitive advantage over *cdi* negative *E. coli* strains.

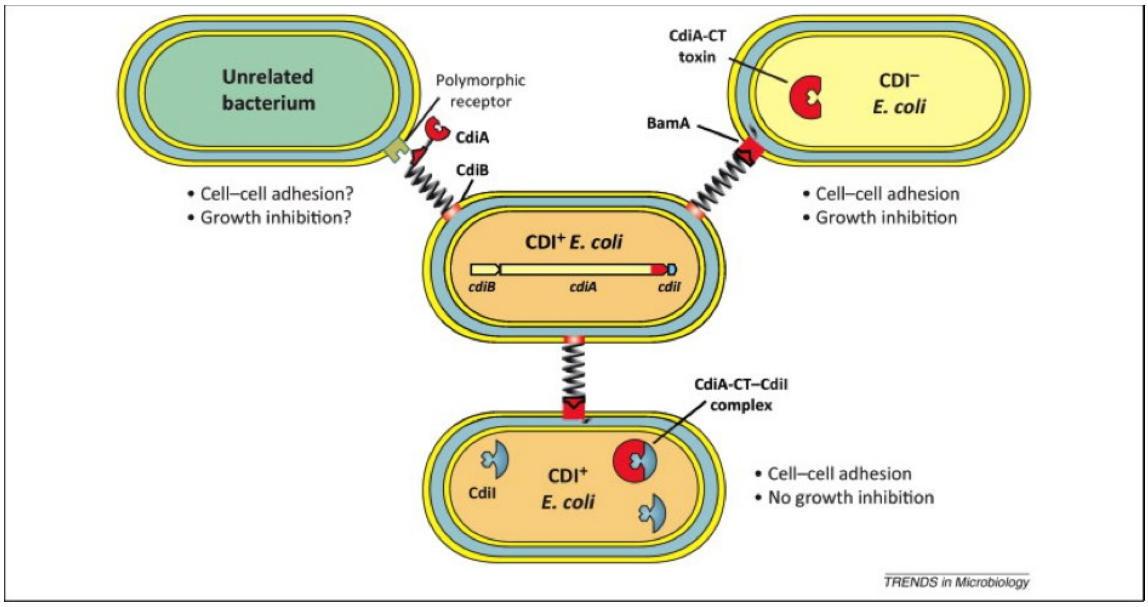


Figure 4.19 Contact dependent growth inhibition
Contact dependent inhibition (CDI) in *E. coli*. CDI^+ cells (centre) express *cdiBAI* gene clusters and present CdiB/CdiA on the cell surface. CdiA binds to receptors on neighbouring cells and delivers a toxin derived from its C terminus, which inhibits the growth of CDI^- cells. Isogenic CDI^+ cells are protected from toxicity by cognate immunity proteins. Reproduced from (Ruhe et al., 2013) with permission.

Similar to *rhs* genes, *cdi* genes are found in many different bacteria, with variation between different strains of a given species (Aoki et al., 2010). While the N termini of CdiA homologues share sequence identity, their C termini, demarcated by a VENN peptide motif, vary greatly, similar to that already described for the highly variable C terminal (Ct) tips of Rhs proteins (Aoki et al., 2010). The Ct tips of CdiA proteins can be exchanged between CdiA homologues from different organisms without loss of activity of the toxin domain, suggesting that CdiAs are modular and capable of delivering different CdiA-Ct toxins (Poole et al., 2011).

The large sequence diversity of CdiA-Ct suggests a broad range of toxin activity, and several have been predicted bioinformatically. These include domains similar to colicins, predicted to have activity similar to their homologous protein (Aoki et al., 2010). CDI toxins have also been predicted to be nucleases, adenosine deaminases, ADP-ribosyl cyclases and metallopeptidases (Iyer et al., 2011; Zhang et al., 2012; Zhang et al., 2011).

CdiABI loci often contain DNA fragments containing additional *cdIA-ct/cdiI* pairs, known as orphan modules, which lack the gene region encoding the N terminal part of the CdiA protein (Poole et al., 2011). These orphan CdiA-Ct lack secretion signals or TPS secretion domains, and are likely not transported. They also lack translational initiation signals suggesting that they are not synthesised. In contrast, it has been shown that orphan CdiI proteins are fully functional, and may persist to confer immunity to multiple CDI toxins (Poole et al., 2011). Expression of orphan CdiA-Ct in *E. coli* leads to toxicity, indicating that the activity of these toxins is conserved. Their presence may indicate alternative reserve toxin domains, with orphan modules being fused to *cdiA* by homologous recombination, resulting in a CdiA protein with an alternative toxic activity and confer a competitive advantage to that bacterium (Poole et al., 2011).

A remarkable observation is that CDI-associated toxin domains are also found in members of the Rhs/YD repeat family, indicating a tight link between these two systems, and cognate immunity genes are also associated with *rhs* genes. The repertoire of toxin-immunity proteins are shared by both CDI and Rhs systems, suggesting that Rhs proteins also mediate bacterial competition. *rhs* loci also encode orphan *rhs-ct/rhsI* modules and are likely capable of toxin exchange by homologous recombination (Poole et al., 2011). A clear difference between CDI and Rhs systems is that *rhs* loci lack the gene encoding the CdiB transporter of the two-partner system, suggesting distinct methods of delivery of Rhs toxins.

4.7.4 Connecting bacterial targeting, Rhs proteins and the T6SS

An example of Rhs mediated toxicity towards bacteria, in the absence of a two-partner delivery system, has recently been shown in the plant pathogen *Dickeya dadantii* 3937 (Koskiniemi et al., 2013; Poole et al., 2011). Three *rhs* genes are encoded by this organism, and two of these inhibit the growth of neighbouring bacterial cells. Strains in which *rhsA* and its cognate immunity genes were deleted exhibited sensitivity to wild type *D. dadantii* during co-culture on solid media, as did strains lacking *rhsB* and its cognate immunity gene, suggesting that both RhsA and RhsB are involved in intercellular competition (Koskiniemi et al., 2013). Reintroduction of the cognate immunity genes protected target cells from growth inhibition, confirming that RhsA and RhsB toxicity is neutralised by cognate immunity proteins. Separation of competing strains by a membrane allowing only secreted substances, but not whole bacterial cells, to pass, abrogated the Rhs mediated toxicity, indicating that this was a contact dependent process (Koskiniemi et al., 2013).

The C termini of RhsA and RhsB show homology to endonucleases, suggesting a mechanism for the observed cytotoxicity. The C terminus of RhsA carries a NS_2 family endonucleases (PF13930), and the C terminus of RhsB contains a HNH endonuclease motif (PF01844) (Koskiniemi et al., 2013). To confirm nuclease activity, the predicted toxins were expressed inside *E. coli* strains stained with DAPI, allowing visualisation of DNA by microscopy. The resulting images are shown in Figure 4.20. Target cells, in which either RhsA or RhsB and their cognate immunity proteins had been deleted were labelled with GFP, and incubated with unlabelled *rhs+* prey cells. DAPI staining showed that 40-50% of target cells lost DNA after 12 hours of culture with prey cells, confirming that RhsA and RhsB mediate toxicity by targeting DNA. Expression of the cognate immunity protein within the target cell prevented the observed DNA loss.

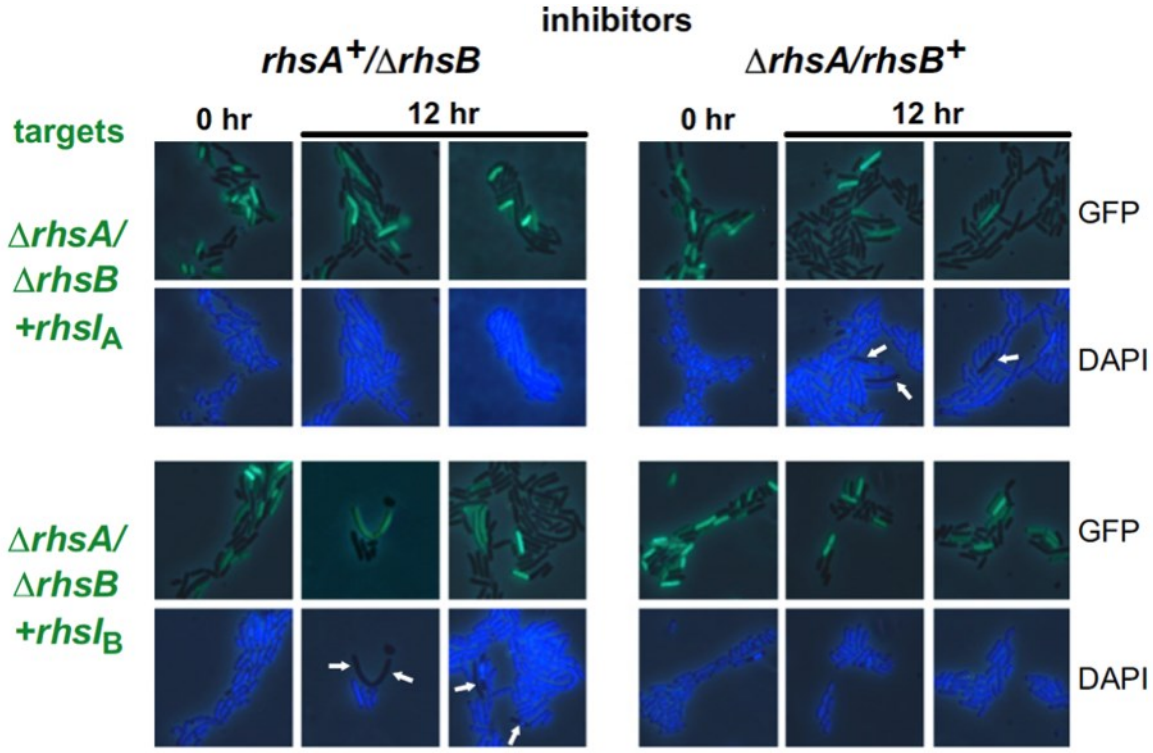


Figure 4.20 RhsA and RhsB from *D. dadantii* degrade the DNA of susceptible neighbouring cells
Fluorescence microscopy of *D. dadantii* 3937 cocultures. Inhibitors expressing RhsA (*rhsA*$^{+}$*/ΔrhsB*) or RhsB (Δ*rhsA/rhsB*$^{+}$) were cocultured at a 1:1 ratio with GFP-labelled target cells lacking both *rhsAI*$_{A}$ and *rhsBI*$_{B}$ loci. Targets were complemented with either *rhsI*$_{A}$ or *rhsI*$_{B}$ where indicated. Samples from 0 and 12 h were stained with DAPI to visualize genomic DNA. Anucleate target cells are indicated by white arrows. Reproduced from (Koskiniemi et al., 2013)

Rhs proteins and T6SS are possibly linked since delivery of RhsA and RhsB from *D. dadantii* was shown to be dependent on *vgrG* genes. Briefly, RhsA and RhsB lack recognisable signal sequences. However, these Rhs are encoded in the vicinity of *hcp* and *vgrG* genes, connecting them to a T6SS-related function. Deletion of both *vgrGa* and *vgrGb*, adjacent to *rhsA* and *rhsB* respectively, abrogated Rhs mediated toxicity (Koskiniemi et al., 2013). Deletion of *vgrGb* alone had no effect, and the toxicity was restored when *vgrGb* alone was reintroduced to the double mutant, indicating that either VgrGa or VgrGb is required for Rhs mediated toxicity in *D. dadantii*. This strongly suggests that RhsA and RhsB are exported using a T6SS mechanism (Koskiniemi et al., 2013).

4.7.5 Rhs14: A putative *P. aeruginosa* Rhs toxin

Considering the connection between *D. dadantii* Rhs proteins and VgrG, and the location of the *rhs14* open reading frame in a *vgrG* cluster; it is tempting to propose that Rhs14 from *P. aeruginosa* PA14 might be a virulence factor and a putative secreted effector of the H2-T6SS, next to which it is encoded. Rhs14 was further characterised by comparison to known Rhs proteins.

Rhs14 is a characteristic Rhs family protein, containing similar domain architecture to the characterised Rhs proteins shown in Figure 4.21. The Rhs14 core domain terminates with PTGWVDPLGL, conforming to the PxxxxDPxGL motif. This motif defines an Rhs14 C terminus tip of 169 amino acids, predicted to have toxic activity, similar to characterised C terminal tips. No putative conserved domains are recognisable within this region (NCBI pBLAST), and no significant structural homology is predicted using a PHYRE search (Kelley and Sternberg, 2009).

Studies of an ABC toxin complex from *Yersinia entomophaga* have provided a remarkable insight into the structure of Rhs proteins (Busby et al., 2013). ABC toxins have been shown to have potent insecticidal activity (Bowen et al., 1998), and are composed of three proteins, A, B and C. The C terminus of the C protein is responsible for cytotoxicity (Lang et al., 2010). A model is proposed where A binds to the surface of target cells, is endocytosed, and forms a transmembrane channel allowing the translocation of the C protein into the cytoplasm, leading to target cell disruption (Lang et al., 2010).

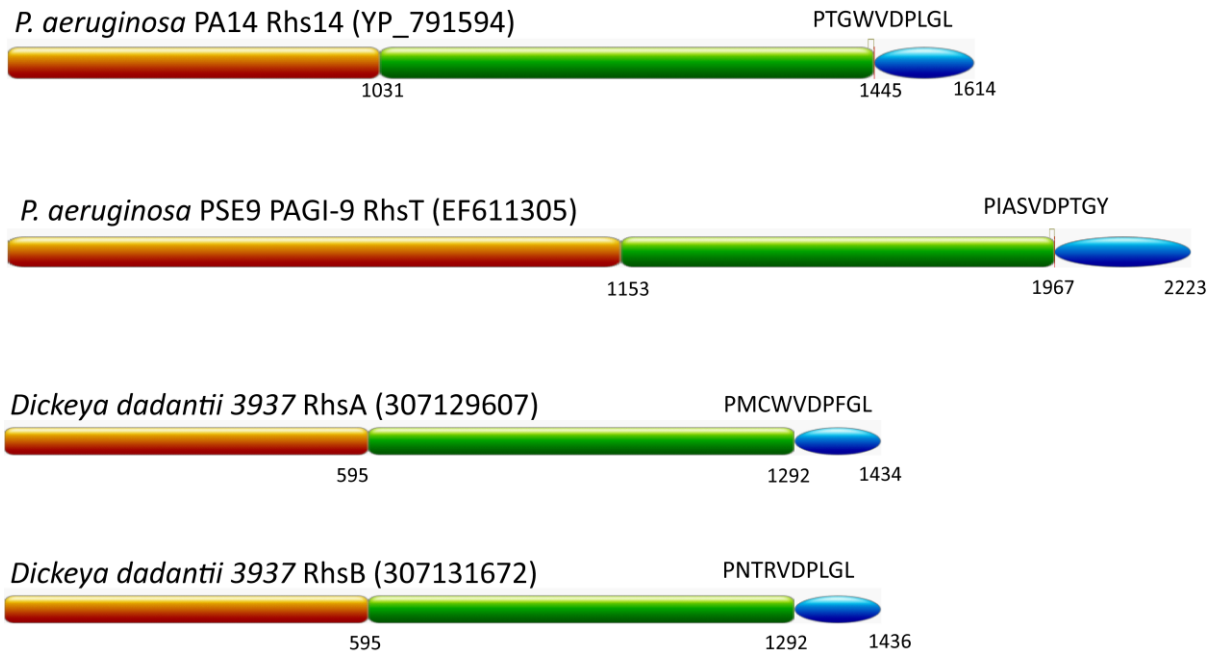


Figure 4.21 Rhs14 domain architecture

Model of *P. aeruginosa* PA14 Rhs14 and the three examples described in the text (*P. aeruginosa* PSE9 RhsT, and *Dickeya dadantii* 3937 RhsA and RhsB). The number of amino acids defining each domain is shown below each model. N terminal domains shown in orange, core domains in green and C terminal domains in blue. The sequence of the PxxxxDPxGL motif defining the end of the core domain is indicated for each protein. Accession numbers are shown in brackets. Protein length is not to scale.

The structure of a complex composed of the BC proteins from *Yersinia entomophaga* was recently reported, and shows an unprecedented large hollow structure, as shown in Figure 4.22A (Busby et al., 2013). The structure is said to resemble the shell of an egg, and has a large hollow central cavity, closed at both ends. It is proposed that this encapsulation device sequesters the cytotoxic C terminal region of the C protein, preventing self-cytotoxicity prior to delivery. An important observation is that the C protein contains Rhs repeats as found in Rhs proteins, and the work by Busby et al. (2013) presents the first structural example of a protein containing such repeats. The observed architecture is likely conserved across Rhs and YD-repeat containing proteins (Busby et al., 2013).

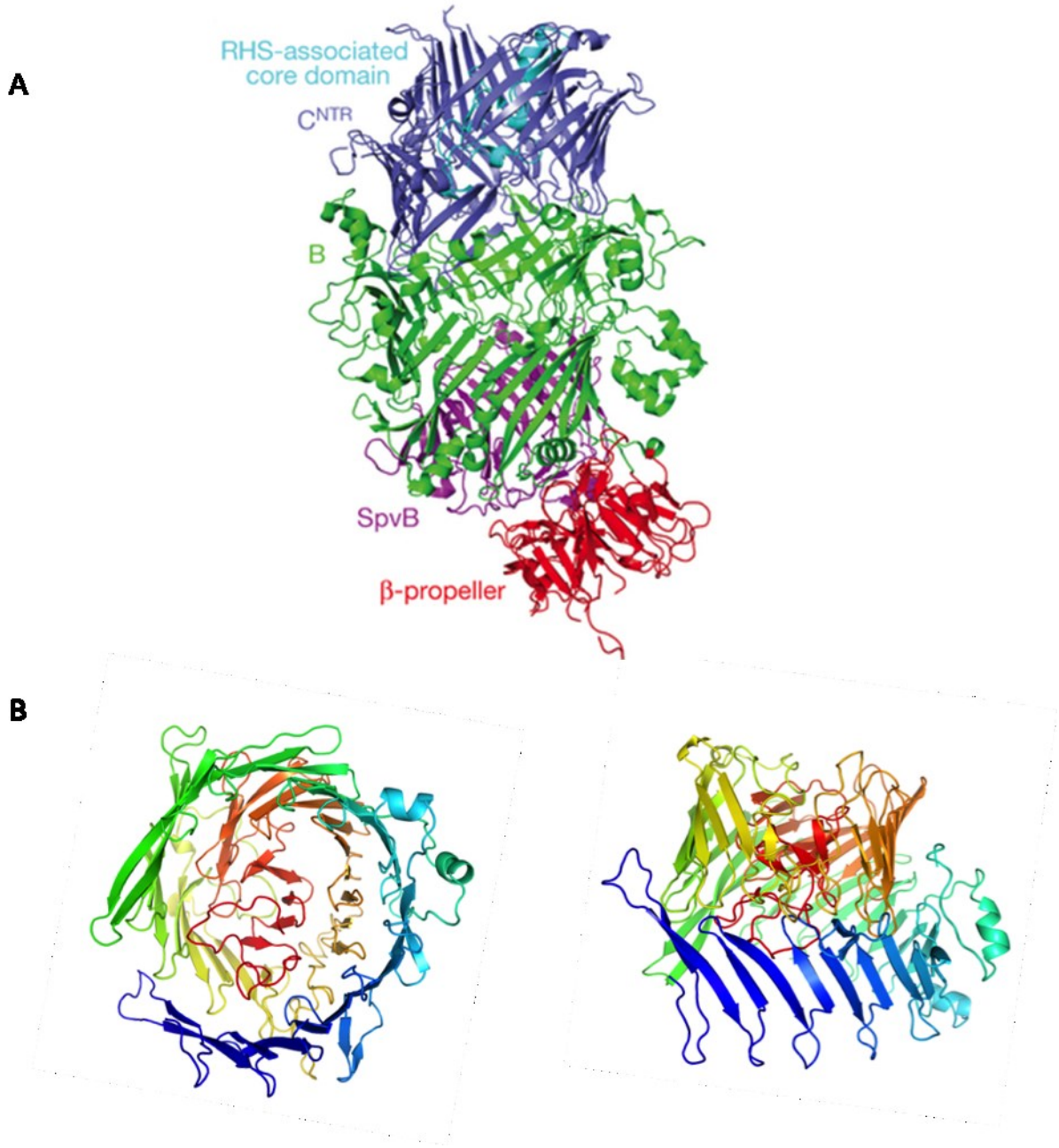


Figure 4.22 Structural predictions for the Rhs14 core domain

The Rhs14 core domain is predicted to adopt a structure homologous to the *Yersinia entomophaga* BC toxin RHS core. A. Side view of the B-C^{NTR} complex of the BC toxin from *Y. entomophaga*. Components of the B proteins are coloured in red purple or green. The CNTR region is coloured blue and the conserved RHS-associated core domain is cyan. Image reproduced from (Busby et al., 2013) with permission from Nature publishing group. B. structural predictions of the Rhs14 core domain, showing 100% structural homology to the region of the *Y. entomophaga* BC toxin coloured in blue and cyan. The Rhs14 model is rainbow coloured, with the blue section representing the N terminus and the red section representing the C terminus of the RHS core. The C terminal of Rhs14 would continue from the red region, and would be located centrally. The left image is looking from the top down, and is rotated 90 degrees to produce a side view (right). Protein structural predictions were made using the PHYRE server (Kelley and Sternberg, 2009)and images generated using PYMOL education build.

The protein sequence of Rhs14 was uploaded to the Phyre server to predict structural homologies (Kelley and Sternberg, 2009). The results show a strong similarity between the Rhs core region of Rhs14 and the structure reported by Busby et al, as shown in Figure 4.22B. Homology was predicted for 38% of the Rhs14 sequence (619 amino acids), corresponding to the central Rhs core. Structural homologies were not found for the N terminal and C terminal regions. The structure of the Rhs14 core was modelled on C terminal region of the *Y. entomophaga* C protein with 100% confidence, indicating that these two proteins are structural homologues. It is very likely therefore that these proteins are also functional homologous, and the core of Rhs14 is important in the encapsulation and delivery of a proposed C terminal Rhs14 tip.

4.7.6 Production and localisation of Rhs14

Based on the above suggestion that Rhs14 may be a T6SS effector, a similar strategy was employed to investigate the production and secretion of Rhs14 as to that used for VgrG14. PA14-DP strain was genetically engineered so that the Rhs14 protein contained a C terminal V5 epitope and poly-histidine tag to allow its detection, in a similar way to that shown for VgrG14-V5, yielding the PA14-DP *rhs14-V5* strain.

The production and secretion of Rhs14-V5 was investigated and the resulting Western blot is shown in Figure 4.23. Hcp2 production and secretion is observed upon arabinose induction, and is not influenced by the genetic manipulation of *rhs14*. A band corresponding to the size of Rhs14-V5 (182 kDa) is observed in samples probed with the anti-V5 antibody suggesting that the chromosomal tagging strategy was successful. However, Rhs14 cannot be detected in the supernatant fraction, as was the case for VgrG14-V5.

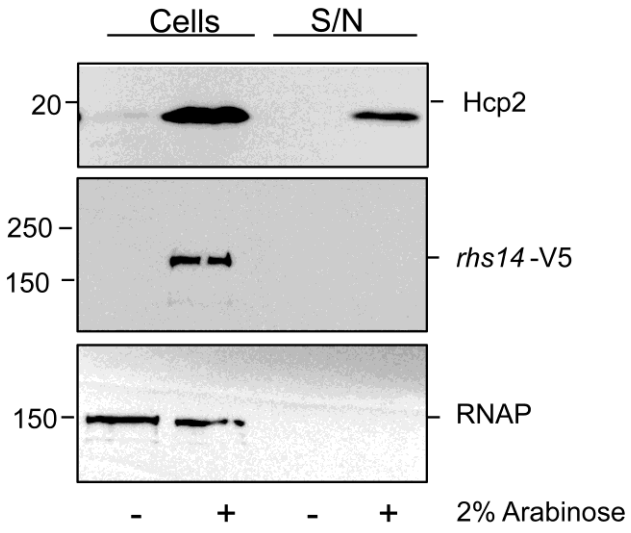


Figure 4.23 V5 tagged Rhs14 is produced but not secreted

Western blot analysis of whole cell extracts and supernatant from PA14-DP strain carrying a chromosomal tagged version of *rhs14* (PA14 DP *rhs14-v5*). The upper blot is probed with anti-Hcp2, the central blot is probed with anti-V5, and the lower blot is probed with anti-RNAP antibody. The molecular weights standards are indicated on the left of each blot, and the expected location of each protein indicated on the right. The type of sample (cells or supernatant) is indicated above each set of blots, and the addition of arabinose to the growth medium is indicated below.

The previously described RhsT protein is surface localised (Kung et al., 2012), therefore it was proposed that Rhs14 may also be located at the cell surface and not secreted. A fractionation experiment was performed to investigate the subcellular location of Rhs14-V5. The resulting western blots shown in Figure 4.24 indicate that the subcellular localisation of Rhs14-V5 is similar to that observed for VgrG14-V5, only clearly detectible in the cytoplasmic fraction. Rhs14-V5 does not co-localise with membrane fractions as would be expected for a surface associated protein, as previously shown for RhsT (Kung et al., 2012).

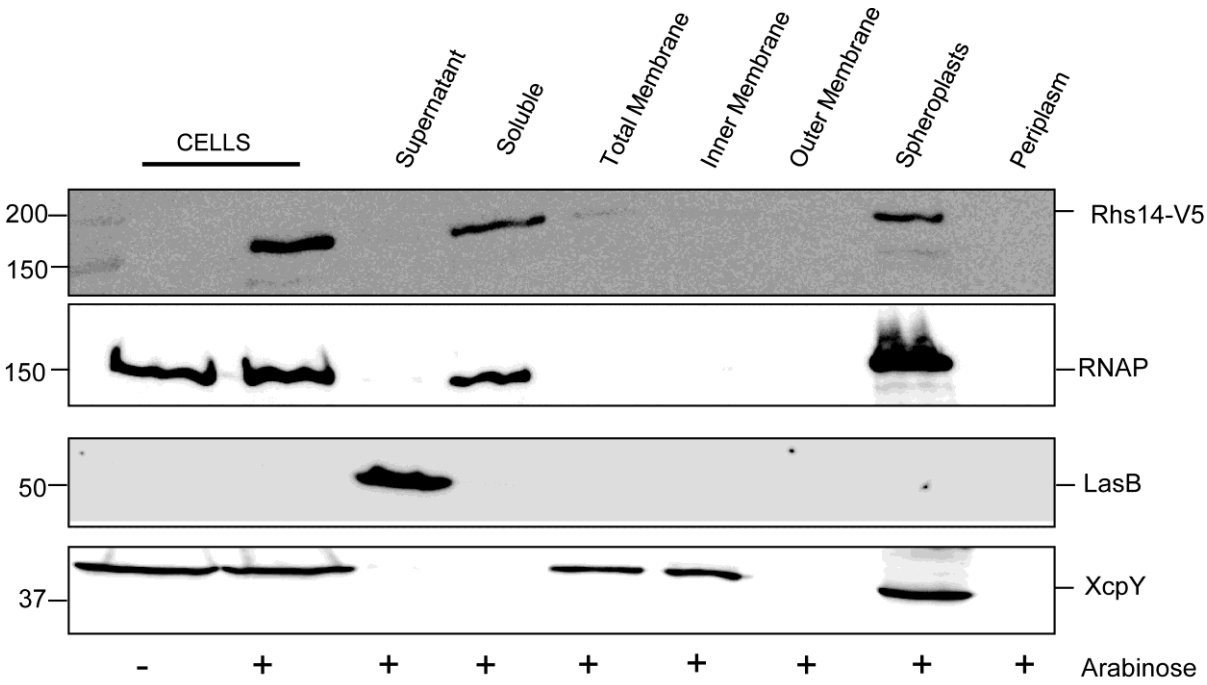


Figure 4.24 Sub cellular fractionation of Rhs14-V5

Fractionation experiments from PA14 DP cells producing V5 tagged versions of Rhs14. Cell fraction indicated above the blots, and the presence or absence of arabinose is indicated below the blots. Samples are probed with (from top to bottom) anti-V5, anti-RNAP, anti-LasB and anti-XcpY, the expected locations of which are indicated to the right of the blots.

4.7.7 Rhs14 transfection

Preliminary experiments have been performed in our laboratory by Dr A. Hachani, investigating the activity of Rhs14 when expressed ectopically in HeLa cells (data not shown). Full length *rhs14* was cloned into the pRK5 mammalian expression vector to allow production in HeLa cells. HeLa cells transfected with the pRK5-r*hs14* vector were cultured on microscope slides for 24 hours, allowing constitutive expression of Rhs14. Rhs14 is proposed to be highly toxic under these conditions, since its expression resulted in the death of HeLa cells, with no viable cells visible by microscopy. Cells expressing non-related proteins showed healthy growth under the same conditions. These observations suggest that Rhs14 may possess eukaryotic cell targeted toxicity, and additional experiments to confirm this observation and subsequently characterise the mechanism of toxicity are required.

4.7.8 Putative Rhs14 immunity protein

A characteristic of contact dependent inhibition and Rhs C-terminal toxic domains is the association of a cognate anti-toxin, which prevents self-intoxication, or intoxication by neighbouring cells. If Rhs14 exhibits toxic activity it would also be expected to be associated with a specific immunity protein. The annotated genome sequence of *P. aeruginosa* PA14 at pseudomonas.com does not show an open reading frame downstream of *rhs14*, where an 800 base pair region of un-annotated DNA is found. This region was analysed by ORF Finder (NCBI) and found to contain a predicted 435 base pairs open reading frame. The DNA and protein sequences of this proposed orf are shown in Figure 4.25. Translation of this sequence yields a 16.5 kDa protein that we propose to be a specific immunity protein for Rhs14. No putative conserved domains are encoded within this protein (NCBI pBLAST), and no significant structural homology is predicted using a PHYRE search (Kelley and Sternberg, 2009), which is not surprising given the diverse nature of immunity proteins. Further experiments to confirm these observations are currently underway in the laboratory.

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agaaaactcatgaaaaccatttataacttcaaacagcgtatcaaagaagaccccgagtac
 R  K  L  M  K  T  I  Y  N  F  K  Q  R  I  K  E  D  P  E  Y
attcgaaaagcccatgaactcactttgaatacaacgaagcctaaagctggccttaaaggg
 I  R  K  A  H  E  L  T  L  N  T  T  K  P  K  A  G  L  K  G
acttatggactactgggctcaaaggaatggtgggacaatcttgaaaacggatcaattcca
 T  Y  G  L  L  G  S  K  E  W  W  D  N  L  E  N  G  S  I  P
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 Q  K  E  I  S  G  T  I  K  K  V  Y  L  T  G  Q  D  N  T  E
gatttcaacacgatagatatagaaacagaaaacaaaaccctctgcacggagggcacttac
 D  F  N  T  I  D  I  E  T  E  N  K  T  L  C  T  E  G  T  Y
acaaacaagaataccgacagaaagcattacgaagcaggaaaaaaaatcaccataaaatat
 T  N  K  N  T  D  R  K  H  Y  E  A  G  K  K  I  T  I  K  Y
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 A  F  D  P  L  K  K  P  K  P  N  G  D  I  D  Y  S  K  I  V
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 V  E  I  L  I  S  E  -  L  K  I  S  L  R  E  G  Y  I  K  N
caaaagacagaagcaaatgaaaacccccctatccatgggaatcatacatcggaaagttgac
 Q  K  T  E  A  N  E  N  P  L  S  M  G  I  I  H  R  K  V  D
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 L  M  T  W  P  N  T  A  G  N  F  P  W  L  R  K  F  T  H  L
gcccaatgagtggaaatttcgctctacccgaaactcccgctcatgacagaaatcactgcc
 A  Q  -  V  E  I  S  L  Y  P  K  L  P  L  M  T  E  I  T  A
tcgccaaccgctgatcgaagctcccc
 S  P  T  A  D  R  S  S
```

Figure 4.25 Putative Rhs14 immunity protein
Genetic sequence of the region downstream of the *rhs14* stop codon on the PA14 chromosome. The open reading frame predicted to encode a Rhs14 immunity protein is highlighted in yellow, and the translated protein sequence is highlighted in cyan.

4.7.9 Proteins encoded by genes adjacent to *rhs14*

In addition to *hcp2*, *vgrG14* and *rhs14*, another gene is encoded within this cluster linked with the PA14 H2-T6SS genes. This gene is located between *vgrG14* and *rhs14*, and is annotated as PA14_43090. The protein encoded by this open reading frame contains a predicted domain of unknown function (DUF4123/PF13503), but the role of this protein and its involvement in the T6SS is not known. There are no predicted transmembrane domains (TMPred server) or secretion signals (Signal P) within this protein.

Analysis of the PF13503 family shows that homologs are found in *V. cholerae*, encoded by VCA0019 and VC1417. Interestingly, VCA0019 and VC1417 are found within *V. cholerae* T6SS cluster, upstream of genes encoding two characterised T6SS toxins, *vasX* and *tseL*, respectively, and downstream of a *vgrG* in each case, as indicated by Figure 4.26. Although the precise role of the protein encoded by PA14_43090 is not known, it is clearly conserved in diverse T6SS clusters, and can be proposed to have a central role in the system. The fact that in each case this gene is encoded between a *vgrG* and a toxin gene suggests that the gene product may interact with one or both VgrG and the toxin, and therefore may be required for their delivery to target cells.

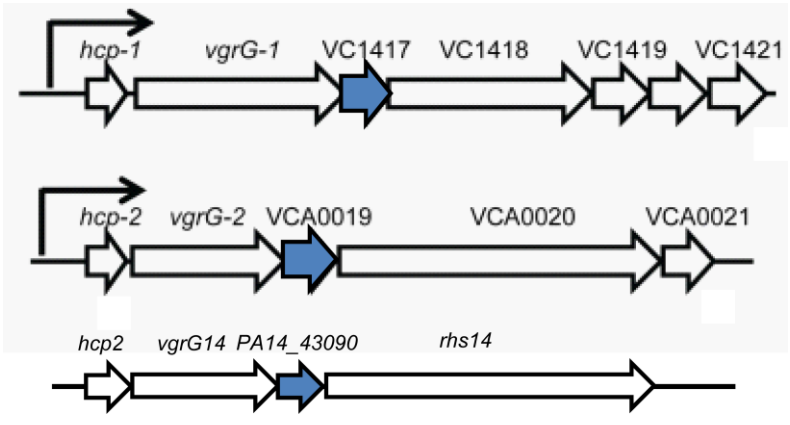

Figure 4.26 Location of PF13504/DUF4123 proteins in *vgrG* islands
The presence of genes encoding proteins with PF13503/DUF4123 domains within *vgrG* islands is shown in blue. Top: vgrG-1 island form *V. cholerae*, middle: vgrG-2island from *V. cholerae*, bottom: the vgrG14 island from *P. aeruginosa* PA14.

4.8 Post-translational regulation of the H2-T6SS

The fact that we were not able to observe VgrG14 or Rhs14 secretion, whereas we expect the H2-T6SS to be assembled because of Hcp2 secretion, led us to consider that the system may not be fully active. As described in section 1.7.5 several T6SS clusters encode for kinase/phosphatase pairs important in post-translational control of the secretion mechanism, and we investigated whether post-translational regulation of the H2-T6SS might play a role in Rhs14 or VgrG14 secretion.

Briefly, a previous study of the H1-T6SS showed that manipulation of the kinase/phosphatase system could activate secretion (Mougous et al., 2007). The H1-T6SS cluster encodes a serine/threonine kinase (PpkA) and a serine/threonine phosphatase (PppA). The target of these two proteins is the FHA domain containing protein, Fha1. Under conditions where the H1-T6SS was active (in a *retS* mutant) Mougous and colleagues showed that deletion of the kinase abrogated Hcp1 secretion due to a decrease in phosphorylated Fha1. Deletion of the phosphatase increased Hcp1 secretion, due to an increase in stable phosphorylated Fha1. (Mougous et al., 2007).

A similar post-translational regulation may occur in the H2-T6SS, since conserved kinase genes (*stk2*), phosphatase (*stp2*) and *fha2* are encoded within the cluster. To investigate the effect of post-translational regulation on the activity of the H2-T6SS the gene encoding the phosphatase, *stp2*, was deleted from the chromosome of both PA14-DP *vgrG14*-v5 and PA14-DP *rhs14*-v5 strains. The deletion of *stp2* should favour Fha2 phosphorylation and therefore the activity of the H2-T6SS. Secretion of each putative substrate was re-evaluated in this background, and the resulting western blots are shown in Figure 4.27.

The *stp2* deletion does not affect arabinose induced VgrG14-V5 production, but also does not promote secretion since VgrG14-V5 is not detectible in the supernatant of this strain (Figure 4.27A). A similar result is seen for Rhs14-V5 (Figure 4.27B), which is not secreted even following deletion of *stp2*. Despite using various approaches, we were not able to demonstrate the secretion of VgrG14-V5 or Rhs14-V5 in this study. However, based on the hypothesis that VgrG14, if similar to other VgrGs, should be recovered from the supernatant, it is still an open possibility that Rhs14 may be a true H2-T6SS substrate, and the addition of the tag may interfere with recognition by the H2-T6SS.

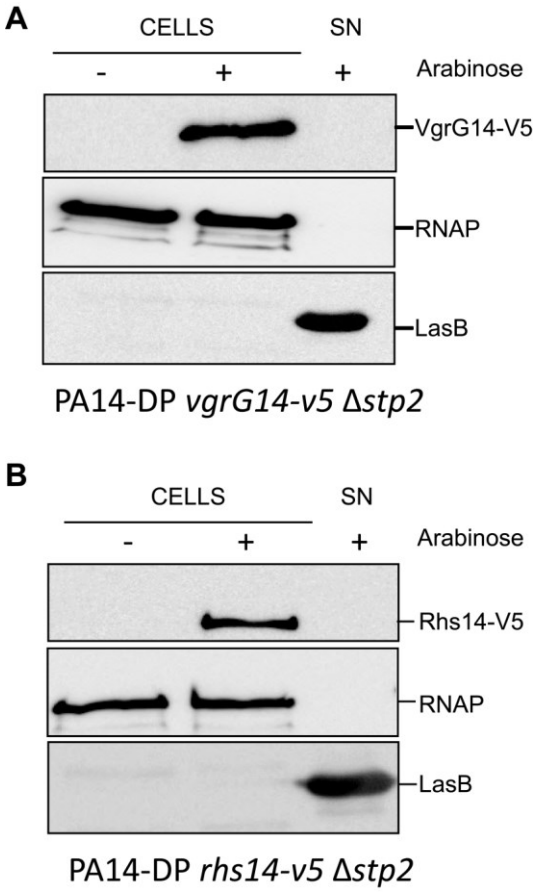

Figure 4.27 Secretion of VgrG14-V5 and Rhs14-V5 in an *stp2* mutant
Western blot analysis of whole cell extracts (Cells) and supernatants (SN) from PA14-DP∆*stp2* strain encoding a chromosomal tagged version of either *vgrG14*(A) or *rhs14*(B). In both cases, the upper blot is probed with anti-V5, the central blot with anti-RNAP (cytoplasmic control), and the lower blot is probed with anti-LasB antibody (a T2SS dependent secreted protein). The expected position of each protein is indicated on the right. The sample type (cells or supernatant), and the absence (-) or presence (+) of arabinose is indicated above each set of blots.

4.9 A role for H2-T6SS: Bacterial Killing

The H1-T6SS is involved in the delivery of toxins and killing of target bacterial cells (Hood et al., 2010). Monitoring bacterial killing has subsequently become a way of monitoring the activity of T6SS, and for investigating the mechanism of the machinery (Hachani et al., 2013). A recent publication has implicated the H2-T6SS in bacterial killing through the discovery of a H2-T6SS dependent toxin, Tle5, in *P. aeruginosa* PAO1, shown to be a toxin lethal to susceptible *P. aeruginosa* strains (Russell et al., 2013). The *E. coli* killing assay developed in our laboratory was therefore used to investigate the role of the *P. aeruginosa* PA14 H2-T6SS in bacterial killing.

An assay was set up to investigate the survival of *E. coli* cells in contact with wild type PA14, or a PA14 strain in which the H2-T6SS cluster, from *hsiA2* to *clpV2*, was deleted, generating PA14 ΔH2. As previously described, the *E. coli* strain carries the pCR2.1 vector, allowing alpha complementation of the *lacZ* gene, allowing it to be detected on X-gal agar plates by visualising the blue colour of the colony (Hachani et al., 2013).

The results of this experiment is shown in Figure 4.28. In figure 4.28A, the top row of images shows X-gal plates inoculated with serial dilutions of monocultures of each strain used in the assay. The two *P. aeruginosa* strains do not generate the blue colour since they lack the *lacZ* gene, while the *E. coli* carrying the pCR2.1 is viable and develops a strong blue colony. The lower images show strains recovered from mix culture competition assay where each *P. aeruginosa* strain was incubated with the *E. coli* reporter strain. In both cases no blue colour is observed following the competition assay, that is upon mixing *E. coli* and *P. aeruginosa*, and colonies show a similar colour to the *P. aeruginosa* monocultures. This suggests that *E. coli* is not recovered when incubated with *P. aeruginosa* PA14, and this phenotype is not associated with the H2-T6SS, since deletion of the cluster has absolutely no effect on the survival of *E. coli*.

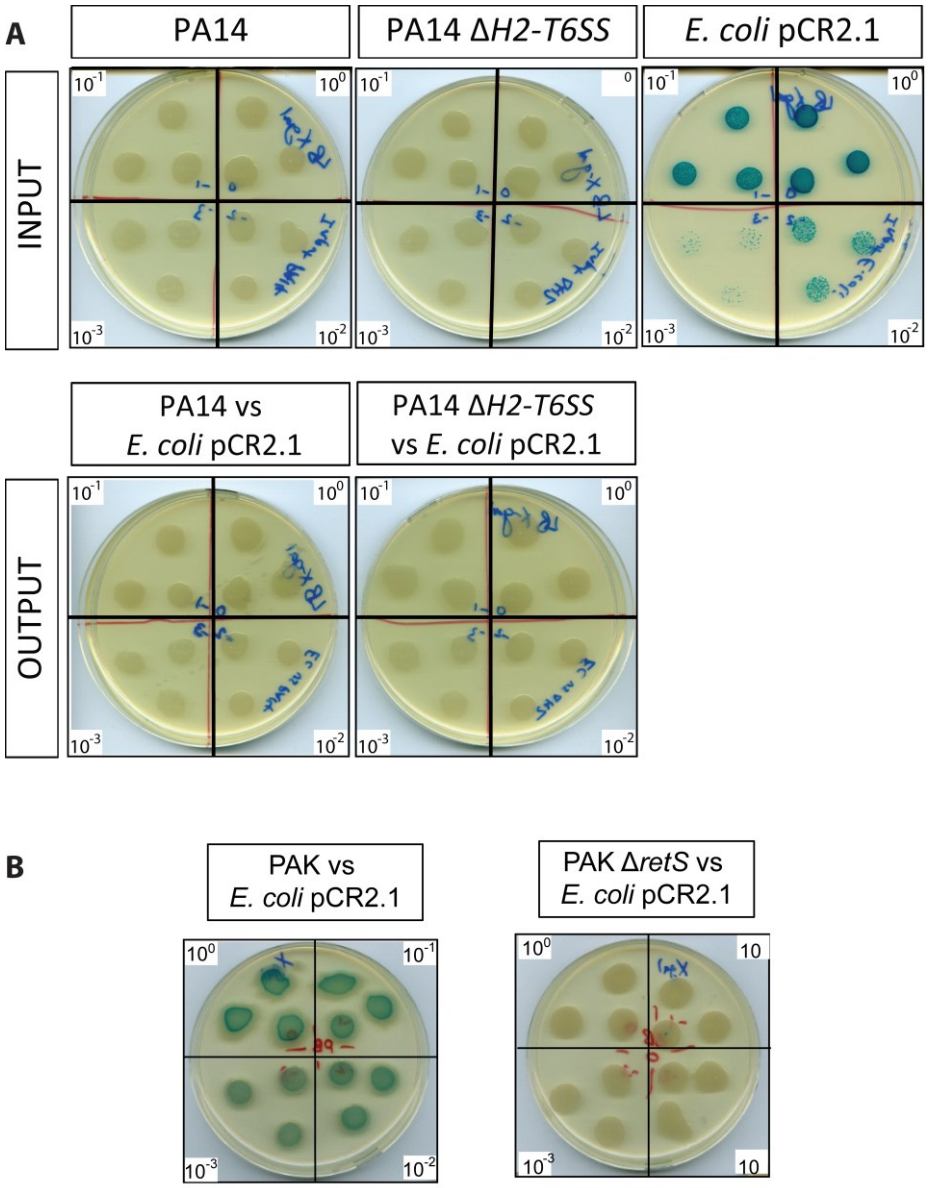

Figure 4.28 *P. aeruginosa* PA14 outcompetes *E. coli* in a H2-T6SS independent manner
A. Top row shows monoculture strains used in this assay, incubated for 5h on LB plates, resuspended, and subsequently plated onto LB X-gal plates in triplicate in a serial dilution from 10^0 to 10^{-3}. Second row shows the resulting colony growth from mixed cultures of two strains, as indicated, which were co-incubated for 5h on LB plates and subsequently plated as described onto LB X-gal plates. B. A typical result from a bacterial competition assay showing survival of E. coli pCR2.1 incubated with wild type PAK, and loss of the *E. coli* population following co-incubation with a *retS* mutant.

This result is different to that previously reported for a different *P. aeruginosa* strain, since *E. coli* is reported to survive competition with *P. aeruginosa* PAK, and is only susceptible to killing when the

H1-T6SS is activated by *retS* deletion, as shown in Figure 4.28B (Hachani et al., 2013). Under the conditions used for this assay PA14 is likely to produce another toxic compound, such as pyocyanin, which is sufficient to outcompete *E. coli*, and therefore will mask any impact of the T6SS.

4.10 A role for H2-T6SS: Bacterial Internalisation

Although recent suggestions that the H2-T6SS is involved in bacterial killing, two publications have previously reported that this system is important in *P. aeruginosa* pathogenesis, and interaction with eukaryotic cells, as described in section 4.1. The work by Lesic and colleagues provided indirect evidence that the H2-T6SS cluster from *P. aeruginosa* PA14 was involved in virulence in different infection models, but did not identify any secreted effectors associated with the process (Lesic et al., 2009). Similarly, Sana *et al*. showed a direct effect of the H2-T6SS system on internalisation of *P. aeruginosa* PAO1 into HeLa cells (Sana et al., 2012), but also no effectors were identified as being responsible for such a phenotype. Here we performed experiments to investigate the role of the PA14 H2-T6SS in cytotoxicity and internalisation of PA14. All tissue culture work described in this section was carried out in collaboration with Dr A. Hachani.

4.10.1 Generation of a non-cytotoxic PA14 strain

As described in section 1.6, PA14 is highly cytotoxic due to the secretion of the T3SS effector ExoU, a potent phospholipase encoded in the pathogenicity island PAPI-1, that causes rapid host cell lysis (Sato et al., 2003; Schulert et al., 2003). A prerequisite to testing the internalisation of *P. aeruginosa* PA14 was thus to engineer a non-cytotoxic strain. Previous studies have indicated that disruption of the *pscC* gene, which encodes the T3SS outer membrane secretin, is sufficient to abrogate the T3SS mediated cytotoxicity of *P. aeruginosa* PA14 (Mikkelsen et al., 2011a; Urbanowski et al., 2007). A strategy was designed to generate a clean deletion of *pscC*, yielding PA14Δ*pscC*.

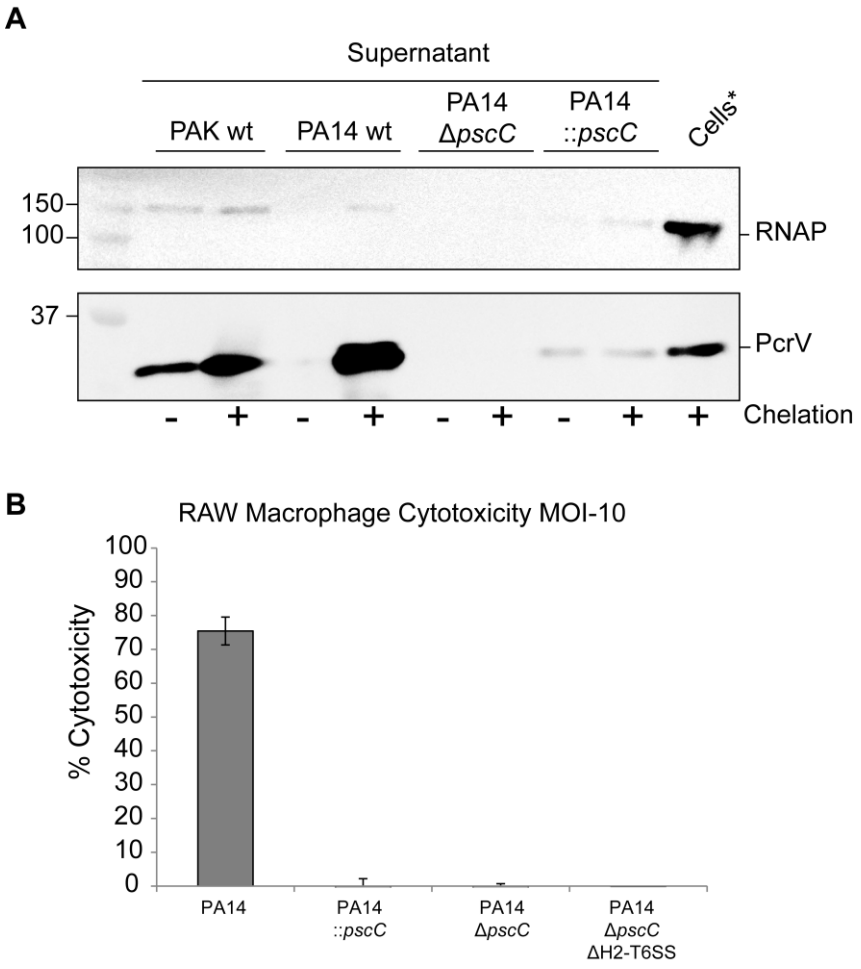

Figure 4.29 Secretion profile and cytotoxicity of a PA14 *pscC* mutant

A. T3SS secretion profile in supernatants of wild type PA14, a clean *pscC* deletion mutant, and a PA14::*pscC* transposon mutant, as indicated above the blots. Blots are probed with anti-RNAP (upper) or anti-PcrV (lower), with the expected position of each protein indicated on the right, and molecular weight markers shown on the left. The presence or absence (+/-) of calcium chelation used to induce the T3SS is indicated below the blots. A whole cell extract of a calcium chelated PAK culture is included as a positive control for RNAP production (*). B. Cytotoxicity assay measuring lactate dehydrogenase release from RAW macrophages infected with strains indicated below the chart at an MOI of 10 during a 3h infection. Percentage of cytotoxicity is shown on the vertical axis. Error bars show standard deviation.

The T3SS phenotype of PA14 Δ*pscC* was tested by isolating supernatants from this strain, and comparing with the transposon mutant from the Ausbel collection, PA14::*pscC* (Liberati et al., 2006), and the wild type PA14 strain. Secretion of PcrV, the tip of the T3SS needle, was used as a reporter to monitor T3SS activity. The resulting western blot using anti-PcrV is shown in Figure 4.29A, and indicates that PcrV is not detected in the supernatant of the PA14 wild type strain. Calcium

chelation is a known trigger for the in-vitro activation of the T3SS, and upon addition of 5 mM EGTA to the growth medium a strong induction of PcrV secretion is then observed in wild type PA14. This effect is abrogated in both the PA14::*pscC* transposon mutant and the clean deletion of PA14Δ*pscC* strain, although there may be a background level of PcrV secretion in the transposon strain that is absent in the clean deletion. For this reason, we use both strains for further investigations.

The cytotoxicity phenotype of the *pscC* mutants was tested by measuring lactate dehydrogenase release from RAW macrophages. The result of this assay can be seen in Figure 4.29B. The PA14 wild type strain is highly cytotoxic, causing 75% LDH release, whereas disruption of *pscC* by transposon insertion or clean deletion totally abrogates this cytotoxicity, confirming that both mutants are T3SS-deficient and that the cytotoxicity of the PA14 strain is entirely mediated by the T3SS. Now that we have a non-cytotoxic PA14 strain available, the role of the H2-T6SS in internalisation can be studied.

4.10.2 Internalisation of PA14

Internalisation into eukaryotic cells is an alternative strategy of infection to cytotoxicity, allowing cells to survive within the nutrient-rich protective environment of host cells, subverting host cell processes to prevent their eradication or evading the host immune system. As described before, the H2-T6SS has been reported to be partly involved in the process since internalisation of a PAO1 Δ*clpV2* mutant into HeLa cells was shown to 75% lower than the wild type strain. This suggested that in this strain the H2-T6SS acts to promote internalisation (Sana et al., 2012).

Similar gentamycin protection assays were performed to confirm the phenotype observed by Sana et al. as shown in Figure 4.30A. The PAO1 Δ*clpV2* strain shows lower levels of internalisation than the parental PAO1 strain, confirming that the H2-T6SS cluster of *P. aeruginosa* PAO1 promotes the internalisation.

Internalisation in HeLa cells was then measured with the *P. aeruginosa* PA14Δ*pscC* strain, and a newly generated double mutant, PA14Δ*pscC*ΔH2, in which the H2-T6SS cluster is deleted in the *pscC* background. Surprisingly, when the H2-T6SS is disrupted, internalisation is increased around 4-fold, which is clearly is different to what is observed in the PAO1 strain, and suggests that in PA14 the H2-T6SS acts to limit internalisation, and not to promote it (Figure 4.30B).

The internalisation phenotype of the H2-T6SS was also tested in the PA14-DP background to investigate the effect of artificial activation of the system on internalisation. The *pscC* mutation was introduced into PA14-DP, generating PA14-DPΔ*pscC*. The levels of internalisation of the PA14-DP Δ*pscC* mutant was equivalent to PA14Δ*pscC*ΔH2 (Figure 4.30B). This suggests that in the original PA14 strain the H2-T6SS may be activated upon contact with HeLa cells, and thus a phenotype can be observed when comparing the H2-T6SS mutant with the parental strain. In the PA14-DPΔ*pscC* strain the H2-T6SS promoter is disrupted, and expression of the H2-T6 SS genes may no longer respond to HeLa cell contact, hence this strain behaves like a H2-T6SS mutant, and display increased internalisation compared to PA14Δ*psc*. Upon addition of arabinose to the PA14-DP Δ*pscC* strain, the level of internalisation drops to levels similar to the PA14Δ*pscC* strain. This indicates that artificial activation of H2-T6SS by arabinose induction results in H2-T6SS activation and mediates prevention of internalisation in HeLa cells, just like the PA14Δ*pscC* strain.

The opposite effects on internalisation observed between PAO1 and PA14 are interesting, and suggest that the H2-T6SS system performs different roles in these two strains. As described in section 4.3, the presence of genes encoding putative effector proteins VgrG14 and Rhs14 in the PA14 cluster may be responsible for this difference, which will need further investigation.

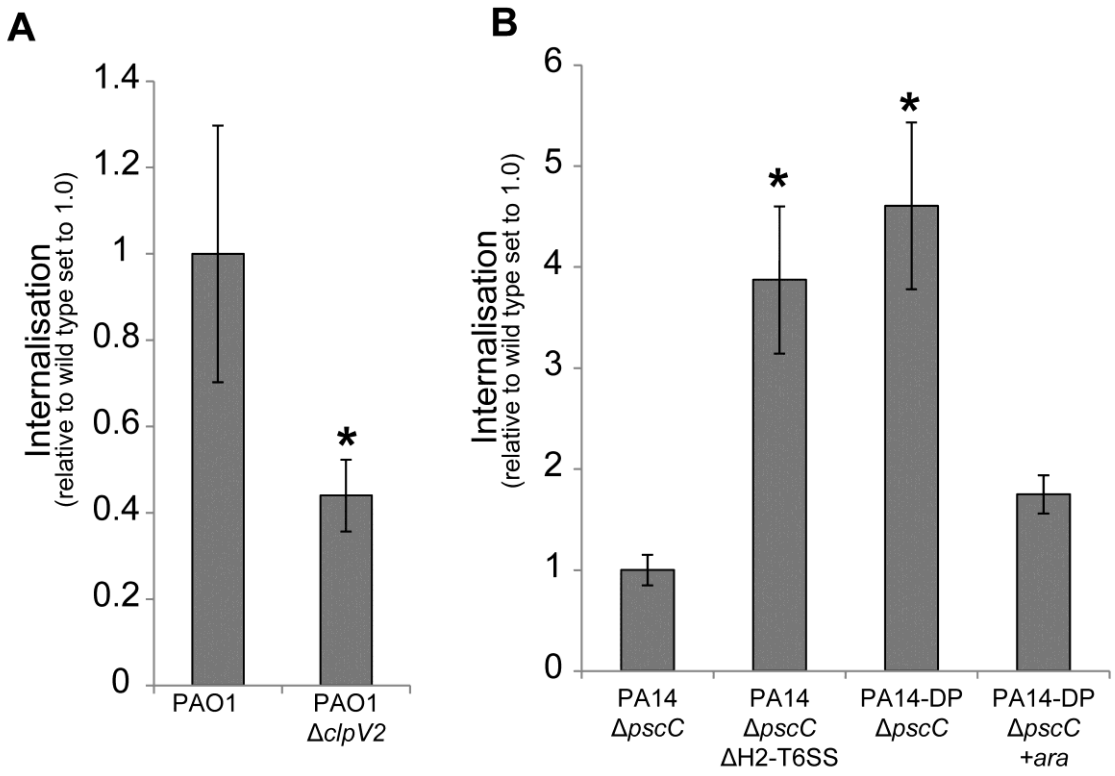

Figure 4.30 The H2-T6SS of PA14 inhibits bacterial internalisation in HeLa cells

A. Relative internalisation in HeLa cells of an isogenic mutant PAO1Δ*clpV2* compared to its parental strain PAO1. (B) Relative internalisation of PA14Δ*pscC*ΔH2-T6SS double mutant, and the PA14-DPΔ*pscC* strain (carrying an inducible H2-T6SS), incubated with (+ara) and without arabinose is shown compared to the parental strain PA14Δ*pscC*. In all cases the internalisation of the isogenic mutants is shown relative to the invasion level exhibited by the corresponding parental strain (normalised to 1.0). The strain tested is indicated below each bar. Error bars show standard deviation of three replicates, and the results shown are representative of three different experiments. * indicates a statistically significant difference compared to the corresponding parental strain (Student's T-test, P<0.001).

4.11 Discussion

This chapter presents a study investigating the activation and function of the second Type VI secretion system (H2-T6SS) from *P. aeruginosa* PA14, and highlights an important feature of this strain; the presence of four additional genes associated with this cluster. This feature is striking, since it includes genes encoding Hcp and VgrG proteins, which are missing in the H2-T6SS of PAO1. The absence of core components from the PAO1 H2-T6SS challenged our concept of how this system was working and which were the true core components. An additional feature specific to the PA14

strain is the presence of a gene, which encodes a member of the Rhs protein family, for which recent studies have shown might be a genuine effector for the T6SS. In this work, the H2-T6SS is genetically engineered by the introduction of arabinose inducible promoters allowing simultaneous activation of both gene clusters and a tight and full control on the expression of these genes. This has successfully allowed the study of the H2-T6SS under standard bacterial culture conditions, and is comparable to what was achieved in other studies with *Citrobacter rodenitum,* for example (Gueguen and Cascales, 2013). Furthermore, this work represents the first study of the H2-T6SS at the protein level and investigating production and secretion of H2-T6SS-related proteins.

4.11.1 Hcp2 and VgrG14 Secretion

Hcp is a core functional component of the T6SS machinery, and its localisation in the supernatant is considered a hallmark of a functioning T6SS. Hcp secretion was a key phenotype in the early characterisation of the T6SS in both *P. aeruginosa* and *V. cholerae* (Mougous et al., 2006; Pukatzki et al., 2006). We demonstrate using our engineered strain that upon arabinose induction Hcp2 secretion is observed, and is dependent on the H2-T6SS cluster. This indicates that in these conditions H2-T6SS assembles into a functional secretion machinery, and confirms a link between the two adjacent gene clusters. Since previous studies on this system have been exclusively performed at the transcriptional level, this is the first report of production and assembly of the H2-T6SS at the protein level.

The Hcp tube is proposed to be topped by VgrG proteins, which puncture the cell membranes, allowing both Hcp and VgrG to be propelled from the cell into the supernatant. An obvious hypothesis was that VgrG14 has such a role and should provide Hcp2 with access to the outside of the cell. While arabinose induction allowed detection of a C terminal tagged version of VgrG14, its presence in the supernatant was not detected. This is somewhat surprising, since it suggests that

Hcp2 is gaining access to the supernatant independent of the puncturing device function of VgrG14, although it might suggest that VgrG14-lacking H2-T6SS, may still be functional using an alternative Hcp/VgrG device, as would be the case in PAO1. However, the lack of VgrG14 in the supernatant may be due to the fact that the amount of VgrG secreted is below the limit of detection. The Hcp tube is a multimeric structure formed of stacks of hexamers, proposed to be topped by a single trimeric VgrG tip, as seen in the corresponding bacteriophage components. It is clear therefore that the abundance of Hcp in the supernatant will be much higher than that of VgrG, which may explain why secretion of VgrG14 is not observed.

Alternatively, the presence of the C terminal V5-his tag could prevent secretion of VgrG14. The C termini of VgrG are amenable to modification, as seen by various C terminal extensions (Section 1.7). VgrG1 in *V. cholerae* encodes an actin crosslinking domain, which is required for cytotoxicity (Pukatzki et al., 2007), and is accommodated by the secretion machinery, whereas VgrG2b of *P. aeruginosa* encodes a large C terminal extension of over 260 amino acids. These examples provided justification that a C terminal V5-his tag of only 30 amino acids would not affect VgrG14 secretion. However, the nature of the V5-his tag may disrupt the secretion process, resulting in VgrG14-V5-his being retained. Synthesis of antibodies directed against purified, full length VgrG14 would allow production and secretion of VgrG14 to be investigated without the need for an artificial tag. It has recently been shown that PAAR domains are able to interact with VgrG proteins, acting as an adaptor, sharpening the T6SS needle and allowing the co-delivery of toxin domains, and the C terminus of the VgrG protein is required for this interaction (Shneider et al., 2013). Introduction of the V5 tag may therefore have interrupted the formation of mature protein complexes and disrupted the secretion process.

An alternative approach to investigate a functional link between Hcp2 and VgrG14 would be to investigate the secretion of Hcp2 in a *vgrG14* deletion mutant, which is currently being performed in

our laboratory. If deletion of *vgrG14* abrogates Hcp2 secretion, then the puncturing activity of VgrG14 is clearly required for Hcp2 secretion, and these two proteins are functionally linked. This approach would provide important information on the role of VgrG14 without the need to detect it in the supernatant, and could confirm a role for VgrG14 in the mechanism of the H2-T6SS.

If Hcp2 secretion is VgrG14 independent then there is a possibility that other VgrG homologs provide the required membrane puncturing activity of the H2-T6SS. There are 11 VgrG encoded by PA14, and three have been associated with the H1-T6SS (Hachani et al., 2011). As described in section 4.6, there are at least 5 VgrGs that are similar to VgrG14 at the protein level, any of which could potentially be associated with the H2-T6SS. Sequential deletion of these *vgrG* and re-evaluation of Hcp2 secretion will provide experimental evidence for a functional link between orphan *vgrGs* and the H2-T6SS cluster. Not only would this approach potentially identify new H2-T6SS associated VgrGs, but it may also uncover new candidate H2-T6SS secreted substrates, since orphan VgrGs are invariably encoded within genetic islands with putative secreted proteins not currently associated with a T6SS system (Barret et al., 2011).

4.11.2 Rhs14 as a putative secreted effector

Production of Rhs14, encoded by the *rhs14* gene found adjacent to the PA14 H2-T6SS, was activated following induction of the divergent promoter. Rhs14 is a member of a diverse family of proteins, which remain poorly characterised. Its location downstream of, and potentially in an operon with, *vgrG14* led to it being proposed as a secreted H2-T6SS effector. However, the presence of Rhs14 could not be observed in the supernatants under the conditions tested, and the role of Rhs14 as a H2-T6SS substrate cannot be confirmed. As suggested for VgrG14, the addition of a V5-his tag to Rhs14 to allow detection may interfere with the secretion process. An alternative approach with peptide antibodies directed against the C terminus of Rhs14 was attempted but did not allow

detection of this protein (data not shown). Antibodies directed against the full-length protein may allow production and secretion to be tested without the requirement for an artificial tag.

The genetic location of the *rhs14* gene strongly suggests that Rhs14 is associated with the H2-T6SS system. This involvement should be investigated by means other than protein secretion assays. An important experiment to perform is to establish if Rhs14 and VgrG14 (and the gene encoded between these two proteins), interact with each other to form a functional complex. Confirmation of a VgrG14-Rhs14 complex could be achieved through bacterial two hybrid analysis or co-purification experiments, and a positive result could indicate that Rhs14 is delivered through interaction with the VgrG puncturing device.

Examples of characterised T6SS systems describe a mechanism of direct effector delivery to eukaryotic or bacterial cells (Hood et al., 2010; Pukatzki et al., 2007). The dynamics observed for H1-T6SS firing is precise and involves cell contact (Basler et al., 2013). With this in mind, the localisation of T6SS effectors into the bacterial supernatant is not likely to be biologically relevant and is likely a waste of resources. As with the H1-T6SS, secretion by the H2-T6SS could be activated upon target detection, ensuring efficient toxin delivery. Although Rhs14 expression is activated via the divergent promoter, additional signals are likely to be required to allow for its delivery into host cells, and to restrict the non-targeted delivery of Rhs14 into the supernatant. This is supported by the fact that a phenotype is observed dependent on the H2-T6SS in the internalisation experiments performed, suggesting that the presence of a eukaryotic cell activates the H2-T6SS.

An alternative approach to testing Rhs14 delivery by the H2-T6SS may be to monitor its direct delivery into eukaryotic or bacterial target cells. Similar experiments have already been performed to monitor the delivery of RhsT into phagocytic cells (Kung et al., 2012). Generating a fusion of Rhs14 to β-lactamase would allow visualisation of translocation of this protein into target cells due

to the change in colour of an intracellular fluorogenic substrate containing a beta lactam ring. Cleavage of the beta lactam ring by the translocated Rhs-β-lactamase fusion will result in a change in the fluorescence resonance energy transfer of the fluorogenic substrate, seen as a colour change by fluorescence microscopy (Charpentier and Oswald, 2004). This direct approach of monitoring T6SS effector delivery may be more biologically relevant than investigating secretion into liquid culture in the absence of a target cell. This experiment could be extended to investigate the translocation of VgrG14 into target cells. If either Rhs14 or VgrG14 are found to be translocated, the dependence of translocation on various components of the H2-T6SS should be investigated using the appropriate *P. aeruginosa* mutants.

The presence of conserved Rhs/VgrG pairs in other organisms suggests a common role for Rhs in the T6SS. There are at least two such pairs in PA14 (VgrG14/Rhs14 and Vgr1c/Rhs). Investigating the delivery of Rhs proteins into eukaryotic cells, the dependency on T6SS clusters, and the effects of such delivery may reveal Rhs proteins to be a broad family of T6SS delivered effectors in addition to the previously described peptidoglycan targeting effectors (Tse) (Russell et al., 2011) and lipid targeting effectors (Tle) (Russell et al., 2013). This role for Rhs proteins has already been suggested in studies of the DNA degrading activity of RhsA and RhsB from *Dickeya dadantii* (Koskiniemi et al., 2013).

The role of the protein encoded by PA14_43090, located between *vgrG14* and *rhs14*, should also be investigated, although it has been overlooked in this study due to the absence of homology with characterised proteins. Its location suggests that PA14_43090 is functionally linked to both VgrG14 and Rhs14, but its role is unknown. Study of the protein encoded by PA14_43090 is essential to fully understand the link between Rhs14 and VrgG14, and how the proteins encoded within this additional gene cluster interact with the H2-T6SS.

4.11.3 Translational regulation of the H2-T6SS

The introduction of the divergent promoter only overcomes transcriptional regulation of the H2-T6SS. It is likely that there are several additional levels of control on the H2-T6SS beyond transcriptional regulation, as is seen for the H1-T6SS, which may be involved in full activation of the system, and necessary for VgrG14 and Rhs14 secretion. The presence of conserved kinase and phosphatase genes in the H2-T6SS suggests that this system may be translationally regulated in a similar manner to the H1-T6SS (Mougous et al., 2007). Manipulating this phosphatase/kinase pathway by deleting the gene encoding the Stp2 phosphatase might have resulted in higher activity of the H2-T6SS. Our data showed it is not the case and the deletion of *stp2* did not result in secretion of VgrG14 and Rhs14. However, this observation alone is not enough to confirm if the *stp*/*stk* system is not involved in regulation of the H2-T6SS and further work will be done.

To confirm a role for *stp2*/*stk2* in the H2-T6SS some alternative strategies should be employed. Since Hcp2 is secreted following arabinose induction of the divergent promoter, its secretion could be used as a reporter for *stp2*/*stk2* activity. In addition to testing if deletion of *stp2* increases the secretion of Hcp2, the effect of *stk2* deletion in reducing Hcp2 secretion should also be investigated. If the amount of Hcp2 secretion is affected by the *stk*/*stp* system, then over expression of the kinase may be an alternative to *stp2* deletion as a means of up-regulating the H2-T6SS.

4.11.4 Alternative approaches for the study of the H2-T6SS

The divergent promoter strategy is limited to activation of genes immediately downstream of the promoters. Other genes associated with the H2-T6SS but encoded at distinct chromosomal locations, will not be activated by induction of the divergent promoter. The expression of the core H1-T6SS cluster, in a *retS* mutant for example, is co-regulated with *vgrG* and toxin genes located

distantly to the H1-T6SS locus. If such additional loci exist for the H2-T6SS, then the divergent promoter construct would be insufficient for their activation, and the H2-T6SS produced under these conditions may not represent a fully assembled system.

A random transposon mutagenesis screen could be employed to identify regulators of the H2-T6SS, screening for changes in the activity of a reporter fusion between β-lactamase and H2-T6SS promoters. Following identification of important regulatory genes, their deletion or overexpression could be used to influence the expression or activity of the H2-T6SS, and allow its study without the requirement of an artificial divergent promoter.

If such regulators are found, and conditions resulting in high H2-T6SS activity demonstrated, then a non-targeted approach should be used to identify proteins secreted by this system. Mass spectroscopy has already been used to identify substrates of the H1-T6SS by Hood et al (2011), and would be a powerful tool for identification of H2-T6SS substrates. Mass spectroscopy analysis could be used to compare the supernatants from cells with active H2-T6SS with those in which the H2-T6SS has been disrupted by gene deletion. This will identify all of the proteins secreted by the H2-T6SS, but relies first on the establishment of conditions in which the H2-T6SS is active. Mass spectroscopy could have been used to analyse the secretome of induced and uninduced PA14-DP cultures, and may have identified novel secreted substrates which were not targeted for analysis by western blot, and without the need for the addition of protein tags. However, since the divergent promoter does not act globally, the result of such an experiment is likely to be an underrepresentation of the H2-T6SS secretome.

4.11.5 The diverse roles of the H2-T6SS

The H2-T6SS has been reported to target bacterial and eukaryotic cells (Russell et al., 2013; Sana et al., 2012). While a defined secreted toxin (Tle5) has been implicated in the H2-T6SS bacterial killing phenotype, no effectors have been identified which are involved in host cell subversion.

While the published data implicates the H2-T6SS in the killing of susceptible *P. aeruginosa* strains, we investigated its ability to kill *E. coli*. The assay employed has previously been successfully used to investigate the role of the *P. aeruginosa* PAK H1-T6SS system in *E. coli* killing. Results presented here indicate that this assay is inappropriate for PA14 due to the fact that *E. coli* is unable to survive co-culture with PA14, while PAK does. The mechanism of PA14 mediated *E. coli* killing is not known. To optimise the assay, conditions need to be found in which *E. coli* can survive co-culture with PA14, such as deletion of the H1-T6SS or other virulence factors which may be responsible for *E. coli* killing. Alternatively, the role of putative PA14 H2-T6SS toxins could be tested in an inter-pseudomonas competition assay.

Investigations into the role of the PA14 H2-T6SS in eukaryotic cell interactions showed that unlike the PAO1 system, the H2-T6SS does not promote the internalisation of PA14 into HeLa cells, but represses it. Due to different phenotypes, it is likely that the H2-T6SS from PAO1 and PA14 secrete different effectors, which have different effects on internalisation. This observation is interesting, and highlights the fact that ultimately the phenotype of a secretion system is mediated by the effectors which it secretes. The work on PAO1 did not identify an effector responsible for the phenotypes observed, which is an important question remaining in the characterisation of the H2-T6SS.

Despite no direct evidence, it is tempting to propose that the PA14 specific VgrG14 and Rhs14 may be responsible for the different internalisation phenotypes observed, since this is the one major difference in the genetic arrangement of these two systems. Experiments could be performed to confirm this hypothesis. Testing the internalisation of PA14 strains in which either *vgrG14* or *rhs14* are deleted would reveal if either is responsible for the internalisation phenotypes of this strain, and confirm their involvement with the H2-T6SS.

Chapter 5 General Discussion

5.1 The Type VI Secretion System

The Type VI Secretion system has, in less than a decade, firmly established itself among the protein secretion systems in Gram-negative bacteria, and attracted the attention of a large scientific community. Since the first reports of the delivery of VgrG and Hcp proteins by the T6SS in 2006 (Mougous et al., 2006; Pukatzki et al., 2006), developments in the field have been rapid. It was soon uncovered that the T6SS resembles an inverted bacteriophage puncturing device, and the molecular mechanisms of toxin delivery are beginning to be elucidated (Leiman et al., 2009). Early reports hypothesised the involvement of the T6SS in bacterial virulence, and while direct roles in pathogenesis have been observed (Pukatzki et al., 2007; Suarez et al., 2010), the activity of the T6SS system is much wider, targeting both eukaryotic and bacterial cells (Hood et al., 2010).

T6SS allow bacteria to disrupt host cell and kill competing bacteria. Such diverse activity is clearly exemplified by *V. cholerae*, which encodes one T6SS which is able to disrupt eukaryotic cell actin (Pukatzki et al., 2007), but also degrade peptidoglycan, inhibiting the growth of competing bacteria (Dong et al., 2013). In the case of *Burkholderia thailandensis*, which is able to target both bacterial and eukaryotic cells, distinct T6SS machineries are employed for each target (Schwarz et al., 2010). Whereas it is clear how disruption of host cell actin is important in subverting host defences, the T6SS may also contribute to the establishment of infections by promoting the colonisation of T6SS+ bacteria by eliminating competitors. This is demonstrated by the importance of the T6SS in the formation of mixed species biofilms, in which *B. thai* requires a functional T6SS to outcompete *Pseudomonas putida* (Schwarz et al., 2010). The influence of T6SS at multiple stages of infection thus amplifies its importance in pathogenesis.

An important step in the characterisation of a secretion system is to identify the proteins that are secreted. The first report of *P. aeruginosa* T6SS dependent toxins was made in 2010 (Hood et al., 2010), and in the last three years significant advances have been made in our knowledge of T6SS secreted toxins. The toxins described so far target conserved molecules among diverse bacteria, including peptidoglycan and phospholipid components of cell membranes, indicating broad susceptibility to the T6SS among bacteria (Russell et al., 2013; Russell et al., 2012).

5.2 *P. aeruginosa* response to subinhibitory antibiotics

T6SSs are generally not active under standard bacterial culture conditions, requiring target cell contact for activation. Study of the first *P. aeruginosa* T6SS cluster, the H1-T6SS, is aided by the fact that manipulation of the RetS/Gac/Rsm regulatory cascade, upon deletion of the *retS* gene, results in a highly active, hyper-secretive, phenotype (Mougous et al., 2006). The precise signals sensed by the components of the cascade that activate the H1-T6SS are not known, and were investigated in Chapter three.

Subinhibitory concentrations of aminoglycosides have been previously shown to activate biofilm formation (Hoffman et al., 2005), and biofilm formation is co-regulated with the H1-T6SS via the RetS/Gac/Rsm cascade (Goodman et al., 2004). It was therefore a unique opportunity to hypothesise that the H1-T6SS may also be activated by subinhibitory concentrations of aminoglycosides. The results obtained indicate that subinhibitory kanamycin concentrations do activate production of core H1-T6SS components (Hcp and VgrG), but secretion and bacterial killing activity were not observed under these conditions. Importantly, the response did require a functional RetS/Gac/Rsm cascade. However, it is still unclear whether it involves a direct sensing of

the antibiotic by the regulatory cascade, or whether antibiotic sensing occurs via another route, which cannot overcome an established RsmA repression exerted on the T6SS.

Comparisons with similar experiments indicate that *P. aeruginosa* does not respond in a predictable way to subinhibitory concentrations of antibiotics from different classes. For example, biofilm formation, previously reported to be induced by aminoglycosides alone, has also been shown to be induced by non-aminoglycosides (Hoffman et al., 2005; Linares et al., 2006). The work presented in this thesis shows that while kanamycin induces production of the H1-T6SS, the closely related aminoglycosides tobramycin and gentamycin, do not. It is thus likely that the responses observed represent more broadly a non-specific stress responses and the mechanistic detail of the signalling cascade might be highly variable and complex depending on the antibiotic used, and unlikely to be directly connected to the structure or function of the antibiotic.

The fact that kanamycin induces production of two core H1-T6SS components, but in these conditions the T6SS does not display secretion activity, remains an interesting and novel observation. Similarly, the H1-T6SS was shown to be up-regulated in response to subinhibitory concentrations of bile (Reen et al., 2012). It is tempting to propose that sensing low-level stresses results in a self-defence response by *P. aeruginosa*, in which the H1-T6SS is primed for activity, and ready to fire whenever *P. aeruginosa* encounters a competing organism.

5.3 Characterising the H2-T6SS

Advances in the study of the second *P. aeruginosa* T6SS cluster, the H2-T6SS, has been slower owing to the fact that the regulatory mechanisms leading to activation of this cluster are not fully characterised. While it is known that quorum sensing and iron limitation increase the expression of the H2-T6SS in *P. aeruginosa* PAO1, it has not been shown that either result in production of an

active system (Lesic et al., 2009; Sana et al., 2012). To overcome repression on the H2-T6SS a promoter swapping strategy was employed, and successfully resulted in production of H2-T6SS proteins. The system was shown to be active, by observing Hcp2 secretion dependent on the H2-T6SS cluster.

While three toxins have been reported so far for the H1-T6SS (Tse1-3) (Hood et al., 2010), only one example of a H2-T6SS dependent toxin, a phospholipase, has been reported (Russell et al., 2013). The work presented in Chapter four of this thesis aimed to uncover additional H2-T6SS dependent toxins, and was focused on a four gene cluster encoded adjacent to the H2-T6SS cluster in *P. aeruginosa* PA14. This cluster contains genes encoding two core T6SS components (*hcp* and *vgrG*), as well as two additional genes, one of which encodes a large protein of the Rhs family (Rhs14), considered to be a putative secreted effector. While production of Rhs14 was observed, its secretion was not. Rhs14 remains to be confirmed as a H2-T6SS dependent effector, but the discovery of a putative immunity protein downstream of *rhs14* suggests it may have toxic activity. Rhs14 has been predicted in this work to structurally resemble a protein encapsulation device, which may be important in the delivery of an Rhs14 C terminal toxin domain (Busby et al., 2013) While further work is required to confirm the secretion of Rhs14 by the H2-T6SS, it is hypothesised that Rhs14 is a novel effector with uncharacterised activity, as experimentally demonstrated for Rhs-proteins from *Dickeya dadantii*, which are likely delivered by the T6SS (Koskiniemi et al., 2013).

The H2-T6SS has been implicated in both eukaryotic cell interaction (Lesic et al., 2009; Sana et al., 2012), and in the secretion of an anti-bacterial phospholipase toxin (Russell et al., 2013). The example given for the *V. cholerae* T6SS indicates that targeting of eukaryotic and bacterial cells by the same T6SS is possible, although mediated by different toxins. To date, no secreted effectors have been uncovered which are responsible for the eukaryotic cell targeting activity of the H2-T6SS.

The H2-T6SS was specifically shown to promote the internalisation of *P. aeruginosa* PAO1 into eukaryotic cells (Sana et al., 2012). The investigations using *P. aeruginosa* PA14 in this thesis have remarkably shown that in this strain the H2-T6SS has the opposite effect, and inhibits internalisation of PA14. The presence of the additional gene cluster adjacent to the H2-T6SS in PA14, including the proposed effector Rhs14, is potentially responsible for the observed differences in the activity of PAO1 and PA14, and this still requires experimental validation.

While much of the work presented in this thesis is focused on single gene manipulation and investigation of the effects, the importance of genome-wide studies should not be overlooked. Global approaches could be utilised to further investigate the T6SS of *P. aeruginosa*. Studies have previously been performed using large sets of genomic data, which revealed the distribution of T6SS clusters in *Pseudomonas* strains (Barret et al., 2011). This analysis also accurately predicted the presence of effector proteins which were subsequently experimentally demonstrated. Comparison of the H2-T6SS gene cluster in a wider set of genomes could reveal more information regarding the presence and conservation of the VgrG14/ Rhs14 containing cluster and may provide an insight into its acquisition. Genomic approaches of *P. aeruginosa* strains could also be used to investigate the presence of other conserved *rhs* genes associated with T6SS cluster to understand the wider association of this putative effector family with *P. aeruginosa* and other T6SSs

Transcriptomics studies would be useful to uncover genes co-regulated with the H2-T6SS clusters, and additional genes important for the function of the H2-T6SS cluster. This is highlighted by the fact that the regulation of the H1-T6SS by the RetS/LadS cascade was identified through microarray studies (Goodman et al., 2004), a powerful tool which also identified co-regulated effector genes. No such regulators of the H2-T6SS are known. Transcriptome analysis of the effect divergent promoter strain constructed in this work has limited value however, since this system is artificial and its effect is not global but limited to a precise chromosomal location. Once conditions are uncovered

in which the H2-T6SS core cluster is fully activated, transcriptome analysis would be useful to discover co-regulated genes, and potential novel effectors. Similarly, proteomic studies could be used to identify H2-T6SS secreted proteins in a non-targeted way, using comparative analysis of conditions in which the system is active and inactive.

5.4 Hcp as a chaperone

During the preparation of this thesis, work published by Silverman and colleagues (2013) presented some interesting observations on the role of the core T6SS component, Hcp. This work suggests a role for Hcp as a receptor and chaperone for T6SS effectors (Silverman et al., 2013). Tse2, a H1-T6SS secreted substrate, was shown to interact with Hcp1. Observation by electron microscopy shows that Tse2 binds within the central pore of the hexameric Hcp ring. Mutation of key residues on the inner surface of Hcp1 hexamers abrogated this binding, and resulted in a loss of Tse2 secretion. It was further shown that Tse1 and Tse3 also required direct interactions with the Hcp1 central pore for secretion (Silverman et al., 2013).

Until now, Hcp has been hypothesised to form a static tail-tube like structure allowing the conduit of secreted substrates, due to its homology to the gp19 component of bacteriophage (Leiman et al., 2009). Hcp1 forms stacked nanotubes in the crystal lattice, and also when tubes are stabilised by the introduction of artificial disulphide bonds (Ballister et al., 2008; Jobichen et al., 2010; Mougous et al., 2006; Osipiuk et al., 2011). However, Hcp nanotubes have not been observed *in vivo*, questioning their biological significance. Silverman et al. thus propose that Hcp is responsible for direct binding of effectors, protecting them from proteolysis, and potentially trafficking them to the T6SS apparatus (Silverman et al., 2013). This potentially changes the current model of T6SS, which hypothesises that upon contraction of the tail-sheath like TssBC components, a VgrG tipped Hcp tube is propelled to the outside of the cell. Silverman et al. suggest that if such a tube does exist,

internally to the TssBC sheath, it would be loaded internally with secreted effectors, and could be considered as a stack of Hcp containing toxins, and not a continuous tube (Silverman et al., 2013). However, the localisation of Hcp within the TssBC sheath is yet to be demonstrated, and advances in this area would clarify the mechanism of T6SS toxin delivery and the role of Hcp.

5.5 Perspectives and future work

In this work the H2-T6SS cluster was investigated using an artificial promoter-swap strategy. While this has provided meaningful new data, the conditions activating the H2-T6SS remain poorly understood in comparison to the H1-T6SS. Experiments are currently underway in our laboratory using non-targeted random transposon mutagenesis and β-lactamase fusions to H2-T6SS promoters, which will potentially uncover major regulators of the H2-T6SS system. Their manipulation may allow constitutive activation of the H2-T6SS and other co-regulated genes, similar to that observed with a *retS* mutant and the H1-T6SS. This approach has several advantages over the promoter-swap strategy since it may allow the discovery of distantly encoded H2-T6SS effectors, which could then be identified using a mass spectroscopy approach, as used in the discovery of the H1-T6SS Tse1-3 toxins (Hood et al., 2010).

While conditions activating the H2-T6SS are sought, further characterisation of Rhs14 could be performed to confirm a role for this protein as a H2-T6SS effector. Preliminary data suggests that transfection of Rhs14 in eukaryotic cells is highly toxic. This effect should be confirmed and the mechanism of Rhs14 toxicity investigated. The expression of Rhs14 in eukaryotic cells should be controlled using an inducible plasmid or by examining cells over a shorter time course, so that the effects of Rhs14 can be observed prior to total cell lysis. The putative immunity protein uncovered in this work could also be used, since co-transfection of Rhs14 and the putative immunity protein should protect HeLa cells from toxicity.

In addition to the effects on eukaryotic cells the role of Rhs14 in bacteria should also be investigated. Preliminary experiments using *E. coli* as a target cell indicated that PA14 kills *E. coli* in a H2-T6SS independent manner, and is not a suitable target strain. Alternatively, an inter-pseudomonas approach could be used, co-incubating PA14 with strains lacking the putative immunity protein. This would confirm if Rhs14 is also capable of bacterial intoxication.

If Rhs14 is found to be an effector of the H2-T6SS, how it is delivered remains an open question. Replacing the C terminal domain with a β-galactosidase reporter would allow direct injection into eukaryotic cells to be investigated, as previously described (Kung et al., 2012), and the dependency of translocation on components of the H2-T6SS should be evaluated. The recent discovery of PAAR domain proteins indicates that they are able to interact with VgrG proteins, sharpening the needle, and suggest that they are co-delivered by injection (Shneider et al., 2013). The interaction of Rhs14 and VgrG14 should be investigated to test if these two components are co-delivered as a complex, and could be address using a bacterial two-hybrid approach. While the RHS core region of Rhs14 has been predicted to form a structure reminiscent of a protein encapsulation device, uncovering the structures formed by C and N terminal domains of Rhs14 and their involvement with the core domain structure may provide and insight into the T6SS delivery of Rhs toxins.

To comprehensively understand the T6SS activity of *P. aeruginosa* it is important to consider the third cluster, the H3-T6SS. The H3-T6SS is not co-regulated with the H1-T6SS, and is likely to be active under distinct conditions. It is likely to have its own effectors, and may target specific hosts. Similarly to the H2-T6SS, conditions activating the H3-T6SS are not known, which has impeded the study of this system. Employing strategies to artificially activate the H3-T6SS by promoter swap or using transposon screens to identify regulators, is likely to also improve our understanding of the third cluster. Ultimately, the full complement of T6SS effectors secreted by all three *P. aeruginosa*

clusters will be understood, revealing how *P. aeruginosa* utilises each cluster differently, and how each system contributes to the success of *P. aeruginosa* as a ubiquitous environmental organism and a prevalent opportunistic pathogen.

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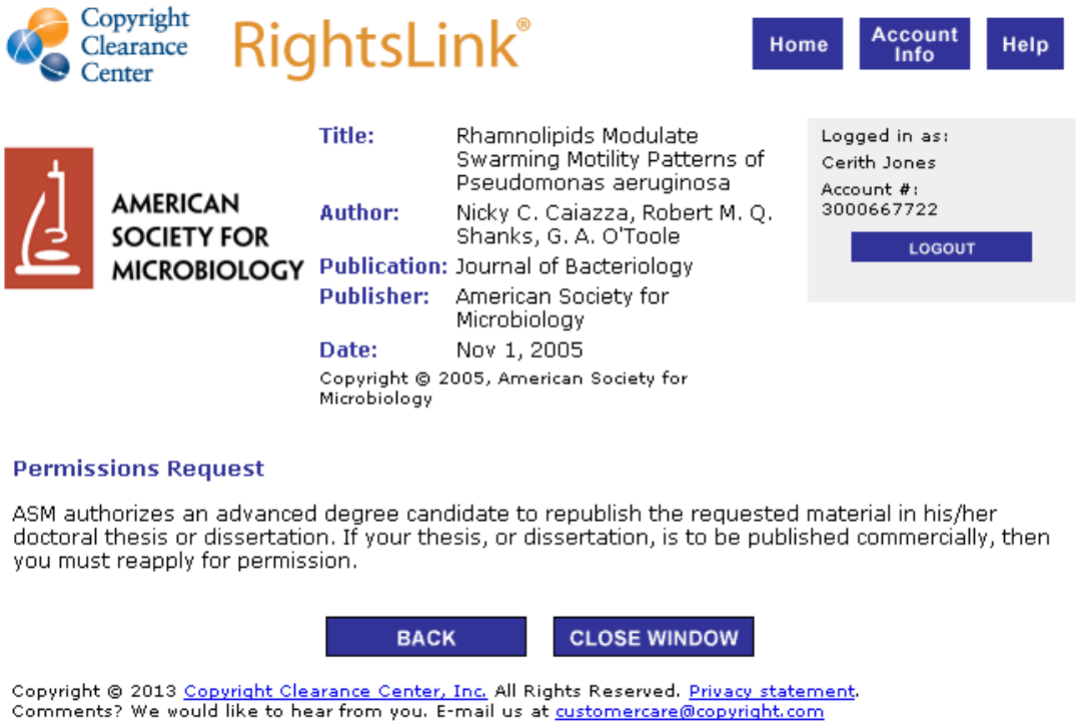
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From: Jones, Cerith

To: 'permissionsfrance@elsevier.com'

I would like to request permission to reproduce a table from one of your publications in my PhD thesis currently being prepared for submission at Imperial College London.

Publication: The Type VI secretion system--a widespread and versatile cell targeting system
Journal : research in microbiology, volume 164, issue 6, July--August 2013, pages 640 to 654.
Authour: Sarah J Coulthurst

I would like to reproduce table 1,'T6SSs reported to have a role in virulence and/or interaction with eukaryotic cells',on page 645 in my thesis entitled 'Activation and functional studies of the Type VI secretion systems in Pseudomonas aerguniosa' in both print and electronic forms

Please let me know if I may obtain the required permission.

Cerith Jones
PhD student
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