The role of small molecule signalling in biofilm migration of *Pseudomonas aeruginosa*

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Laura Nolan
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Certificate of Original Authorship

I certify that the work in this Thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the Thesis has been written by me. Any help that I have received in my research work and the preparation of the Thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the Thesis.

Signature of Student:

Laura Nolan

Date: 22/1/2014

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List of abbreviations

Abbreviation	Meaning
ABS	Adult bovine serum
AC	Adenylate cyclase
AHL	*N*-acyl homoserine lactones
AHS	Adult serum albumin
AMP	Adenosine 5´ monophosphate
Ap^R	Ampicillin resistant
ASGM1	Glycolipid asialoGM1
ATP	Adenosine 5' triphosphate
BM	Base Media
BMA	Base media agar
Bp	Basepairs
BSA	Bovine serum albumin
CAMHB	Cation-adjusted mueller hinton broth
cAMP	3'-5'-cyclic adenosine monophosphate
CAUTI	Catheter-associated urinary tract infections
c-di-GMP	Bis-(3´-5´)-cyclic dimeric guanosine monophosphate
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis transmembrane conductance regulator
CLSM	Confocal scanning light microscopy
CRP	Catabolic repressor protein
DAG	Diacylglycerol
DGC	diguanylate cyclase
diH_2O	Deionised water
DKP	Diketopiperazine
DMSO	Dimethyl sulfoxide
DSF	Diffusible signal factors
DTT	Dithiothreitol
eATP	Extracellular adenosine 5' triphosphate
eDNA	Extracellular deoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assays
EPS	Extracellular polymeric substances
g	Grams
GlcNAc	*N*-acetyl glucosamine
hr	Hour/s
HCl	Hydrochloric acid
Hpt	Histidine phosphotransfer
icAMP	Intracellular 3'-5'-cyclic adenosine monophosphate
Ig	Immunoglobulin
Kb	Kilobases

kg	Kilograms
Km^R	Kanamycin resistance
9L	Litre
LB	Luria Bertoni
LBA	Luria Bertoni agar
LCFA	Long-chain fatty acid
LPS	Lipopolysaccharide
M	Molar
Min	Minute/s
MCP	Methyl-accepting chemotaxis protein
MDCK	Madin-Darby Canine Kidney
mL	Millilitres
mM	Millimolar
mm	Millimetres
MQ	MilliQ
ms	Milliseconds
Na_2EDTA	EDTA disodium salt dehydrate
nM	Nanomolar
nm	nanometres
NO	Nitric oxide
OMV	outer-membrane vesicle
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween 20 (0.05%)
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDE	phosphodiesterase
PE	Phosphatidylethanolamine
PIA	Pseudomonas isolation agar
PQS	*Pseudomonas* quinolone signal
PVDF	Polyvinylidene fluoride
QS	Quorum sensing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Second/s
SEM	Standard error of mean
SNP	Single nucleotide polymorphism
SOLiD	Sequencing by Oligonucleotide Ligation and Detection
Spt	Serine phosphotransfer
T3SS	Type III secretion system
TBE	Tris Borate EDTA

TCA	Tricarboxylic acid
TE	Tris-EDTA buffer
TEM	Transmission electron microscopy
Tet^R	Tetracycline resistance
tfp	Type IV pili
TLR	Toll-like receptor
Tpt	Threonine phosphotransfer
Vfr	Virulence factor regulator
μM	Micromolar

Abstract

Pseudomonas aeruginosa is a Gram-negative pathogen which exploits damaged epithelium to cause acute and chronic infections in a range of immunocompromised individuals. The chronic nature of infections caused by *P. aeruginosa* is often associated with the formation of biofilms. Extension and retraction of type IV pili (tfp) mediates a form of surface translocation, termed twitching motility, which is involved in active biofilm expansion and sessile biofilm formation. In *P. aeruginosa* the biogenesis, assembly and twitching motility function of tfp is controlled by a number of complex regulatory systems, however the signals that these systems respond to are not well characterised. The aim of this Thesis was to understand how intracellular and extracellular signals control *P. aeruginosa* twitching motility-mediated biofilm expansion.

In this Thesis five independent *fimL* mutants, that had presumably acquired extragenic suppressor mutations which restored twitching motility ability, were characterised. All *fimL* revertants were found to have increased levels of intracellular cyclic AMP (icAMP). While an extragenic suppressor mutation in the cAMP phosphodiesterase CpdA was shown to be responsible for the increase in icAMP levels and restoration of twitching motility in one *fimL* revertant, the site of suppressor mutation(s) in the remaining four revertants was not identified. These results suggest that twitching motility reversion in *fimL* mutants occurs via at least two mechanisms and that an increase in icAMP levels is correlated with twitching motility.

Extracellular ATP (eATP) is released by damaged epithelial cells which acts as a "danger" signal to recruit host immune system factors to repair the damage. As *P. aeruginosa* has a propensity for damaged epithelia the effect of eATP on *P. aeruginosa* biofilm expansion and formation was investigated. The results presented in this Thesis demonstrate that eATP inhibits *P. aeruginosa* twitching motility-mediated biofilm expansion and stimulates sessile biofilm formation, which may provide a potential advantage for *P. aeruginosa* within an infection setting. Additionally, our results suggest that high levels of endogenously-produced bacterial eATP acts to coordinate *P. aeruginosa* multicellular behaviours.

This Thesis also reports the identification of a novel extracellular signal *N*-acetylglucosamine, which stimulates *P. aeruginosa* twitching motility. Additionally, the twitching motility response of *P. aeruginosa* to the host derived signals serum albumin, mucin and oligopeptides was characterised in detail. These analyses implicated the CheW-homolog, ChpC which is a component of the Chp chemosensory system, in this response.

Overall the results presented in this Thesis provide insight into the regulation *P. aeruginosa* twitching motility by a number of intracellular and extracellular signals. Our results suggest that the adaptive response of *P. aeruginosa* to these signals is likely to have significant implications in the success of this pathogen within an infection setting.

Chapter One

General Introduction

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen which grows in soil and marine environments and infects many organisms including plants, nematodes, insects and animals (Rahme et al., 2000, Mahajan-Miklos et al., 2000). This pathogen has a propensity for damaged epithelium which it is able to exploit to cause both acute and chronic infections with high levels of morbidity and mortality (Kazmierczak et al., 2001). *P. aeruginosa* is implicated in both community-acquired infections such as ulcerative keratitis, which occurs mainly in contact lens wearers (Bourcier et al., 2003, Alexandrakis et al., 2000), otitis externa (Roland and Stroman, 2002, Clark et al., 1997), infections of the skin and soft-tissue (Lipsky et al., 2010), as well as in hospital-acquired infections, such as pneumonia (Kollef et al., 2005, Arruda et al., 1999), urinary tract infections (Aguilar-Duran et al., 2012), surgery-wound site infections (Anderson et al., 2007) and in some cases bacteremia (Wisplinghoff et al., 2004). *P. aeruginosa* commonly causes serious, and often chronic, infections in those with cystic fibrosis (CF) (Høiby et al., 2010b), and also in immunocompromised individuals, such as those with AIDS, patients undergoing cancer chemotherapy and those with severe burns (Lyczak et al., 2000, Tatterson et al., 2001). *P. aeruginosa* also causes ventilator-acquired pneumonia (VAP), which is the leading cause of nosocomial infection in trauma patients that have had prolonged periods of mechanical ventilation (Berra et al., 2010).

Many strains of multidrug resistant *P. aeruginosa* exist today and this number is predicted to increase in the coming decades making treatment with current antibiotics extremely challenging (Nordmann et al., 2007). The success of this organism in various environments is attributed to its broad metabolic versatility (Stover et al., 2000) and its ability to produce many cell-associated and secreted virulence and survival factors (Van Delden and Iglewski, 1998).

1.2 *P. aeruginosa* virulence mechanisms

P. aeruginosa is capable of causing both acute and chronic infections. Comparison of chronically adapted and acute infection isolates reveals significant genotypic and phenotypic differences, which generally relate to virulence factor production. Typically, strains isolated from acute infections express a large number of cell associated and secreted virulence factors, while those associated with chronic infections produce fewer virulence factors, and are more likely to adopt a sessile mode of life (Smith et al., 2006, Gellatly and Hancock, 2013).

1.2.1 *P. aeruginosa* virulence mechanisms associated with acute infections

1.2.1.1 Secreted virulence factors

The production of a variety of secreted virulence factors contributes to the acute nature of infections caused by *P. aeruginosa* (Van Delden and Iglewski, 1998).

1.2.1.1.1 Exotoxin A and proteases

In vivo studies have demonstrated that production of exotoxin A, elasatase and alkaline protease are all essential for *P. aeruginosa* virulence in a number of animal models (Nicas and Iglewski, 1985). Exotoxin A is responsible for local tissue damage, bacterial invasion (Woods and Iglewski, 1983), possible immunosuppression (Vidal et al., 1993) and inhibition of protein synthesis leading to cell death (Wick et al., 1990). *P. aeruginosa* also produces a number of proteases including LasB and LasA elasatase, and alkaline protease (Morihara and Tsuzuki, 1977). Both LasB and LasA are involved in the destruction of human lung tissue, with LasB also being able to interfere with host defense mechanisms (Heck et al., 1990, Jacquot et al., 1985, Hong and Ghebrehiwet, 1992, Morihara et al., 1979, Johnson et al., 1982) and cause the pulmonary haemorrhages of invasive *P. aeruginosa* infections (Galloway, 1991). The role of alkaline protease in tissue invasion is unclear, however the production of this protease appears to be important in establishment and development of corneal infections in a mouse model (Howe and Iglewski, 1984).

1.2.1.1.2 Pyocyanin

Another important secreted virulence factor is the redox-active phenazine pyocyanin which is responsible for the green-blue colour of *P. aeruginosa* cultures and colonies. Pyocyanin is required for full virulence in animal models, and most likely within human infections (Mahajan-Miklos et al., 1999, Lau et al., 2004). Once secreted, this compound is able to cross host cell membranes and cause production of reactive oxygen species (ROS) which expose the cell to oxidative stress (Rada and Leto, 2009). The overall effect of pyocyanin production on the host is widespread including stimulation of the host proinflammatory response, and increased secretion of antibacterial compounds (Rada and Leto, 2013).

1.2.1.1.3 Type III secretion system

The type III secretion system (T3SS) is a complex needle-like machine on the bacterial surface which is able to secrete and deliver bacterial effectors directly into the host cell

cytoplasm (Galan and Wolf-Watz, 2006). While the T3SS is not absolutely required for infection, this system greatly enhances the acute nature of *P. aeruginosa* infections in a number of animal models (Hauser, 2009) for instance in *P. aeruginosa* systemic infections (Vance et al., 2005) and acute corneal disease (Lee et al., 2003). In *P. aeruginosa* ExoS, ExoT, ExoY and ExoU, are the main T3SS effectors, which are injected into the host cell (Finck-Barbancon et al., 1997, Yahr et al., 1996a, Yahr et al., 1998, Yahr et al., 1996b). The T3SS effectors have a widespread effect on the host including inhibition of wound repair, modulation of the host's immune response and perpetuation of tissue injury, which increases the susceptibility of the host to further infection by *P. aeruginosa* and other pathogens (Engel and Balachandran, 2009).

1.2.1.2 Cell surface virulence factors

In addition to secreted virulence factors, *P. aeruginosa* also possesses a number of cell-associated virulence factors which contribute to an acute infection state.

1.2.1.2.1 Type IV pili and flagella

Two major cell-surface associated virulence factors are the single polar flagellum and the multiple polar type IV pili (tfp) possessed by *P. aeruginosa*, which both act as adhesins and mediators of bacterial motility. Flagella are involved in swimming motility, as well as tethering and adhering to epithelial cells (Feldman et al., 1998). They are also able to elicit an immune response in eukaryotes via interactions with the Toll-like receptors (TLR) 5 and 2 (Adamo et al., 2004). Non-flagellated *P. aeruginosa* mutants are defective in a respiratory acute infection model (Feldman et al., 1998) and thus flagella have been considered as interesting immunotherapy targets (Landsperger et al., 1994). Tfp are involved in a flagellum-independent form of translocation termed twitching motility, as will be discussed in detail in later sections of this Thesis introduction, which allows the rapid colonisation of surfaces (Whitchurch, 2006). Like flagella, tfp are also involved in epithelial cell adherence (Hahn, 1997, Gupta et al., 1994), with both tfp-mediated motility and adherence being crucial for *P. aeruginosa* virulence (Farinha et al., 1994, Comolli et al., 1999, Tang et al., 1995). Tfp have also been used as targets for anti-pseudomonal immunotherapy (Hertle et al., 2001).

1.2.1.2.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major structural component of the outer membrane of *P. aeruginosa* which is also critical for virulence (Tang et al., 1996). LPS promotes a strong inflammatory response within a eukaryotic host, via the action of the O-antigen and lipid A

components of LPS. This triggers production of proinflammatory cytokines and chemokines, inflammation and ultimately endotoxic shock (Gupta et al., 1994, Hajjar et al., 2002, Backhed et al., 2003, Wieland et al., 2002).

1.2.2 *P. aeruginosa* virulence mechanisms associated with chronic infections

In contrast to *P. aeruginosa* strains isolated from acute infections, the strains of *P. aeruginosa* isolated from chronic infections often lack most of the inflammatory features associated with acute infections (Hogardt and Heesemann, 2010). Two well-studied chronic infection scenarios are that of *P. aeruginosa* within the lungs of CF patients (Gilligan, 1991, Bodey et al., 1983, Yu and Head, 2002) and within the urinary tract of catheterised patients (Mittal et al., 2009, Stickler, 2008).

1.2.2.1 Chronic infections in cystic fibrosis patients

CF is an autosomal recessive disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) which causes dehydration of the lung epithelium, and ultimately results in impairment of mucociliary clearance from the lungs (Collins, 1992). The inability to clear infecting bacteria and other microbes stimulates a strong inflammatory response, which contributes to lung tissue damage and exposure of the basement membrane (Høiby et al., 2010b), allowing attachment to and infection of the lung epithelium by opportunistic bacteria such as *P. aeruginosa* (Ramphal and Pyle, 1983). The combination of *P. aeruginosa* infection and lung tissue inflammation are the main causes of morbidity and mortality in CF patients (Yu and Head, 2002).

Several studies reveal that CF-adapted *P. aeruginosa* strains lack a number of secreted and cell-associated virulence factors. Mutations affect the secretion of virulence factors such as elastase and pyocyanin, as well as T3SS-mediated toxin effector secretion (Nguyen and Singh, 2006). Acquisition of insertions and mutations in a number of genes have been shown to inhibit assembly of the cell-surface associated tfp and flagella structures in these chronically adapted strains (Kresse et al., 2003, Luzar et al., 1985, Mahenthiralingam et al., 1994). Additionally, mutations resulting in modifications in the lipid A moiety and O-antigen component of LPS have been shown to decrease resistance of these bacteria to antimicrobials and to cause a decreased immunostimulatory response in CF patients (Ernst et al., 2007, Kresse et al., 2003, Spencer et al., 2003, Hancock et al., 1983).

Over production of the exopolysaccharide alginate by *P. aeruginosa* mucoid strains is correlated with worsening clinical prognosis for the CF patient (Ryder et al., 2007). This mucoid phenotype most frequently arises as a result of mutations in the negative regulator *mucA* (Martin et al., 1993a). The role of alginate as a virulence factor in chronic infections is multifactorial. Alginate has been reported to have a role in adhesion, as mucoid strains have been reported to have increased adherence to mouse tracheas (Ramphal and Pier, 1985). Additionally, alginate is able to act as a capsule-like barrier for *P. aeruginosa* which assists in combatting the consequences of inflammation by scavenging free radicals released from activated macrophages and providing protection for bacteria from phagocytic clearance (Govan and Deretic, 1996). The protective nature of alginate also appears to be relevant for antibiotic treatment, as mucoid *P. aeruginosa* strains appear to be more resistant to some antibiotics (Hentzer et al., 2001), an effect which can be reversed to some extent by the addition of alginate lyase to degrade alginate (Alkawash et al., 2006).

1.2.2.2 Chronic catheter-associated urinary tract infections

P. aeruginosa is also commonly associated with chronic catheter-associated urinary tract infections (CAUTIs). CAUTIs are the most common nosocomial infection and present a significant economic burden in developed countries (Nicolle, 2013). The process of urinary tract catheterisation often causes mucosal layer damage, which increases the chance of bacterial colonisation (Mittal et al., 2009). After initial infection bacteria are able to migrate at the interstitial space between the epithelial surface of the urethra and the catheter, resulting in colonisation along the length of the catheter and within the bladder (Stickler, 2008). Long term catheterisation often results in chronic infection of the bladder and urinary tract, which is typically not resolved by antibiotic treatment (Donlan, 2001b).

While a number of factors contribute to the chronic nature of *P. aeruginosa* infections within the lungs of CF patients and in CAUTIs, one key component is the innate propensity of *P. aeruginosa* to attach to and proliferate on the surface of epithelial cells and catheters (Kurosaka et al., 2001). This attachment and proliferation results in the formation of complex sessile bacterial communities, termed biofilms, and is associated with the chronic nature of infection (Costerton, 1999).

1.3 Bacterial biofilms

Bacterial biofilms are complex structures composed of matrix-encased microcolonies which are separated by open water channels, allowing the flow of nutrients, waste and signalling molecules (Stoodley et al., 2002). The self-produced matrix is composed of extracellular polymeric substances (EPS), the major components being polysaccharides, proteins and nucleic acids (Stoodley et al., 2002). Observations of bacteria living in association with surfaces have been documented as early as the 1930's (Henrici, 1933, ZoBell, 1933, Zobell and Allen, 1935), however it was not until the late 1970's that it was demonstrated that surface-associated, sessile bacteria actually predominated over planktonic cells in an aquatic environment (Geesey et al., 1978). Following on from this work, the same trend was also observed in other natural environments as well as in medical and industrial settings (Costerton et al., 1995). Despite these observations, the dogma that bacteria mainly exist as planktonic, free-living cells was still widely accepted for the most part of last century. Studies in the last 30 years have characterised this surface-associated mode of bacterial life, and allowed a greater understanding of the molecular mechanisms involved in forming biofilms. Remarkably, as our understanding of bacterial biofilms increased, it has become apparent that there is actually evidence for biofilms in the early fossil record, particularly in hydrothermal vents (Rasmussen, 2000, Reysenbach and Cady, 2001, Westall et al., 2001). This implies that the biofilm mode of life is an integral and ancient characteristic which is likely to have contributed to the survival and evolution of bacteria in such a wide range of natural environments.

Bacterial biofilms form in both natural and clinical settings. The physical location of the biofilm, as well as the composition and virulence state of the bacterial community, generally determines whether the interaction will be problematic or beneficial for the local environment and/or host (Hall-Stoodley et al., 2004).

1.3.1 Bacterial biofilms in natural environments

Bacterial biofilms form in a range of natural settings including hydrothermal hot springs, freshwater rivers, and areas of the ocean (Decho, 2000, Hall-Stoodley et al., 2004). In the environment, biofilms are associated with numerous symbiotic relationships, for instance with coral and plants, with varied outcomes for the non-bacterial host (Danhorn and Fuqua, 2007, Webster et al., 2004). In human industrial processes, such as biological wastewater treatment and bioremediation, the formation of bacterial biofilms is advantageous (Singh et al.,

2006, Nicolella et al., 2000). However in other cases, surface-associated bacteria are detrimental, for instance in the bio-fouling of ship hulls and other submerged objects in a marine environment, and in affecting mass and heat transfer within pipes and tubes (de Carvalho, 2007). Treatment with biocides is often ineffective at eradicating bacterial biofilms in these settings (de Carvalho, 2007).

1.3.2 Bacterial biofilms in clinical settings

In humans, bacterial biofilms exist on almost all mucosal surfaces (Costerton et al., 1995). The bacterial composition of these biofilms can determine the presence or absence of disease, for example within the oral cavity (Sbordone and Bortolaia, 2003), vagina (Hammill, 1989) and gastrointestinal tract (Scharl and Rogler, 2012). The triggers which stimulate the transition from a bacterial biofilm having a benign, or even beneficial effect on the human host to being problematic, are not well understood. However it appears that shifts in the bacterial composition, which change the resistance and virulence profile of the biofilm, as well as changes in environmental signals generated from within the biofilm and exogenous sources are involved (Donlan, 2001a, Hall-Stoodley et al., 2004).

Bacterial biofilms can be composed of human commensal bacteria, or of opportunistic pathogens which colonise the host (Donlan, 2001a). These biofilms become a serious problem when they interfere with normal bodily functions. There are a number of examples of this including chronic lung biofilm infections leading to airway obstruction; urinary tract infections and infective kidney stones, which can obstruct urine flow, causing inflammation and recurrent infection; and infective endocarditis which disrupts heart valve function (Donlan, 2001a). Bacterial biofilms are also problematic when they develop on implanted devices such as catheters, prosthetics, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts and endotracheal tubes (Hall-Stoodley et al., 2004). Formation of such biofilms on biotic or abiotic surfaces within a host commonly leads to local tissue damage and/or inflammation. Depending upon the degree of tissue damage and the immune state of the host this may result in detachment of cells or cell aggregates and subsequent entry into the bloodstream (Donlan, 2001a).

Treatment of biofilms made up of pathogens such as *P. aeruginosa* is especially problematic as the bacteria within these biofilms are inherently more resistant to host immune system factors and antibiotics than planktonic cells (Høiby et al., 2010a). In the case of some antibiotics the concentration necessary to inhibit bacteria in a biofilm can be up to 1000-times

more than that required to inhibit the same bacterial strain in a planktonic state (Costerton et al., 1999, Simoes, 2011). A number of different factors can confer this resistance, for instance the composition and depth of the biofilm (Gordon et al., 1988) and the physiology of bacteria within the biofilm (Fux et al., 2005, Brown et al., 1988, Lewis, 2005). Due to the intrinsic resistance of bacteria within biofilms, these infections are often chronic (Costerton et al., 1999). For this reason, when antibiotic treatment has been shown to be an ineffective treatment for infected implanted devices and kidney stone infections, the only remaining course of action is physical removal of the device or kidney stones which is highly invasive and increases the chance of further infection (Donlan, 2001a, Parsek and Singh, 2003).

1.3.3 Laboratory models of bacterial biofilms

Despite the wide range of environments in which bacterial biofilms form, laboratory models are able to closely reflect the biofilms which form in both natural and clinical settings (Hall-Stoodley et al., 2004). Sessile biofilms, also termed fully-hydrated or submerged biofilms as they form in well hydrated environments, are the best studied kind of bacterial biofilm. The formation and developmental sequence of these biofilms by pure cultures of proteobacteria and by mixed-species populations in natural ecosystems is represented in Figure 1-1. Essentially, sessile biofilms form by initial attachment of bacteria to a surface, followed by production of the EPS matrix and microcolony formation, which results in a stronger, irreversible surface attachment. At this point the biofilm begins to develop mushroom-like structures which are a characteristic of mature biofilms. Eventually the dispersal of single cells from the biofilm occurs which allows bacteria to colonise other environments (Stoodley et al., 2002). These biofilms appear to be most similar to the biofilms which form within a cystic fibrosis (CF) patient lung infection, or within quiescent marine environments (Stoodley et al., 2002, Moreau-Marquis et al., 2008).

Other kinds of biofilms which form within natural and clinical settings, can also be modelled in the laboratory. For example, active biofilm expansion in semi-hydrated systems can occur at the air/agar interface of an agar plate to generate a colony biofilm which is similar to biofilm formation on any semi-solid biotic or abiotic surface (Branda et al., 2005). Additionally active biofilm expansion at the interface between agar and a petri-dish base, or a catheter and epithelial tissue, results in formation of an interstitial biofilm (Donlan, 2001b). Furthermore, formation of a pellicle at the air/liquid interface of a laboratory broth culture or

floating aggregates within a broth are related to biofilms which form within wastewater treatment systems (Nicolella et al., 2000).

This Thesis introduction will focus on sessile, surface attached biofilms which form within hydrated environments and actively expanding biofilms which form in semi-hydrated environments.

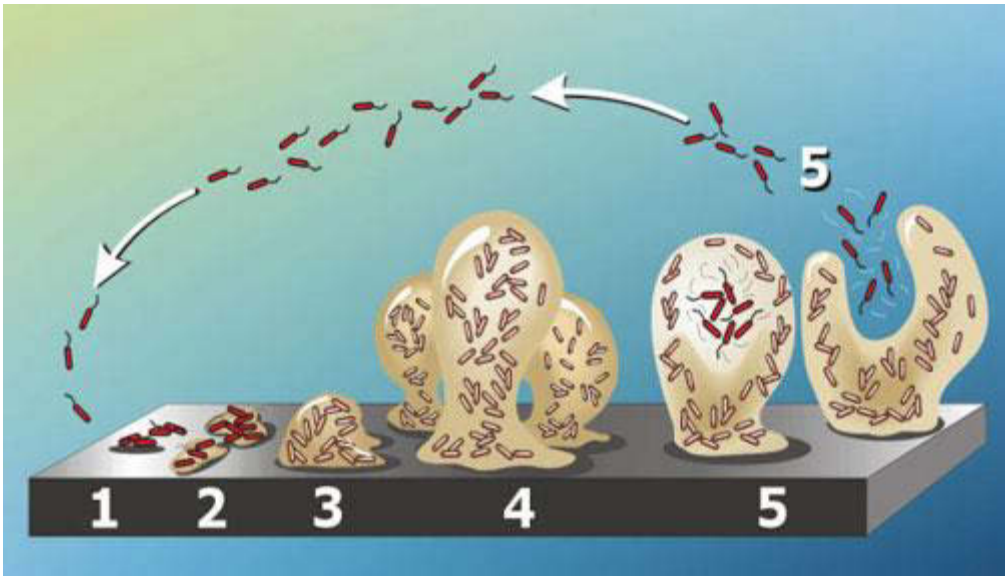

Figure 1-1. Classic model of fully-hydrated biofilm development.

Stage 1 – initial attachment to a surface; stage 2 – production of EPS; stage 3 and 4 – development of the biofilm to form mushroom-like structures; and stage 5 – dispersal of single cells from the biofilm. (Adapted from Stoodley et al., 2002).

1.4 *P. aeruginosa* sessile biofilms

1.4.1 Sessile biofilm constituents

P. aeruginosa sessile biofilms are complex, multi-layered structures, with the main EPS constituents being DNA, polysaccharides and proteins (Sutherland, 2001). As will be described below, each of these components have varied roles in the formation and structure of these biofilms.

1.4.1.1 Extracellular DNA

Extracellular DNA (eDNA) has been shown to be important in the initial stages of *P. aeruginosa* sessile biofilm formation in both the laboratory reference strain PAO1 (Whitchurch et al., 2002b), and in clinical *P. aeruginosa* isolates (Nemoto et al., 2003). DNase treatment

was shown to dissolve young PAO1 biofilms, but had little effect on established biofilms, suggesting that eDNA is a significant component in young PAO1 biofilms, while in older biofilms additional components are more important (Whitchurch et al., 2002b). Microscopic investigations have revealed that eDNA within *P. aeruginosa* biofilms is primarily located in the outer sections of the mushroom stalk structures, forming a distinct barrier between the stalk and mushroom cap-forming bacteria (Allesen-Holm et al., 2006) This eDNA is most similar to whole chromosomal DNA (Allesen-Holm et al., 2006), however the exact mechanism for release of this DNA is undefined. Lysis of a subpopulation of bacteria within the biofilm and/or lysis of membrane vesicles containing DNA has been proposed as one mechanism (Allesen-Holm et al., 2006) as has active secretion (Hara, 1981). Electron microscopy images showing intact *P. aeruginosa* cells with cell-associated extracellular double-stranded DNA prompted the suggested active secretion mechanism, however this has only been demonstrated within planktonic cultures and from the images presented, it is unclear whether the DNA is being excreted or taken up by the cell (Hara, 1981).

1.4.1.2 Exopolysaccharides

Alginate, Psl and Pel are the three main exopolysaccharides that are produced as a part of the EPS matrix of *P. aeruginosa* biofilms (Ryder et al., 2007).

1.4.1.2.1 Alginate

The existence of mucoid *P. aeruginosa* variants within biofilms in CF patient airways is due to mutation in *mucA* (Martin et al., 1993a) and results in overproduction of the exopolysaccharide, alginate (Høiby et al., 2010b). This high molecular weight acetylated polymer is composed of nonrepetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids. While alginate is not absolutely required for biofilm formation it appears to contribute to biofilm architecture in mucoid (*mucA*$^-$) strains of *P. aeruginosa* (Nivens et al., 2001, Wozniak et al., 2003, Stapper et al., 2004). Studies of isogenic alginate biosynthesis mutants of the *P. aeruginosa* laboratory strains PAO1 and PA14 demonstrated that biofilms formed by these mutants still contained what appeared to be exopolysaccharide matrix material, suggesting that alginate was not the sole biofilm exopolysaccharide (Wozniak et al., 2003). Subsequent to this discovery, two additional exopolysacchrides, Psl and Pel were identified within the *P. aeruginosa* EPS matrix (Jackson et al., 2004, Friedman and Kolter, 2004).

1.4.1.2.2 Psl

Psl is a mannose- and galactose-rich exopolysaccharide, the production of which is encoded by the polysaccharide synthesis locus (PA2231-PA2245) (Ma et al., 2007, Matsukawa and Greenberg, 2004, Jackson et al., 2004). Interestingly, this exopolysaccharide is not made in *P. aeruginosa* strain PA14 as the *psl* operon is incomplete (Lee et al., 2006). However, in strain PAO1 and ZK2870 this exopolysaccharide is required for bacterial cell adherence to a substratum, acting as a scaffold which holds biofilm cells together within the matrix, and ultimately maintains biofilm structure throughout sessile biofilm development (Ma et al., 2009, Matsukawa and Greenberg, 2004). The production of Psl is also required for colonisation of both airway epithelial cells and mucin-coated surfaces, which suggests that Psl may be an important component in the early stages of biofilm formation within the lungs of CF individuals (Ma et al., 2006). Recent work has demonstrated that in early biofilm development *P. aeruginosa* deposits a trail of Psl as it moves across a surface, which then influences the surface motility of cells that encounter these trails (Zhao et al., 2013). Interestingly, eDNA and Psl localise to distinct areas of the biofilm EPS during development (Ma et al., 2009).

1.4.1.2.3 Pel

The cluster of genes *pelABCDEFG* (PA3064-PA3058) encode the factors which are involved in production of a glucose-rich exopolysaccharide Pel, which contributes to the EPS matrix of pellicle biofilms, which form at air-liquid interfaces. While Pel is produced by *P. aeruginosa* strains PA14, PAK and PAO1 and is important in the formation of pellicle biofilms in these strains, the role of this exopolysaccharide in sessile biofilms appears to be strain specific. In strain PA14 this gene cluster produces the major structural component of *P. aeruginosa* strain PA14 sessile biofilms, which presumably compensates for the lack of Psl in this strain (Colvin et al., 2011). However, Pel appears to only be involved in the later maturation stages of hydrated PA14 biofilm formation suggesting that other factors are involved in the initial attachment stages (Friedman and Kolter, 2003, Friedman and Kolter, 2004). In *P. aeruginosa* strain PAK, Pel is involved in both the attachment and maturation stages, however it appears that tfp in combination with Pel are important in the initial attachment stages (Vasseur et al., 2005). Pel is not required for either the attachment or development stages of PAO1 hydrated biofilms (Colvin et al., 2011). Remarkably, the presence of Pel within PA14 hydrated biofilms appears to provide some protection against aminoglycoside antibiotics,

which may contribute to the observed increased antibiotic resistance of *P. aeruginosa* biofilms (Colvin et al., 2011).

1.4.1.3 Proteins and outer membrane vesicles

Protein is another major component of *P. aeruginosa* biofilms, however there is limited knowledge as to the identity of the specific proteins present within the EPS matrix. One identified protein is CdrA which, when secreted, appears to interact with the Psl polysaccharide to promote biofilm formation on surfaces and auto-aggregation in broth culture (Borlee et al., 2010). In another recent study of the overall protein content of the biofilm matrix, a large number of additional proteins were identified which, in some cases, were associated with outer-membrane vesicles (OMVs) (Toyofuku et al., 2012). OMVs have also been shown to be important structural components of the biofilm matrix in laboratory grown biofilms as well as biofilms obtained from natural environments (Schooling and Beveridge, 2006).

1.4.2 Regulation of sessile biofilm formation in *P. aeruginosa*

A number of regulatory systems control *P. aeruginosa* sessile biofilm formation including the quorum sensing (QS) system (Davies, 1998, Diggle et al., 2003), a number of two component sensor/regulator systems (Mikkelsen et al., 2011), and the Wsp chemosensory system, which is composed of homologs of the Che chemosensory system of *Escherichia coli* (Hickman et al., 2005). These systems modulate the levels of small intra and intercellular signals, which repress factors associated with an acute infection mode, such as flagella and tfp, and production of secreted and cell-associated virulence factors, and stimulate production of factors associated with chronic infection, such as production of EPS.

1.4.2.1 Intercellular signals

1.4.2.1.1 Quorum sensing

A large number of Gram-negative bacteria produce and utilise *N*-acyl homoserine lactones (AHLs) to control and regulate gene expression in a cell density-dependent manner referred to as quorum sensing (QS) (Withers et al., 2001, Whitehead et al., 2001). In *P. aeruginosa* this system produces the main intercellular signals that are involved in regulating *P. aeruginosa* sessile biofilm formation. QS is mediated by the diffusion of signalling molecules into the external environment, the release of which corresponds to specific population cell densities. Las and Rhl are two complete QS systems in *P. aeruginosa*, existing

in a hierarchy where the Las system controls expression of the Rhl QS system activator, RhlR (Pesci et al., 1997, Latifi et al., 1996). The synthases LasI and RhlI produce the AHL signalling molecules, 3-oxo-C_{12}-HSL and C_4-HSL respectively, which go on to induce their respective LuxR-type transcriptional regulators, LasR and RhlR, resulting in the activation of numerous QS-controlled genes (Juhas et al., 2005, Pesci et al., 1997). Together, the Las and Rhl systems directly or indirectly regulate expression of approximately 10 % of the *P. aeruginosa* genome (Schuster and Greenberg, 2006).

A third QS system utilises a 2-alkyl-4-quinolone (AQ) based signalling molecule, 2-heptyl-3-hydroxy-4-quinolone, designated the *Pseudomonas* quinolone signal (PQS). This signal is synthesised by gene products encoded by the *pqsABCD* operon and *pqsH* which are also involved in production of other AQs, including 2-heptyl-4-quinolone (HHQ) (Wade et al., 2005, Deziel et al., 2005). HHQ is the immediate precursor of PQS and is also considered to be a functional QS molecule (Xiao et al., 2006). The production of PQS occurs at the onset of stationary phase, with this production operating between the two other QS systems, Las and Rhl, in *P. aeruginosa* (McKnight et al., 2000, Diggle et al., 2003). PQS binds to MvfR (PsqR), which together control expression of more than 140 genes, the majority of which are co-regulated by the Rhl system (Deziel et al., 2005). Unlike the HSL autoinducers from the Las and Rhl systems, which are able to mediate cell-cell signalling via diffusion into the extracellular environment, it appears that intercellular transport of PQS may occur within membrane vesicles (Mashburn and Whiteley, 2005).

The Las but not Rhl system is involved in biofilm formation in *P. aeruginosa* (Davies, 1998). A *lasI* mutant is able to undergo the initial stages of biofilm formation and produce wildtype levels of EPS, however differentiation to form a mature biofilm does not occur. The biofilms formed by these mutants are also sensitive to detergent-induced disruption (Davies, 1998). Exogenous addition of the autoinducer 3-oxo-C_{12}-HSL is able to restore both the ability of *lasI* mutants to form mature biofilms and resist detergent-induced disruption (Davies, 1998). A role for QS-controlled release of eDNA within sessile biofilm development has been reported (Barken et al., 2008). Studies of a mixed population of wildtype and *pilAlasRrhlR* mutant cells revealed that while wildtype cells formed normal mushroom cap structures on top of other wildtype cells, the same cap structures did not form on top of *pilAlasRrhlR* mutants. As a *pilAlasRrhlR* mutant is deficient in eDNA release it was concluded that QS-dependent eDNA release in initial microcolony formation is required for subsequent development of mushroom-shaped structures in *P. aeruginosa* sessile biofilms (Barken et al., 2008).

PQS is also involved in sessile biofilm formation, however its role is less clear. Addition of exogenous PQS is able to stimulate attachment and biofilm formation of a growing planktonic culture of *P. aeruginosa* (Diggle et al., 2003). The molecular basis for this is unknown, however it has been suggested that it may be due in part to stimulation of PA-IL lectin production (Diggle et al., 2003), which plays a role in biofilm formation (Diggle et al., 2006). PQS levels often correlate with levels of autolysis in later stage growth of *P. aeruginosa*, however it is unclear as to how this may contribute to biofilm formation (D'Argenio et al., 2002).

1.4.2.1.2 Cyclic dipeptides

Diketopiperazines (DKPs), which are a part of the cyclic dipeptide family of molecules, have been shown to act in a manner similar to the AHL QS molecules of *P. aeruginosa* (Holden et al., 1999). Two different DKPs have been identified in the supernatant of *P. aeruginosa*, cyclo(ΔAla-L-Val) and cyclo(L-Pro- L-Tyr) which appear to bind and activate LuxR receptors (Holden et al., 1999). A role for these molecules in *P. aeruginosa* biofilm formation has been suggested, due to their ability to activate QS components, and the potential role of cyclic dipeptides in biofilm formation in *Vibrio vulnificus* (Park et al., 2006). The specifics of this role are however unclear, particularly as these molecules are unable to regulate QS-related mechanisms in the absence of the AHL QS signalling molecules, and the fact that very high concentrations of these molecules are required for LuxR-dependent QS activation (Holden et al., 2000).

1.4.2.1.3 *cis*-2-decanoic acid

Diffusible signal factors (DSF) are intercellular bacterial signals that are implicated in production of multiple virulence factors and in biofilm formation (Jimenez et al., 2012). Since the initial discovery of the first DSF signal family molecule, *cis*-11-methyl-2-dodecenoic acid in the plant pathogen, *Xanthomonas campestris* (Barber et al., 1997), a large number of DSF signals have been identified and characterised (He and Zhang, 2008). This includes *cis*-2-decenoic acid which has been shown to induce dispersal of established *P. aeruginosa* biofilms as well as biofilms from a wide range of other species (Davies and Marques, 2009). Additionally, *cis*-2-decenoic acid has been shown to increase the resistance of *P. aeruginosa* to polymyxin antibiotics (Ryan et al., 2008). While the exact mechanism of action of this DSF-like molecule is yet to be uncovered in *P. aeruginosa*, a sensor kinase, PA1396, has been shown to be essential for the response to this molecule (Ryan et al., 2008).

1.4.2.2 Intracellular signals

In addition to intercellular signals, a number of intracellular signals are involved in sessile biofilm formation. As outlined below, alterations in the intracellular levels of these signals results in transitions between sessile and motile modes of life in *P. aeruginosa*.

1.4.2.2.1 Bis-(3´-5´)-cyclic dimeric guanosine monophosphate

Bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP) is an important second messenger signal which controls a range of functions within the bacterial cell, including virulence (Dow et al., 2006, Tamayo et al., 2007, Moscoso et al., 2011), cell cycle progression (Duerig et al., 2009) and antibiotic production (Fineran et al., 2007). c-di-GMP is also important in biofilm formation, as changes in intracellular levels of this second messenger can mediate transitions between a motile planktonic state and a sessile biofilm mode of life (Simm et al., 2004). In *P. aeruginosa* intracellular c-di-GMP levels are regulated by diguanylate cyclases (DGCs) containing GGDEF domains, which synthesise c-di-GMP, and phosphodiesterases (PDEs) which possess EAL domains, and degrade c-di-GMP. In *P. aeruginosa* 39 genes have been identified which encode DGCs and PDEs or proteins containing GGDEF and/or EAL domains (Kulesekara, 2006). One DGC, WspR, is linked to the Wsp chemosensory system, which is composed of homologs of bacterial chemotaxis proteins (Hickman et al., 2005). The Wsp system regulates the generation and degradation of c-di-GMP in response to currently unknown environmental signals (D'Argenio et al., 2002, Hickman et al., 2005).

In *P. aeruginosa* an increase in intracellular c-di-GMP levels stimulates production of extracellular matrix proteins which increases biofilm formation (Borlee et al., 2010, Hickman and Harwood, 2008, Hickman et al., 2005, Ueda and Wood, 2009). This c-di-GMP-dependent effect occurs at both the transcriptional and post-translational levels. An increase in c-di-GMP levels relieves transcriptional repression of *cdrA* and the *pel* and *psl* operons by binding to the regulator FleQ, which increases biofilm formation (Hickman and Harwood, 2008, Borlee et al., 2010). Production of the Pel exopolysaccharide is also dependent upon one of the gene products in the *pel* operon, PelD binding to c-di-GMP (Lee et al., 2007) (Hickman et al., 2005). In a further layer of complexity, the Las QS system has also been shown to exert an effect on c-di-GMP levels via controlling tyrosine phosphatase TpbA (tyrosine phosphatase related to biofilm formation)-based activation of the *pel* locus (Ueda and Wood, 2009). Additionally, Psl

stimulates c-di-GMP production by two DGCs SadC and SiaD, which in turn further increases biofilm formation in a forward-feed loop (Irie et al., 2012).

1.4.2.2.2. 3'-5'-cyclic adenosine monophosphate

3'-5'-cyclic adenosine monophosphate (cAMP) is another important bacterial second messenger, which is involved in virulence factor regulation in *P. aeruginosa* (Smith et al., 2004, Fuchs et al., 2010a) (see more in section 1.7.1.3 and 1.7.2.1). In *P. aeruginosa* cAMP is synthesized by two adenylate cyclases, CyaA and CyaB, with the latter contributing the majority of intracellular cAMP (icAMP) levels (Fulcher et al., 2010, Wolfgang et al., 2003). The 3'-5' nucleotide PDE CpdA serves to reduce the levels of icAMP by hydrolyzing the phosphodiester bond to release the biologically inactive 5'-AMP (Fuchs et al., 2010a). An increase in icAMP levels via CyaA has been reported to be involved in glucose starvation-mediated biofilm dispersal (Huynh et al., 2012). While the role of CyaB was not investigated it has been proposed to have a similar role as CyaA in biofilm dispersal (Huynh et al., 2012). The authors propose that an increase in icAMP levels promotes expression of proteins involved in remodelling biofilm cells, which would prepare the cell for the transition from a sessile to a motile mode of life. The cAMP PDE in *Serratia marcescens*, CpdS has been reported to be involved in biofilm formation in this organism (Kalivoda et al., 2013). A decrease in icAMP levels by CpdS has been shown to promote biofilm formation via production of Type I fimbriae (Kalivoda et al., 2013), which are cellular appendages involved in surface attachment and biofilm development (Vallet et al., 2001). However, the role of the *P. aeruginosa* cAMP PDE CpdA in icAMP-modulated sessile biofilm development has not been investigated.

1.4.2.2.3 Nitric oxide

Nitric oxide (NO), or other reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$), produced during anaerobic respiration, have been shown to induce dispersal of mature *P. aeruginosa* biofilms (Barraud et al., 2006). The involvement of intracellular NO was confirmed by biofilm dispersal studies of a mutant unable to generate NO through anaerobic respiration (nitrite reductase mutant, Δ*nirS*) which did not disperse, and a mutant which was unable to reduce NO (NO reductase mutant, Δ*norCB*) which exhibited greatly enhanced dispersal (Barraud et al., 2006). In addition to intracellular NO, exogenous addition of sublethal concentrations of the NO donor, sodium nitroprusside (SNP), has been shown to induce dispersal (Barraud et al., 2006). The NO-mediated biofilm dispersal phenotype has been shown to involve the chemotaxis transducer protein BdlA (biofilm dispersion locus) which is thought

to interact with the c-di-GMP regulatory network, resulting in an overall decrease in intracellular c-di-GMP levels that triggers biofilm dispersal and promotion of motility (Barraud et al., 2009).

1.4.2.3 Extracellular signals

In addition to inter and intracellular signals produced by bacteria within biofilms, a range of signals which originate from extracellular sources affect *P. aeruginosa* sessile biofilm formation.

1.4.2.3.1 Alterations in nutrient source

Variations in the normal progression of *P. aeruginosa* sessile biofilm formation have been observed when the carbon source is varied. The characteristic mushroom-like structures seen in Figure 1-1 form when the biofilm is grown in minimal glucose media, however, when citrate, casamino acid or benzoate are used as the main carbon source the biofilms that form are flat and densely packed (Klausen et al., 2003b). The reason for these differences appears to be due to the Crc (catabolic repression control) protein (O'Toole et al., 2000). This protein is involved in repressing *P. aeruginosa* metabolism of carbohydrates like glucose and mannitol, when tricarboxylic acid (TCA) cycle intermediates are present (Wolff et al., 1991). A *crc* mutant is defective in biofilm formation which appears to be associated with the inability of a *crc* mutant to undergo a form of tfp-mediated surface translocation, termed twitching motility (O'Toole et al., 2000) (see more in section 1.7.1.6). Additionally, the involvement of the QS system in biofilm formation is thought to be somewhat dependent upon alterations in carbon source (Shrout et al., 2006). Interestingly, these authors demonstrated that quorum sensing was only important in the early stages of biofilm formation, as well as a form of flagellum-mediated motility, swarming motility, when cells were grown on succinate but not on glutamate or glucose (Shrout et al., 2006).

1.4.2.3.2 Mucin

Mucin glycoproteins consist of a polypeptide core with numerous *N*-acetyl glucosamine (GlcNAc)-linked saccharide side chains, each of which contains 8 to 10 sugars. These glycoproteins are major components of epithelia mucosal secretions which, when combined with effective mucociliary clearance, aid in the host defence against invading pathogens. At least 16 distinct mucins have been identified in humans. The expression of these mucins varies between different tissues, with the gastrointestinal tract having the highest and most diverse

expression profile (Linden et al., 2008). *P. aeruginosa* is able to bind human respiratory mucins (Vishwanath and Ramphal, 1984). This preferential binding is thought to be important in initial colonisation of *P. aeruginosa* in CF patient lungs (Ramphal et al., 1991), however reports which contradict this proposed involvement have been published (Sajjan et al., 1992). Mucin has been reported to both stimulate (Landry et al., 2006) and repress (Caldara et al., 2012) *P. aeruginosa* sessile biofilm formation. Laudry et al. (2006) suggest that the observed stimulation of sessile biofilm formation is associated with both a specific adhesion-mucin interaction, which may act to repress swimming motility. Conversely, the results of Caldara et al. (2012) suggest that the observed repression of sessile biofilm formation is associated with a stimulation in swimming motility. Caldara et al. (2012) propose that the differences reported in both studies may be due to the state of the mucins in each experimental setup. Their study used mucins within a hydrogel, which is thought to maintain a more native confirmation of the glycoprotein (Yeung et al., 2012), whereas Laundry et al. (2006) used mucins absorbed onto a two-dimensional surface, which is thought to affect biophysical properties of mucin such as viscoelasticity and lubricity (Caldara et al., 2012).

1.4.2.3.3 Extracellular ATP

Adenosine triphosphate (ATP) is the universal intermediate of energy synthesis and a key molecule in all aspects of intracellular metabolism in living cells. More recently the role of this molecule as an extracellular signalling molecule in eukaryotes has begun to be more widely accepted. ATP is a part of the eukaryotic purinergic signalling system, which uses purines and pyrimidine nucleotides as signalling molecules. These nucleotides are released from cells via a number of mechanisms, including both lytic and non-lytic pathways (Burnstock and Verkhratsky, 2009, Yin et al., 2007). Cell damage and stress caused by infecting pathogens can cause this nucleotide release, which results in recruitment of host immune system factors to clear the infection (Di Virgilio, 2007, Burnstock, 2007). Within human cells extracellular ATP (eATP) is sensed by a range of P2X and P2Y purinergic receptors which have been identified in virtually all human tissues and cell types (Burnstock and Verkhratsky, 2009). Interestingly, eATP has recently been shown to stimulate sessile biofilm formation in *Escherichia coli, Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Staphylococcus aureus* and increase adherence of *A. baumannii* to human epithelial cells *in vitro* (Xi and Wu, 2010). It appears that the observed stimulation of sessile biofilm formation may, at least in part, be due to eATP-induced cell lysis and subsequent eDNA release, with this eDNA assisting in sessile biofilm formation (Xi and Wu, 2010).

1.4.2.3.4 Iron

Iron within the environment of a bacterium often exists within ferric oxide hydrate complexes, or bound to extracellular iron carrier proteins termed siderophores (Miethke and Marahiel, 2007). A number of pieces of evidence suggest that iron limitation has an inhibitory effect on sessile biofilm formation and as discussed further in section 1.7.3.1.4 a number of these iron-related effects on sessile biofilm formation are also dependent upon the effects of iron limitation on tfp-mediated twitching motility. The role of iron levels in sessile biofilm formation was first demonstrated from the observed inhibition of *P. aeruginosa* biofilm formation in the presence of the mammalian iron binding protein lactoferrin (Singh et al., 2002). Further evidence was supplied in later reports of biofilm formation defects in *P. aeruginosa* Fur (ferric dependent repressor)-dependent iron scavenging mutants (Banin et al., 2005), as well *P. aeruginosa* mutants that are unable to synthesise the siderophore pyoverdin (Patriquin et al., 2008).

1.4.2.3.5 D-amino acids

The exogenous addition of D-amino acids has been shown to stimulate *P. aeruginosa* biofilm dispersal (Kolodkin-Gal et al., 2010). This study also demonstrated that endogenously produced D-amino acids (D-leucine, D-methoinine, D-tyrosine and D-tryptophan) from *Bacillus subtilis* were involved in biofilm dispersal. The observed dispersal effect was shown to be due to D-amino acid-dependent release of amyloid fibres which link cells within the biofilm matrix together (Kolodkin-Gal et al., 2010). While the effect of endogenously produced D-amino acids on *P. aeruginosa* biofilm dispersal was not investigated in this study, a number of bacteria have been shown to produce D-amino acids and thus this has been suggested as a widespread signal for biofilm dispersal (McDougald et al., 2012).

1.5 *P. aeruginosa* semi-hydrated biofilms

Clearly, *P. aeruginosa* sessile biofilms are complex structures which form by the coordinated action of a number of regulatory systems as well as numerous intracellular, intercellular and extracellular signals and factors. In addition to the sessile biofilms which form within hydrated environments, *P. aeruginosa* is able to form biofilms within semi-hydrated settings. As is the case for sessile biofilms, the formation of semi-hydrated biofilms is controlled by a range of complex regulatory systems, as well as a number of intracellular and extracellular signalling molecules. The mechanism of *P. aeruginosa* semi-hydrated biofilm

formation will be outlined in detail below, and will also include a comprehensive description of the regulation of this process.

1.5.1 Formation and constituents of semi-hydrated biofilms

Within a semi-hydrated environment, *P. aeruginosa* is able to form biofilms on the surface of solidified nutrient media (Figure 1-2a) and at the interstitial space between solidified nutrient media and another surface, such as a coverslip or petri dish base (Figure 1-2b) (Semmler et al., 1999). Both of these semi-hydrated biofilms can undergo active expansion by a form of flagellum-independent translocation termed twitching motility. Twitching motility allows *P. aeruginosa* to rapidly colonise solidified nutrient media surfaces to form these semi-hydrated biofilms. Detailed analyses of biofilms formed at the interstitial space between a solidified nutrient media-coated microscope slide and a coverslip revealed that this active expansion results in development of a vast, intricate lattice-like network of interconnected trails (Semmler et al., 1999) (Figure 1-3). This study demonstrated that this highly organised, multicellular behaviour was shown to arise through the collective behaviour of individual cells within the biofilm, initiated by migration of rafts at the leading edge of the biofilm that appeared to lay down a trail along which cells were observed to preferentially migrate (Semmler et al., 1999). Recent work has revealed that this preferential migration occurs along a network of furrows which are created by the leading edge rafts of cells (Gloag et al., 2013). Additionally, it has been shown that eDNA facilitates the efficient flow of cells through this furrow network by maintaining coherent cell alignments, ensuring that cells continue to actively expand away from the main colony (Gloag et al., 2013).

While the extracellular matrix of *P. aeruginosa* sessile biofilms is composed of a number of components, only eDNA has been shown to be a significant component within the extracellular environment of *P. aeruginosa* semi-hydrated biofilms (Gloag et al., 2013). As semi-hydrated biofilms lack the three-dimensional structures which are characteristic of sessile biofilms, it is likely that eDNA alone is sufficient to allow efficient formation of these biofilms.

1.6 Twitching motility

The term twitching motility was first used in 1961 by Lautrop to describe flagellum-independent surface motility in *Acinetobacter calcoaceticus* as he observed that cells within a suspension appeared to move in a somewhat ‘jerky’ fashion (Lautrop, 1962). Twitching motility occurs in a wide range of bacteria. The most extensive studies have been carried out

in *P. aeruginosa*, *Neisseria gonorrhoeae* and *Myxococcus xanthus*, with this form of motility in *M. xanthus* being referred to as gliding motility (Whitchurch, 2006, Semmler et al., 1999). Twitching motility appears to be largely restricted to Gram-negative bacteria, mainly the β, γ and δ subdivisions of the Proteobacteria (Henrichsen, 1975, Mattick, 2002).

Both organic and inorganic surfaces such as epithelial cells, nutrient agar, plastics, glass and metals support twitching motility, with the only requirement that these surfaces be slightly wet, but not fully-hydrated (Whitchurch, 2006). Active expansion of *P. aeruginosa* on the surface of agar results in the formation of flat, spreading colonies which have a characteristic rough appearance and a small outer twitching zone, which when visualised macroscopically appears as a "ground glass" edge (Figure 1-2a). At the interstitial space between agar and a petri dish base active expansion produces a large twitching zone resembling a halo (Figure 1-2b) (Semmler et al., 1999, Whitchurch, 2006).

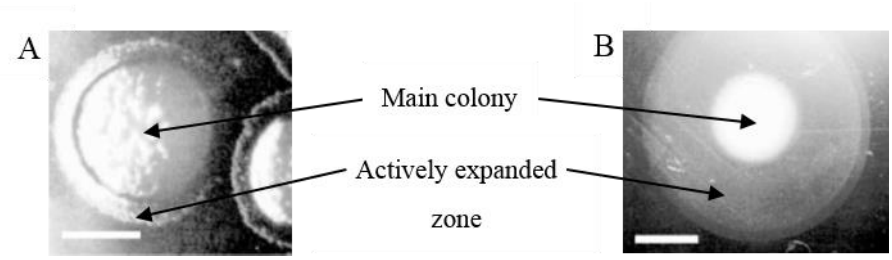

Figure 1-2. Twitching motility-mediated biofilm expansion in semi-hydrated environments.

Macroscopic images showing semi-hydrated biofilm formation as either a colony (A) or interstitial (B) biofilm. The main colony and the regions formed by active expansion are indicated. The bar represents 5 mm. (Adapted from Semmler et al., 1999).

When *P. aeruginosa* interstitial biofilms are visualised microsopically, three major micromorphological patterns are observed as shown in Figure 1-3. These biofilms are made up of a dense multilayered main colony which extends into a lattice network of cell trails typically 1-5 cells in diameter and finally, at the extreme edge of growth, produces leading rafts which are usually 10-50 cells in diameter (Semmler et al., 1999).

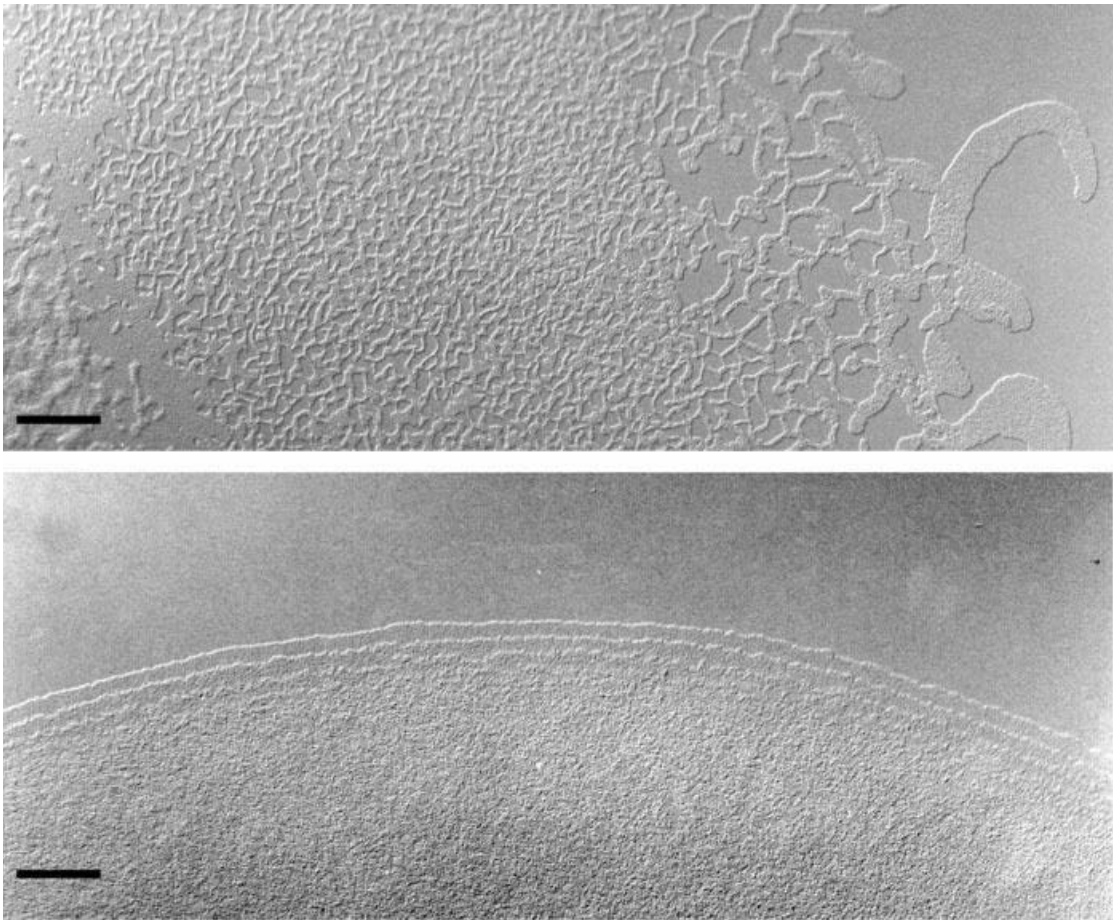

Figure 1-3. Microscopic view of *P. aeruginosa* interstitial biofilms formed by wildtype and a non-twitching mutant.

The top panel shows a microscopic view of the twitching zone of *P. aeruginosa* showing the expansion of the interstitial biofilm away from the main colony (far left), which is initiated by leading rafts (far right), and results in formation of a distinct lattice-like network. The bottom panel shows the edge of a colony formed by a non-twitching *pilA* mutant. Scale bar = 50 µm. Taken from Semmler et al., (1999).

1.6.1 Type IV pili

Active expansion via twitching motility to form this zone involves the extension and retraction of type IV pili (tfp) by individual bacterial cells within the community (Merz et al., 2000, Skerker and Berg, 2001). Tfp are long surface filaments produced at one or both cell poles by a range of Gram-negative bacteria (Mattick et al., 1996), as shown in Figure 1-4. Tfp are also a major *P. aeruginosa* adhesin, being important in epithelial cell adherence in culture (Woods et al., 1980, Doig et al., 1988, Chi et al., 1991, Comolli et al., 1999) and in virulence

(Sato et al., 1988, Hahn, 1997), as well as serving as receptors for certain bacteriophages (Bradley and Pitt, 1974).

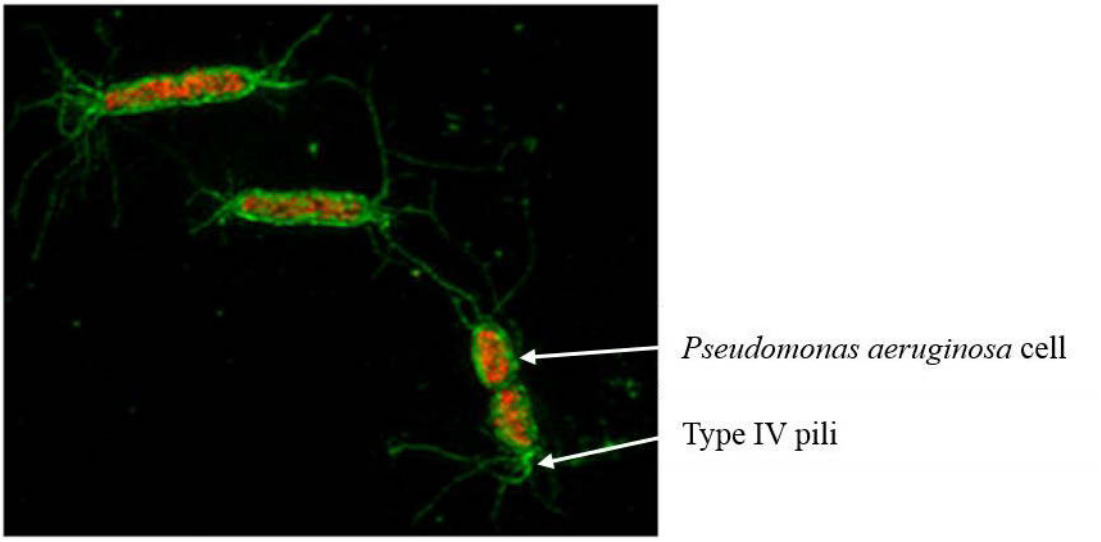

Figure 1-4. **Hyper-piliated *P. aeruginosa* cell.**

Cells stained with antisera to type IV pili (green); DNA stained with DAPI (red). OMX 3D-SIM, University of Technology Sydney, Microbial Imaging Facility (L. Turnbull and C. B. Whitchurch).

As twitching motility is a collective bacterial behaviour, and usually involves cell-to-cell contact, cells in isolation are rarely seen to undergo twitching motility unless they are within a certain distance of other cells (up to several μm), which corresponds to the length of tfp (Semmler et al., 1999). Studies of fluorescently labelled pili in *P. aeruginosa* showed that pili extend and retract at a rate of approximately 0.5 μm/sec (Skerker and Berg, 2001), which is similar to the rates observed for twitching motility in culture (Semmler et al., 1999). Remarkably, individual pili on the same cell body are able to extend and retract independently of one another (Skerker and Berg, 2001). It has been demonstrated that cell movement actually occurs as a result of tfp surface attachment and subsequent retraction, with tfp extension only being a means of tethering the cells to the surface. The force exerted by retraction of an individual pili is estimated to be around 10 pN (Skerker and Berg, 2001).

1.6.1.2 Tfp structure

Tfp are typically 5-7 nm in diameter and extend up to several μm in length. They are composed of individual PilA protein monomers, also called pilin (Sastry et al., 1985). Pilin have a short positively charged leader sequence with a conserved and highly hydrophobic amino terminal domain, which forms the core of the assembled tfp structure (Sastry et al.,

1985). The central and carboxy-terminal two thirds of the PilA monomer are relatively hydrophilic and contain the hypervariable region, which is the major site of structural and antigenic variation between strains (Sastry et al., 1985, Hazes et al., 2000). The truncated structure of the *P. aeruginosa* strain PAK PilA protein has been solved (Hazes et al., 2000). Fibre models based upon X-ray and electron microscopy data predict that PilA monomers are packed into a helical structure of 5 subunits per turn. The core of the tfp structure is composed of a parallel, overlapping coiled-coil made up of the hydrophobic N-terminal regions of the PilA monomer subunits. The outside surface of the tfp structure is composed of a scaffold of β-sheets packed flat against the hydrophobic core region, with the outermost layer composed of the hypervariable region which is exposed, and an extended C-terminal tail (Craig et al., 2003) .

1.6.1.3 Tfp biogenesis and assembly

In *P. aeruginosa* the components involved in biogenesis and assembly of tfp are encoded by *pilA*, *B*, *C*, *D*, *E*, *F*, *M*, *N*, *O*, *P*, *Q*, *T*, *U*, *V*, *W*, *X*, *Y1*, *Y2*, *Z*, and *fimT*, *U*, *V* (Whitchurch, 2006), with a number of these being homologous to protein secretion and DNA uptake proteins in various bacteria (Whitchurch et al., 1991, Hobbs and Mattick, 1993, Mattick and Alm, 1995). Tfp assembly requires pilin leader sequence cleavage and N-terminal methylation by the pre-pilin peptidase PilD (Nunn and Lory, 1991), with these processed pilin being assembled on the base of the minor pilins (PilE, V, W, X and FimU) by the combined action of the ATPase PilB and the cytoplasmic membrane protein PilC (Alm and Mattick, 1995, Alm et al., 1996b, Nunn et al., 1990, Takhar et al., 2013). The pilus is extruded through the outer membrane by a pore composed of multimeric PilQ (Martin et al., 1993b). PilF is involved in insertion and multimerisation of PilQ to allow formation of the secretin pore (Koo et al., 2008, Watson et al., 1996b), with multimeric PilQ being stabilised by the lipoprotein PilP, and PilM, PilN and PilO (Ayers et al., 2009). Formation of the secretion pore also requires the action of FimV (Wehbi et al., 2010, Semmler et al., 2000). The PilMNOPQ complex also appears to facilitate movement of the pilus through the periplasm and fixes the pilus to the cell envelope (Tammam et al., 2013). TonB3, which is a part of a major active import system in *P. aeruginosa*, has wildtype intracellular pili levels, but very low surface-associated pili levels. This suggests that this protein also has a role in the transport and secretion of the pilus across the outer membrane (Huang et al., 2004). TonB3 has also recently been shown to associate with PocA and PocB, which together form a membrane-associated complex that appears to be required for localisation of the tfp (Cowles et al., 2013).

Pilus extension is mediated by the combined activity of PilZ, FimX, and the ATPase PilB, while the ATPases PilT and/or PilU are responsible for pilus retraction (Alm et al., 1996a, Nunn et al., 1990, Huang et al., 2003, Kaiser, 2000, Merz et al., 2000, Whitchurch and Mattick, 1994, Whitchurch et al., 1991, Burrows, 2005). Recent data suggests that the cytoplasmic membrane protein PilC also interacts with PilT to mediate pilus retraction (Takhar et al., 2013). The mechanism of pili retraction is thought to be mediated by disassembly of the pilus structure by PilT (Kaiser, 2000), which is estimated to occur at a rate of around 1000 pilin subunits/sec (Merz et al., 2000). Pili extension is thought to be mediated by the reverse effect of PilB on the pilin subunits (Whitchurch et al., 1991). Additionally, the disassembly of the tfp structure into individual PilA subunits is thought to allow recycling for the assembly of new tfp (Skerker and Berg, 2001). *P. aeruginosa* PilY1 is homologous to the *N. gonorrhoea* pilin cap protein, PilC, which in this strain prevents complete pilin retraction by PilT (Alm et al., 1996b), however the role of PilY1 in *P. aeruginosa* tfp biogenesis/assembly has not been fully characterised. PilY2 also appears to play a role in tfp biogenesis, however the specifics of this involvement remain unclear (Alm et al., 1996b).

1.7 Regulation of twitching motility

1.7.1. Regulatory components

A number of complex regulatory systems control the biogenesis and assembly of the tfp and regulate its twitching motility function in response to mostly unknown environmental signals. These systems include two component sensor-regulator pairs, the putative Chp chemosensory system, the global virulence factor regulator Vfr, and a large number of other proteins.

1.7.1.1 Two component sensor-regulators

Transcription of *pilA* in *P. aeruginosa* is controlled by the RpoN-dependent two-component sensor-regulator pair, PilS/PilR (Hobbs et al., 1993, Ishimoto and Lory, 1989). PilS is a transmembrane histidine kinase protein located at the cell poles (Boyd, 2000) which, upon detection of a currently unknown environmental signal activates PilR, leading to transcriptional activation of *pilA* (Hobbs et al., 1993). Interestingly, the signal sensed by PilS has been proposed to be PilA itself (Burrows, 2012), however this has not been directly demonstrated. The atypical sensor-regulator pair FimS/AlgR is also required for twitching motility (Whitchurch et al., 1996). Unlike PilS/PilR, this sensor-regulator pair has wildtype cellular

PilA levels, demonstrating a lack of involvement in PilA expression. However, mutants of this regulator pair lack surface assembled pili which suggests a role in tfp biogenesis and assembly (Whitchurch et al., 1996). FimS appears to be incapable of autophosphorylation, and so it has been proposed that FimS phosphorylation occurs by an unknown upstream signalling pathway. Alternatively, or additionally, FimS may be capable of dephosphorylating AlgR, which has been phosphorylated by another pathway (Whitchurch et al., 1996). Interestingly, AlgR but not FimS, is also involved in production of the exopolysaccharide, alginate by mucoid *P. aeruginosa* strains (Whitchurch et al., 1996). However, the role of AlgR in tfp biogenesis and assembly and twitching motility is not mediated via its role in the control of alginate biosynthesis (Whitchurch et al., 2002a) and instead is controlled by the direct activation of the *fimU-pilVWXY1Y2E* operon by AlgR (Belete et al., 2008).

1.7.1.2 The Chp system

The Chp system in *P. aeruginosa* is a highly complex putative chemosensory system which is encoded by the *pilGHIJK-chpABC* gene cluster (Darzins, 1993, Darzins, 1994, Darzins, 1995, Whitchurch et al., 2004). This gene cluster is related to the components of the Che chemosensory signal transduction system in *E. coli* which in this organism is involved in regulation of flagella-mediated chemotaxis in response to chemical attractants and repellents in the environment (Baker et al., 2006, Wadhams and Armitage, 2004). The core signalling components of the Chp system include a putative histidine kinase encoded by *chpA* (Whitchurch et al., 2004) which is likely associated with the inner membrane (Bertrand et al., 2010). ChpA is significantly more complex that its *E. coli* homolog, CheA, as it contains not one but six histidine phosphotransfer (Hpt) domains, as well as two novel serine and threonine-containing phosphotransfer (Spt, Tpt) domains and a CheY-like receiver domain at its C-terminus (Whitchurch et al., 2004). This suggests that ChpA is receiving and integrating multiple inputs and/or transmitting multiple outputs. A *chpA* mutant shows defects in expression of a range of virulence factors, suggesting a role for this protein in integration and regulation of a range of signals during host colonisation (Whitchurch et al., 2004). ChpA is thought to be coupled to a predicted methyl-accepting chemotaxis protein (MCP) receptor, PilJ, by one of two CheW adaptor protein homologues, PilI and ChpC (Whitchurch et al., 2004). As a *chpC* mutant has wildtype twitching motility levels it has been suggested that while PilI links ChpA to PilJ, ChpC may serve to link other MCPs to ChpA, allowing the input of multiple environmental signals into the Chp system (Whitchurch et al., 2004).

It is likely that components of the Chp system associate at the polar regions of the cell in *P. aeruginosa* as the MCP PilJ from *P. aeruginosa*, as well as MCPs in other organisms, have been shown to localise here (Maddock and Shapiro, 1993, Alley et al., 1992, DeLange et al., 2007). The system is thought to receive currently unknown environmental signals which likely results in PilJ undergoing a conformational change, causing ChpA to be autophosphorylated, with the resulting phosphates being transferred to two CheY-like response regulators, PilG and PilH (Darzins, 1993, Darzins, 1994). Phosphorylated PilG is thought to interact with a tfp motor complex made up of PilZ and ATPase PilB, to mediate tfp extension (Guzzo et al., 2009, Bertrand et al., 2010). While the diguanylate phosphodiesterase FimX has previously been thought to associate with the tfp motor complex along with PilZ and PilB based upon observations in *X. campestris* (Guzzo et al., 2009), a recent study suggests that this interaction is unlikely to occur in *P. aeruginosa* (Qi et al., 2012). Conversely, phosphorylated PilH is thought to interact with the ATPases PilT and PilU to mediate tfp retraction (Hahn, 1997, Bertrand et al., 2010). Adaptation to the unknown environmental signal is mediated through methylation of PilJ by the competing activities of the methyltransferase PilK and the methylestrase ChpB (Whitchurch et al., 2004, Darzins, 1995). The Chp system is thought to regulate the motors which control tfp extension and retraction to allow twitching motility to occur (Winther-Larsen and Koomey, 2002) however, it is not known specifically how the Chp system controls twitching motility in response to environmental cues, and what these cues are. A diagram highlighting the features of the Chp system is shown in Figure 1-5.

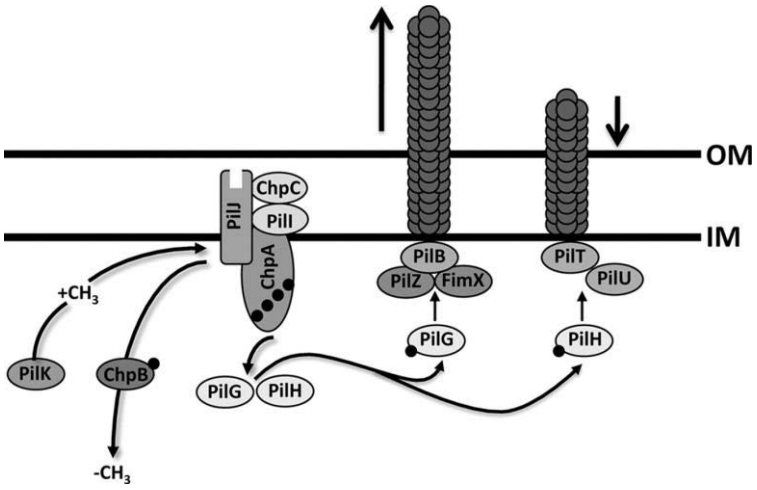

Figure 1-5. Diagram showing the key features of the Chp chemosensory system in *P. aeruginosa*.

The arrows indicate the direction of motion of the Type IV pili. (Adapted from Bertrand et al., 2010).

1.7.1.3 Virulence factor regulator, Vfr

In *P. aeruginosa*, the virulence factor regulator Vfr, is homolgous to the *E. coli* catabolic repressor protein (CRP) (West et al., 1994). Vfr along with its allosteric regulator cAMP, activates expression of *P. aeruginosa* tfp biogenesis and assembly genes (Wolfgang et al., 2003) and so is necessary for twitching motility (Beatson et al., 2002a). Vfr however, does not control expression of the tfp subunit *pilA*, as *vfr* mutants produce wildtype levels of cellular PilA protein (Beatson et al., 2002a). The low levels of surface tfp in *vfr* mutants indicates that Vfr controls twitching motility via expression of components required for assembly of the tfp on the cell surface (Beatson et al., 2002a, Wolfgang et al., 2003). Vfr also controls expression of at least 200 other virulence genes including genes involved in type III secretion systems (T3SS), flagella-mediated motility, and quorum sensing systems (Albus et al., 1997, Dasgupta et al., 2002, Wolfgang et al., 2003).

1.7.1.4 FimL

FimL is necessary for twitching motility, and while *fimL* mutants have wildtype levels of intracellular tfp, surface associated tfp levels are decreased, suggesting a role for FimL in tfp assembly (Whitchurch et al., 2005). FimL is homologous to the N-terminal domain of ChpA, except that the putative histidine and threonine phosphotransfer sites have been replaced with glutamine, indicating that this protein likely works alongside the Chp system (Whitchurch et al., 2005). *fimL* and *vfr* mutants share a number of virulence-associated phenotypes including increased levels of autolysis, reduced levels of surface-assembled tfp, and decreased production of T3SS effectors (Whitchurch et al., 2005). Interestingly, expression of *vfr in trans* is able to complement these virulence-associated phenotypes in a *fimL* mutant and it has therefore been suggested that FimL controls these cellular processes at least in part through regulation of *vfr* expression (Whitchurch et al., 2005). Taken together, these results suggest that FimL and Vfr are components of a common pathway which act to control a number of virulence mechanisms in *P. aeruginosa*.

It has also been observed by Whitchurch *et al.* (2005) that *fimL* mutants quickly revert to wildtype twitching motility phenotypes by the acquisition of an extragenic suppressor mutation(s) that results in an increase in production of icAMP and Vfr. Identification of the currently unknown site(s) of suppressor mutation in these twitching motility revertants would improve our understanding of how cAMP and Vfr interface with FimL and Chp system to regulate twitching motility in *P. aeruginosa*.

1.7.1.5 Polyphosphate kinase, Ppk

Inorganic phosphate (poly P) is found in every cell in nature and has varied biological roles. Poly P, which is synthesised from ATP by the polyphosphate kinase, Ppk was first shown to be important in regulating *Escherichia coli* adaption to nutritional and environmental stresses, as well as for survival in stationary phase bacteria (Kornberg et al., 1999). Ppk has been shown to be involved in twitching motility of *P. aeruginosa* (Rashid and Kornberg, 2000). As *ppk* mutants possess wildtype levels of surface and cell-associated pili it appears that the twitching motility defect arises at the level of tfp regulation. It has been suggested that the observed effect on twitching motility may arise due to involvement of poly P and/or Ppk in phosphotransfer reactions within the Chp system (Rashid and Kornberg, 2000).

1.7.1.6 Catabolic repression control protein, Crc

As mentioned in section 1.4.2.3.1 above, Crc is involved in regulation of carbon source utilisation and sessile biofilm formation of *P. aeruginosa* (O'Toole et al., 2000). A *crc* mutant has decreased levels of *pilA* and *pilB* transcription, as well as decreased levels of surface-associated tfp and twitching motility. Based upon these results, it appears that the *crc* mutant defect in sessile biofilm formation is largely due to a lack of fully functional tfp (O'Toole et al., 2000). It is not clear exactly how Crc nutrient sensing is linked to its role in twitching motility and biofilm formation, however it appears that Crc may be a part of a signal transduction pathway which regulates twitching motility and biofilm formation of *P. aeruginosa* in response to nutritional signals (O'Toole et al., 2000).

1.7.1.7 FimX

FimX is an interesting protein which has a number of apparently varied roles within *P. aeruginosa* cells. This polar protein is required for twitching motility and possesses a CheY-like response regulator domain, DUF1 (or GGDEF) and DUF2 (or EAL) domains, and a PAS-PAC domain (commonly involved in environmental sensing) (Huang et al., 2003). *fimX* mutants have wildtype cellular but reduced surface pilin levels, suggesting a role for FimX in surface assembly of tfp (Huang et al., 2003). The CheY-like domain appears to be involved in bi-polar localisation of tfp and in the uni-polar localisation of FimX within *P. aeruginosa* cells (Huang et al., 2003, Kazmierczak et al., 2006). While not possessing any diguanylate cyclase activity, the GGDEF and EAL domains are required for phosphodiesterase activity of FimX towards c-di-GMP in the presence of guanosine triphosphate (GTP) (Kazmierczak et al., 2006).

FimX also appears to be involved in sensing tryptone and mucin, two environmental signals which stimulate twitching motility of *P. aeruginosa* (Huang et al., 2003) (see more in section 1.7.3.1 below).

1.7.1.8 WaaL

waaL encodes a functional O-antigen ligase in *P. aeruginosa* which has also been shown to be required for twitching motility (Abeyrathne et al., 2005). Transmission electron microscopy (TEM) reveals that *waaL* mutants have decreased levels of surface-assembled tfp (Abeyrathne et al., 2005). While the reduced levels of surface tfp would contribute to the observed twitching motility defect it has also been suggested that the absence of O-antigen in a *waaL* mutant affects surface wettability, contributing the observed decrease in twitching motility (Abeyrathne et al., 2005).

1.7.2 Intracellular signalling molecules

While a number of intercellular signals affect *P. aeruginosa* sessile biofilm formation, including quorum sensing, there are no known intercellular signals which control twitching motility-mediated active biofilm expansion. However, modulation of the levels of a few intracellular signals are involved in controlling this process.

1.7.2.1 Cyclic-AMP

As mentioned above, cAMP is an allosteric activator of Vfr, which controls a wide range of important cellular functions including twitching motility (Wolfgang et al., 2003, Beatson et al., 2002a). Given that a large number of these functions contribute to virulence it is imperative that cAMP homeostasis is tightly controlled. This is achieved by the adenylate cyclases (ACs) CyaA and CyaB, which synthesise icAMP, with the contribution of CyaB being significantly greater than that of CyaA (Wolfgang et al., 2003, Fulcher et al., 2010) and the 3'-5' nucleotide PDE CpdA which serves to reduce the levels of icAMP by hydrolyzing the phosphodiester bond to release the biologically inactive 5'-AMP (Fuchs et al., 2010a). In addition to the direct activity of these enzymes, icAMP levels are also modulated by a number of other proteins. This includes positive regulation of *cpdA* expression by Vfr in response to elevated icAMP (Fuchs et al., 2010a), as well as suggested post-translational modulation of CyaB activity by FimL (Inclan et al., 2011). Additionally, the Chp system is implicated in modulation of icAMP levels based upon observations of reduced icAMP levels in *pilG*, *pilI*,

pilJ, and *chpA* mutants and increased icAMP levels in *pilH*, *pilK* and *chpB* mutants (Fulcher et al., 2010). While modulation of icAMP levels by the Chp system is thought to be involved in controlling tfp production (Fulcher et al., 2010), the connection between icAMP levels and twitching motility is less clear. Some studies have reported a correlation between icAMP levels and twitching motility in *P. aeruginosa* strains PAO1 and PA103 (Inclan et al., 2011), while others have reported cAMP-independent control of twitching motility by the Chp system in *P. aeruginosa* strain PAK (Fulcher et al., 2010). While it is clear that modulation of icAMP levels by a number of regulatory components and systems is important in controlling a number of virulence processes in *P. aeruginosa*, including tfp production and twitching motility, a great deal still remains to be uncovered regarding the nature of the interactions between these regulatory components and their specific contribution to virulence.

1.7.2.2 Cyclic-di-GMP

As discussed in section 1.4.2.2.1 above, the role of the second messenger c-di-GMP in sessile biofilm formation has been well-studied, revealing that an increase in c-di-GMP levels stimulates sessile biofilm formation, while a decrease is associated with swimming motility (Simm et al., 2004). How c-di-GMP specifically affects twitching motility is less clear, however there are two proteins required for twitching motility and tfp biogenesis, FimX and PilZ, which appear to have some role in these processes. As mentioned above, FimX possesses some c-di-GMP PDE activity in the presence of GTP (Kazmierczak et al., 2006) and appears to be involved in the twitching motility response to environmental signals (Huang et al., 2003). The non-catalytic EAL domain binds to c-di-GMP with high affinity, which results in a conformational change in the N-terminal CheY domain of FimX which is responsible for polar localisation of FimX (Qi et al., 2011, Navarro et al., 2009). This has been suggested to allow linkage between sensing of extracellular cues and intracellular c-di-GMP levels to modulate *P. aeruginosa* twitching motility (Qi et al., 2011). When PilZ was identified in *P. aeruginosa* and shown to be involved in tfp biogenesis (Alm et al., 1996a) it had a novel sequence. Subsequently, homologous PilZ domains have been identified in a large and diverse family of proteins, and designated as a c-di-GMP binding adapter domain (Amikam and Galperin, 2006). Ironically, unlike the majority of proteins in this family, *P. aeruginosa* PilZ does not bind c-di-GMP which appears to be due to critical structural differences in the N-terminal c-di-GMP binding region of the *P. aeruginosa* PilZ protein (Merighi et al., 2007, Li et al., 2009). Studies in *Xanthomonas* spp. demonstrate that PilZ interacts with both PilB and the non-functional EAL domain of FimX, suggesting that together, these three proteins may regulate PilB function

and thus twitching motility in these bacterial species in a c-di-GMP-dependent manner (Guzzo et al., 2009). As mentioned in section 1.7.1.2 PilZ and FimX do not appear to associate with one another in *P. aeruginosa* (Qi et al., 2012). Thus, while intracellular c-di-GMP levels and c-di-GMP-related proteins clearly have a role in environmental sensing and twitching motility in *P. aeruginosa*, a large number of questions still remain regarding the proteins involved, as well as the specific role of this second messenger in twitching motility.

1.7.3 Extracellular signals

The regulatory systems and proteins described above are all thought to be involved in either sensing environmental signals and/or in linking the environmental sensing domains of these systems to the components and small signalling molecules which modulate twitching motility in *P. aeruginosa*. While the role of a number of these proteins in tfp biogenesis and assembly is well characterised, what remains to be further elucidated is how these regulatory systems interface with one another to detect changes in the environment and mediate changes in tfp expression and/or twitching motility function. Given that a number of these proteins possess sensing domains and/or are a part of a putative chemosensory system, it is surprising that only a small number of environmental signals have been identified which affect twitching motility. As described below these signals originate from both exogenous and from endogenous sources.

1.7.3.1 Exogenous signals which affect twitching motility

At a uniform concentration within the growth media mucin, bovine serum albumin (BSA) and tryptone stimulate twitching motility, while NaCl, KCl, KNO_3, sucrose, glucose, polyvinylpyrrolidone and high osmolarity conditions have an inhibitory effect (Huang et al., 2003). The cellular components that detect these compounds are currently unknown, however, as mentioned above it appears that FimX may be involved in responding to mucin and tryptone (Huang et al., 2003). The relevance of a number of these signals in controlling active biofilm expansion within an infection setting is discussed below.

1.7.3.1.1 Oligopeptides

As described in section 1.2.2 above, *Pseudomonas aeruginosa* is commonly implicated in chronic UTIs in patients with indwelling urinary catheters (Mittal et al., 2009). The formation of biofilms along the length of these catheters is well documented, with active biofilm expansion implicated in colonisation along the length of these devices (Stickler, 2008,

Donlan, 2001b, Sabbuba et al., 2002). Human urine is a complex mixture of organic and inorganic constituents, which includes a large number of oligopeptides (Kentsis et al., 2009, Putnam, 1971, Lutz et al., 1972). An increase in twitching motility has been reported when *P. aeruginosa* is exposed to high levels of tryptone (Huang et al., 2003). As tryptone is an assortment of oligopeptides this suggests that the observed twitching motility response of *P. aeruginosa* to tryptone may be relevant in active biofilm expansion along the length of urinary tract catheters.

1.7.3.1.2 Mucin

As described in section 1.4.2.3.2 above, mucin glycoproteins have an important role in the host immune response to infectious agents (Linden et al., 2008). Mucin has been shown to stimulate *P. aeruginosa* twitching motility (Huang et al., 2003) however, tfp are not required for binding mucin (Ramphal et al., 1991). Instead it appears that FliD, the flagella cap protein, is involved in mucin adhesion (Ramphal, 1999). It has also been shown that both *P. aeruginosa* flagella and tfp bind to the host cell glycolipid asialoGM1 (ASGM1), with the binding of flagella stimulating expression of mucin-encoding genes in epithelial cells (McNamara and Basbaum, 2001, Comolli et al., 1999). The effect of tfp binding to ASGM1 on mucin production has not been investigated. In any case, host production of mucin in conjunction with mucociliary clearance, acts to remove infecting pathogens (Linden et al., 2008). As mentioned above, mucin has been shown to both stimulate and inhibit *P. aeruginosa* sessile biofilm formation however, the inhibitory effect demonstrated by Caldara et al., (2012) is likely to more accurately reflect the *in vivo* role of mucin in sessile biofilm formation (Caldara et al., 2012, Landry et al., 2006). Thus, in an infection setting, where high levels of mucin are present, *P. aeruginosa* sessile biofilm formation is likely to be inhibited, and active biofilm expansion stimulated, which may accelerate *P. aeruginosa* surface colonisation of infected tissue (Huang et al., 2003). This effect may be even more pronounced within CF patient lungs due to the overproduction of mucin (Li et al., 1997).

1.7.3.1.3 Phosphatidylcholine

Phosphatidylcholine (PC) is a major constituent of animal membranes, and a minor constituent of some bacterial membranes (Geiger et al., 2013). Phopholipases C (PLCs), which degrade PC, are widespread in prokaryotic organisms and have diverse biological roles. For instance, the PLC from *Listeria monoctyogenes* contributes to cell-to-cell spread of this pathogen in infected eukaryotic cells (Marquis et al., 1997), while in *Clostridium perfringens*

a homologous PLC, the α toxin, is the major cytotoxic agent that contributes to gas gangrene (Awad et al., 1995). In *P. aeruginosa* PC degradation by the phospholipase C PlcB releases diacylglycerol (DAG). The long-chain fatty acid (LCFA) moiety of DAG has been shown to affect twitching motility of *P. aeruginosa*, with motility occurring up PC gradients (Barker et al., 2004). Interestingly, the levels of PC within the lungs of CF patients are increased compared to healthy individuals (Meyer et al., 2000), which suggests that PC-stimulated active biofilm expansion may be important in *P. aeruginosa* CF lung colonisation.

1.7.3.1.4 Serum Albumin and Lactoferrin

BSA has been shown to stimulate twitching motility of *P. aeruginosa* (Huang et al., 2003). More recently, adult human serum (AHS) and adult bovine serum (ABS) have been shown to inhibit *P. aeruginosa* sessile biofilm formation on plastic surfaces and intravenous catheters, with some of this inhibition being attributed to the action of serum albumin (Hammond et al., 2010). This same study also demonstrated that ABS and BSA stimulated twitching motility, in agreement with the results of Huang et al. (2003) for BSA. Hammond et al., (2010) suggest that the inhibition of ABS and BSA on sessile biofilm formation is in part due to the stimulatory effect that both of these components have on twitching motility. A similar effect has been also observed for lactoferrin from human serum (Singh et al., 2002). Singh et al., (2002) demonstrate that lactoferrin inhibits *P. aeruginosa* biofilm formation by chelating free iron. The opposite effect was observed on twitching motility - as levels of free iron decreased, twitching motility increased (Singh et al., 2002). While it is unknown how *P. aeruginosa* senses these serum components and/or iron levels to mediate the observed biofilm and twitching motility effects, the results of these three studies suggest important implications for pathogenesis and the host immune response. Albumin, lactoferrin, and potentially other serum components, may provide a protective effect for the host by inhibiting *P. aeruginosa* sessile biofilm formation and stimulating twitching motility-mediated biofilm expansion on mucosal surfaces. It has been suggested that by inhibiting the formation of biofilms the host immune system may then have a better chance of removing the invading bacteria (Singh et al., 2002). Interestingly, there is evidence to suggest that lactoferrin may be inactivated by proteolytic cleavage within CF patient lungs (Britigan et al., 1993) which may also contribute to *P. aeruginosa* pathogenesis in CF lung infections (Singh et al., 2002).

1.7.3.2 Endogenous signals which affect twitching motility

Another source of signals that control twitching motility-mediated active biofilm expansion which has not been widely considered is from within *P. aeruginosa* biofilms themselves. The AHL and 4-quinolone signalling molecules produced by the quorum sensing system of *P. aeruginosa* are important in sessile biofilm formation (Davies, 1998) however, these same molecules are not involved in controlling twitching motility (Beatson et al., 2002b). At present, only one endogenously produced signalling molecule has been shown to control twitching motility-mediated biofilm expansion of *P. aeruginosa*. Phosphatidylethanolamine (PE) is the main phospholipid in bacterial cell membranes (Geiger et al., 2013). As is the case for PC (section 1.7.3.1.3), *P. aeruginosa* exhibits directional twitching motility towards the LCFA moiety of PE purified from its own membrane (Kearns et al., 2001, Miller et al., 2008). At present, no other endogenously produced molecules have been shown to control twitching motility of *P. aeruginosa*.

1.8 The role of twitching motility in sessile biofilm formation

While twitching motility is clearly important in *P. aeruginosa* twitching motility-mediated biofilm expansion, twitching motility is also involved in a number of the stages of sessile biofilm formation. Time lapse microscopy studies by O'Toole et al., (1998) first demonstrated that twitching motility of strain PA14 was involved in microcolony formation, which occurs in the early stages of sessile biofilm formation. Subsequent longer term studies of sessile biofilm formation within a flow system uncovered more detail on the involvement of twitching motility in this process. Time lapse confocal laser scanning microscopy (CLSM) with fluorescently labelled *P. aeruginosa* wildtype and non-twitching mutants was used to investigate the involvement of twitching motility in sessile biofilm formation within a flow system, over a longer time frame (Klausen et al., 2003a). This study revealed that the mushroom structures, characteristic of mature biofilms, formed in a sequential process. This process was initiated by clonal growth of a non-motile subpopulation to form the mushroom stalk, after which motile bacteria climbed the stalks via twitching motility to form the mushroom cap structure. The Chp system appears to play a minor role in these tfp-mediated stages of sessile biofilm formation, as *pilH* and *chpA* mutants showed some defects in cap formation compared to wildtype (Barken et al., 2008).

1.9 Thesis aims

P. aeruginosa is an important opportunistic pathogen which causes serious and often chronic infections in immunocompromised individuals and those with cystic fibrosis. The chronic nature of infection is associated with the formation of biofilms, which represent a protected mode of life for bacteria. *P. aeruginosa* is able to form sessile biofilms in hydrated settings and is also able to undergo twitching motility-mediated biofilm expansion within semi-hydrated environments. A number of complex regulatory systems, as well as small intra and intercellular signals, and environmental signals are involved in controlling the formation of both of these kinds of biofilms. Twitching motility is a form of tfp-mediated surface translocation which is involved in some of the stages of sessile biofilm formation and in twitching motility-mediated biofilm expansion. In *P. aeruginosa* this form of motility is regulated by a number of complex systems which translate changes in levels of extracellular signals into alterations in twitching motility-mediated biofilm expansion, however the environmental signals that these systems respond to are mostly unknown. Additionally, how each of these regulatory systems interact with one another to mediate changes in twitching motility is complex and not well understood.

The overall aim of this Thesis is to gain insight into the intracellular and extracellular signals which control twitching motility, and to better understand the interactions between the systems which sense and respond to these signals. Chapter 3 of this Thesis describes the characterisation of a number of *fimL* mutants, which had previously been reported to have regained some twitching motility ability via acquisition of unknown suppressor mutation(s) (Whitchurch et al., 2005). This chapter presents results which identify one site of suppressor mutation, and demonstrate that an increase in icAMP levels is a general mechanism of twitching motility reversion which occurs via at least two distinct mechanisms. Overall these results shed light on the complex interplay between icAMP levels, FimL and twitching motility in *P. aeruginosa*. Chapter 4 of this Thesis characterises the effect of extracellular ATP (eATP) on *P. aeruginosa* twitching motility-mediated biofilm expansion. Chapter 5 goes on to demonstrate that eATP also affects sessile biofilm formation. The results presented suggest an important role for eATP in *P. aeruginosa* biofilm formation and expansion within an infection setting. Chapter 6 presents results on the identification of a novel extracellular signal, *N*-acetylglucosamine, which stimulates *P. aeruginosa* twitching motility. This chapter also provides a detailed characterisation of the twitching motility response of *P. aeruginosa* to the

host-derived signals mucin, albumin and oliopeptides and links the observed twitching motility response to the Chp chemosensory system as well as to modulation of icAMP levels.

Chapter Two

General Materials and Methods

2.1 Media and Buffers

2.1.1 ELISA Coating buffer

ELISA coating buffer was prepared by dissolving 15 mM Na_2CO_3 (Sigma Aldrich) and 35 mM of $NaHCO_3$ (Sigma Aldrich) into Baxter's water (Baxter Healthcare, Pty. Ltd.) and the pH adjusted to 9.6 with 1 M HCl. The solution was then filter sterilised with a 0.22 µm membrane filter (Millipore Ltd.) and stored at 4 ºC for no longer than 2 weeks.

2.1.2 Na_2EDTA

A 0.5 M solution of EDTA disodium salt dihydrate (Na_2EDTA, Sigma-Aldrich) was prepared by mixing 0.5 M of the solid in deionised water (diH_2O) and then raising to pH 9 by addition of NaOH pellets, allowing the solid to dissolve, before lowering the pH to 8 with 10 M HCl. The solution was sterilised in an autoclave at 121 ºC and 15 kPA for 20 min (unless otherwise stated, all autoclave settings will remain the same as those stated here), and stored at room temperature.

2.1.3 Tris Borate EDTA (TBE)

A 10x stock solution of TBE was prepared by dissolving a premixture of 0.89 M tris base, 0.89 M boric acid and 0.02 M 0.5 M EDTA (Medicago AB) in diH_2O. The solution was autoclaved and stored at room temperature. A 0.5x working solution was prepared by diluting the stock with diH_2O, then stored at room temperature.

2.1.4 Phosphate Buffered Saline (PBS) and PBS Tween 20

PBS was prepared by dissolving a premixture of 0.14 M NaCl, 0.03 M KCl and 0.01 M phosphate buffer, at pH 7.4 (Medicago AB) in diH_2O. The solution was then sterilised by autoclaving and stored at room temperature. PBS Tween 20 (PBST) was prepared by the addition of Tween 20 (0.05% v/v) to PBS after autoclaving. The solution was stored at room temperature.

2.1.5 Tris-Cl

A 1 M solution of Tris-Cl was made by dissolving 121.1 g/L of Tris base in diH_2O and adjusting the pH to 6.8, 7.5, 8 or 8.2 with 10 M HCl. The solution was then autoclaved and stored at room temperature.

2.1.6 SDS PAGE loading buffer

SDS PAGE loading buffer for use in whole cell and surface Westerns was prepared by combining 2.4 mL of 1 M Tris-Cl (pH 6.8) (section 2.1.5), 3 mL of sodium dodecyl sulphate (SDS) (20 %) (Sigma-Aldrich) 3 mL glycerol (100 %) (Amresco LLC) 1.6 mL 2-mercaptoethanol (100%) (Sigma-Aldrich) 0.006 g bromophenol blue (Sigma-Aldrich) and adding diH_2O to a final volume of 10 mL. The solution was stored at 4 ºC.

2.1.7 NuPAGE® MES SDS running buffer for Westerns

20x NuPAGE® MES SDS running buffer (Life Technologies Corporation) was diluted to 1x prior to use in SDS PAGE in MilliQ (MQ) water. The buffer was used immediately.

2.1.8 Tris-EDTA (TE) buffer

A 1 M solution of Tris-Cl (prepared as in section 2.1.5) and a 0.5 M solution of EDTA (prepared as in section 2.1.2) were diluted in 100 mL of diH_2O so that the final concentrations of Tris-Cl and EDTA were 10 mM and 1 mM, respectively. The solution was adjusted to pH 8 with 10 M NaOH, the solution autoclaved, and stored at room temperature.

2.1.9 Sodium chloride solution

A 6 M saturated solution and a 5 M solution of NaCl was prepared by mixing, respectively, 350.64 g/L or 292.2 g/L of NaCl (Sigma-Aldrich) with diH_2O. The solution was autoclaved and stored at room temperature.

2.1.10 Bacterial lysis solution

Bacterial lysis solution was prepared by mixing 1 M Tris-Cl (as prepared in section 2.1.5), 0.5 M Na_2EDTA (as prepared in section 2.1.2) and 6 M NaCl (as prepared in section 2.1.9) with diH_2O so that the final concentrations were 10 mM, 2 mM and 400 mM, respectively. The solution was autoclaved and stored at room temperature.

2.1.11 Proteinase K storage solution

Storage solution for Proteinase K was prepared by combining 1 M Tris-Cl at pH 7.5 (as prepared in section 2.1.5), 6 M NaCl (prepared in section 2.1.9), 0.5 M Na_2EDTA (as prepared in section 2.1.2), 1 M $CaCl_2$ (Sigma-Aldrich) and 1 M Dithiothreitol (DTT) so that the final concentrations were 50 mM, 100 mM, 0.1 mM, 10 mM and 1 mM, respectively, also with the

addition of glycerol (50% v/v) and Triton X-100 (0.1%, Sigma-Aldrich). Proteinase K stock was made up at a concentration of 10 mg/mL in storage solution and stored at -20 ºC.

2.1.12 Low salt Luria-Bertoni (LB) Broth and Agar (LBA)

Low salt LB was composed of 10 g/L of Tryptone (Oxoid), 5 g/L of yeast extract (Oxoid) and 5 g/L NaCl dissolved in diH_2O, autoclaved and then cooled before use. LBA was made with the addition of 1.5% w/v bacteriological grade agar (Oxoid) which was cooled to 55 ºC before the addition of heat labile additives such as antibiotics and then poured into 92 mm Petri dishes (Sarstedt, Nümbrecht, Germany).

2.1.13 Base Media (BM) Broth and Agar (BMA)

Base media was composed of 5 g/L of yeast extract (Oxoid) and 5 g/L NaCl dissolved in diH_2O, autoclaved and then cooled before use. BMA was made with the addition of 1.5% w/v bacteriological grade agar (Oxoid) which was cooled to 55 ºC before the addition of heat labile additives such as antibiotics and then poured into 92 mm Petri dishes (Sarstedt, Nümbrecht, Germany).

2.1.14 SOB media

SOB media was prepared by combining 20 g/L of Tryptone (Oxoid), 5 g/L of yeast extract (Oxoid) and 0.5 g/L NaCl dissolved in diH_2O, with the pH being set to 7.5 by addition of KOH. The media was then sterilised by autoclaving and cooled before use. $MgSO_4$ and $MgCl_2$ in MQ water, which had been filter sterilised (0.22 μm membrane filter), were added at a final concentration of 10 mM and 4.6 mM, respectively to the media post autoclaving. The media was then stored at room temperature.

2.1.15 RF1 and RF2 buffer

RF1 was prepared by dissolving 0.1 M RbCl (Sigma-Aldrich), 0.06 M $MnCl_2.2H_2O$ (Sigma-Aldrich), 0.03 M KOAc (Sigma-Aldrich), 0.02 M $CaCl_2.2H_2O$ (Sigma-Aldrich) and 7 % w/v glycerol in MQ water, with the pH being adjusted to 5.8 with 2 M acetic acid. The solution was then sterilized though a 0.22 μm filter and stored at 4 ºC.

RF2 was prepared by dissolving 5.3 mM MOPS (Amresco LLC), 8.3 mM RbCl, 0.07 M $CaCl_2.2H_2O$, 7 % w/v glycerol in MQ water, and the pH adjusted to 6.8 with 10 M NaOH. The solution was then sterilized though a 0.22 μm filter and stored at 4 ºC.

2.1.16 Difco™ *Pseudomonas* Isolation Agar F (PIA)

PIA (Difco, BD Biosciences) was made according to the manufacturer's instructions. In brief, 45 g/L of powder was mixed with diH_2O and 0.02% v/v glycerol. This was then autoclaved, cooled to 55 ºC and then poured into plastic 92 mm Petri dishes and allowed to set at room temperature.

2.1.17 LBA with 5% Sucrose

LBA with 5% sucrose (Sigma-Aldrich) was prepared for use in the *P. aeruginosa* conjugation to counter-select strains with vectors containing the *sacB* gene. This media was made by preparing a stock solution of 0.73 M sucrose, which was filter sterilized using a 0.22 μm pore size filter. LBA was prepared as described previously except the NaCl was omitted and the volume adjusted to 80% of the final volume. After the media had cooled to 55 ºC, sucrose stock solution was added so that the final concentration of sucrose was 0.15 M (20% of the final volume). The media was poured into 150 mm Petri dishes (BD Falcon®) and cooled overnight.

2.1.18 Cation-Adjusted Mueller Hinton Broth (CAMHB)

21 g/L of Mueller-Hinton Broth powder (Oxoid) was dissolved in diH_2O. The cations in the powder varied depending on the batch. Using the analysis breakdown on the package, 1 M $CaCl_2$ (Sigma-Aldrich) was added so that the final Ca^{2+} concentration was between 20–25 mg/L and 1 M $MgCl_2$ (Sigma-Aldrich) was added so that the final Mg^{2+} concentration was between 10–12.5 mg/L. The solution was autoclaved then allowed to cool before use and stored at room temperature.

2.1.19 Gellan Gum-solidified nutrient media

Gellan gum-solidified nutrient media was prepared by addition of 4 g/L of Tryptone (Oxoid), 2 g/L of yeast extract (Oxoid), 2 g/L NaCl and 1 g/L $MgSO_4.7H_2O$ (Sigma-Aldrich) and dissolved in diH_2O. The solution was then heated and stirred on a magnetic stirrer and 8 g/L Gellan Gum (MP Biomedicals) was added slowly to ensure it completely dissolved. Upon boiling and becoming clear the media was sterilised by autoclaving and stored at room temperature.

2.1.20 Gellan Gum-solidifed base media

Gellan gum-solidified BM was prepared as for the gellan gum-solidified nutrient media (section 2.1.19), however without the addition of tryptone. Gellan gum solidified BM supplemented with mucin (0.05 % (w/v)) (Sigma-Aldrich) and tryptone (3 % (w/v)) (Oxoid) was also prepared in the same manner as gellan gum-solidified base media. Gellan gum solidified BM supplemented with bovine serum albumin (BSA) (Research Organics, USA) was prepared as follows: BSA (3.5% (w/v)) was dissolved in BM, filter sterilised (0.22 μm membrane filter, Millipore Ltd) and added to autoclave-sterilised gellan-gum solidified BM at a final concentration of 0.1% (w/v).

2.1.21 Twitching Assay Agar

LBA and BMA were made as indicated in section 2.1.12 and 2.1.13 with the exception of 4 g/L of bacteriological agar. Exactly 10 mL were added to 92mm plastic Petri dishes and allowed to set.

2.2 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in the materials and methods sections for each results chapter.

2.3 Bacterial culture conditions

2.3.1 Storing and reviving bacteria

Overnight broth cultures of bacterial were mixed with 15% v/v glycerol (Amresco LLC) and stored at −80 ºC. Bacteria were revived from the frozen glycerol stock by streaking some of the frozen culture onto an LB agar plate (supplemented with appropriate antibiotics) and incubating at the appropriate temperature overnight.

2.3.2 *E. coli* culture conditions

E. coli strains were cultured in LB. Cultures were incubated overnight at 37 °C shaking at 250 r.p.m. After being cooled to 55 °C, media was supplemented where appropriate with antibiotics at the following concentrations: 50 μg/mL ampicillin (Astral Scientific), 50 μg/mL

kanamycin sulphate (Astral Scientific), 10 μg/mL gentamicin sulphate (Sigma-Aldrich) and 5 μg/mL tetracycline hydrochloride (Sigma-Aldrich).

2.3.3 *P. aeruginosa* culture conditions

All *P. aeruginosa* strains were cultured in 2 mL of LB or CAMHB in 14 mL loosely capped polypropylene tubes (BD Falcon®) and cultured at 37 °C overnight in an orbital shaker at 250 r.p.m. For phenotypic assays where larger volumes (up to 10 mL) of culture were needed, the strain was inoculated in a 50 mL tube (BD Falcon®). The tube was then sealed in Aeraseal™ (Excel Scientific Inc., Victorville, CA, USA) to allow for sufficient air flow, and cultured at 37 ºC shaking at 250 r.p.m. Where appropriate, media was supplemented with antibiotics at the following concentrations: 200 μg/mL carbenicillin (Sigma-Aldrich), 100 μg/mL tetracycline hydrochloride, 100 μg/mL gentamicin sulphate.

2.3.4 Preparation of competent *E. coli* cells

E. coli competent cells were prepared as follows: *E. coli* was inoculated into 4 mL SOB media (as prepared in section 2.1.14) and grown overnight at 37 ºC, with shaking at 250 r.p.m. These overnight cultures were then added to 500 mL of fresh SOB media and grown at 37 ºC, with shaking at 250 r.p.m for 3 hr. The cultures were then divided into 50 mL aliquots and incubated on ice for 30 min. The cells were then pelleted by centrifugation (3, 000 *g* for 10 min at 4 ºC). Each pellet was resuspended in 8 mL RFI buffer (as prepared in section 2.1.15) by swirling, and all resuspended cells pooled and left on ice for 15 min. The pooled cells were then pelleted by centrifugation (3, 000 *g* for 10 min at 4 ºC) and the pellet resuspended in 20 mL RF2 buffer (as prepared in section 2.1.15). The resuspended cells were left on ice for 15 min and 500 μl aliquots transferred into 1.5 mL eppendorf tubes. These aliquots were snap frozen in a dry ice/95 % ethanol bath and stored at -80 ºC. The transformation frequency was calculated for competent cells using the equation:

Transformation frequency = (# of colonies/pg plasmid DNA) x (10^6 pg/μg) x (1000 μL/μL cells plated) x (dilution factor)

2.3.5 Preparation of *P. aeruginosa* competent cells

2.3.5.1 Electrocompetent cell preparation

P. aeruginosa electrocompetent cells were prepared as follows: Overnight 10 ml CAMHB cultures of *P. aeruginosa* were harvested by centrifugation at 3,000 *g* for 10 min at 4 °C. The cell pellet was washed 4 times in 1 ml of ice-cold 300 mM sucrose (Sigma-Aldrich) and finally resuspended in 200 µl of ice-cold 300 mM sucrose. 300 ng of plasmid DNA was added to 80-100 µl of electrocompetent cells, incubated at room temperature for 5 min, and then transferred to a 1 mm gap-width electroporation cuvette. After applying a pulse (settings: 25 µF; 200 Ω; 2.5 kV on a BTX 600 Electro Cell Manipulator; Harvard Apparatus Inc.) the cells were added to 1 ml of CAMHB with 0.2 % glucose and incubated at 37 °C, shaking, for 1 h and then plated onto LB agar containing appropriate antibiotic selection.

2.4 Molecular Biology techniques

2.4.1 Genomic DNA preparation

500 µL of an overnight *P. aeruginosa* culture was pelleted by centrifugation (16, 200 *g* for 3 min at room temperature). 1 µL of 50 µg/mL Proteinase K was added to 300 µL of lysis solution (prepared as in section 2.1.10) and used to resuspend the pelleted cells. This was incubated at 65 ºC for 45 min with vortexing every 5 min. After incubation, 500 µL of 6 M NaCl (prepared in section 2.1.9) was added to the cell lysate which was then vortexed well. The cell lysate was then centrifuged (10, 000 *g* for 10 min at 4 ºC). The supernatant volume was then transferred to an eppendorf tube and combined with 2x volume of isopropanol (Sigma-Aldrich) with the contents of the tube mixed by 40 rounds of inversion. The DNA was pelleted by centrifugation (10, 000 *g* for 10 min at 4 ºC) and the pellet then washed twice with 75 % ethanol (Sigma-Aldrich). The washed pellet was then resuspended in TE buffer (as prepared in section 2.1.8).

2.4.2 Oligonucleotides

Oligonucleotides were purchased from Invitrogen (Life Technologies Coorporation) and were reconstituted with Baxter's H_2O to a final concentration of 100 mM and stored at -20 °C. Oligonucleotides for PCR were thawed and made to a concentration of 10 mM with Baxter's H_2O and stored at -20 °C. Oligonucleotides used in this study are listed in Table 2-1.

2.4.3 Polymerase chain reaction (PCR)

Primers for flanking regions for *cpdA, cyaA, cyaB* and *pilGH* were designed using MacVector (Oxford Molecular Group) (see Table 2-1) and amplified by PCR using 2.5 U of *Pfu Turbo* DNA Polymerase (Agilent Technologies), 1x *Pfu Turbo* buffer, 0.2 mM dNTPs (purchased as individual nucleotides from Promega from which a 5 mM mix was made and stored frozen at -20 ºC), dimethyl sulfoxide (DMSO) (1.5% v/v, Sigma-Aldrich) and 0.2 µM of each primer (as prepared in section 2.4.2). PCR reactions were performed on an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) using the following cycling conditions: An initial 95 ºC for 2 min, followed by 30 cycles of 95 ºC for 30 sec; annealing temperature as indicated in Table 2-1 for 30 sec; extension at 72 ºC for 1 min/kb. A final extension at 72 ºC for 10 min followed. The products were stored at 4 ºC.

Table 2-1. Relevant characteristics of oligonucleotides used in this Thesis.

Oligo	Sequence (5' – 3')	Description	Annealing Temperature
cpdA_F	CATCGGGAACGGGCTAATG	Amplify coding and upstream region of *cpdA*, producing a 1.3 kb product	57 ºC
cpdA_R	GTAGACCCGCACTTCCAGCC		57 ºC
cyaA_F	CTGAGCGGACGGAAAGTC	Amplify coding and upstream region of *cyaA*, producing a 3.1 kb product	55 ºC
cyaA_R	CAGCGAGCAGGGTAATAC		55 ºC
cyaB_F	GCATCGGTCTCTTCTTGTTC	Amplify coding and upstream region of *cyaB*, producing a 1.6 kb product	62 ºC
cyaB_R	GTTTCGGCGGAGGAGTTC		62 ºC
pilGH_F	TCCGGGCATTATGGATAGAG	Amplify coding and upstream region of *pilGH*, producing a 1.3 kb product	55 ºC
pilGH_R	AACCGCAGAGGTCCATGAT		55 ºC

2.4.5 PCR clean up

PCR cleanup was performed using a HiYield™ Gel/PCR DNA mini kit (Real Biotech Corporation). Cleanup was performed as per the manufacturer's instructions, and products stored in the supplied TE buffer at 4 °C.

2.4.6 Isolation of DNA from agarose gels

DNA fragments were isolated by running products (as described below in section 2.5) on a 1% agarose gel. Fragments were then visualised with a UV light source and excised with a scalpel. DNA was extracted from the gel using a HiYield™ Gel/PCR DNA mini kit (Real Biotech Corporation) as per the manufacturer's instructions.

2.4.7 PCR product cloning

To ligate PCR products into the pGEM-T-Easy vector (Promega), a 3' non-template A tail was generated by incubating 7 µL of PCR product with 1 µL 10× Taq buffer, 1 µL 2 mM dATP and 1.25 U of Taq DNA polymerase at 70 ºC for 30 min. The A-tailed product was then ligated into pGEM®-T Easy Vector using the pGEM®-T Easy Vector System 1 (Promega) according to the manufacturer's instructions.

2.4.8 Restriction endonuclease digestion

Restriction enzymes were purchased from New England Biolabs and stored at −20 ºC. All reactions were incubated at 37 ºC for 2−20 h. Each reaction consisted of 2 µL of 10× buffer, 1 µL of enzyme, 2 µL of 1 mg/mL BSA (if required) and 1 µg of DNA and the volume was made up to 20 µL using diH_2O. The digest was run on an agarose gel (see section 2.5) to test for complete digestion before the enzyme was inactivated. If a double digest was performed, 1 µL of both enzymes was added to the reaction and a compatible buffer chosen.

2.4.9 Isolation of plasmid DNA

Plasmids were isolated from overnight cultures using the QIAprep® Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. Plasmid DNA was eluted in TE buffer and stored at 4 °C.

2.4.10 Antarctic phosphatase treatment and ligation

To prevent cut vectors from re-ligating, the cut plasmid vectors were treated with Antarctic phosphatase (New England Biolabs). Up to 1 µg of cut vector DNA, 5 µL of 10× Antarctic phosphatase buffer and 5 U of Antarctic phosphatase was made to a volume of 50 µL with sterile diH_2O. The reaction was incubated for 30 min at 37 ºC and then heat inactivated at 65 ºC for 30 min.

Ligations were performed using a T4 DNA ligase kit (New England Biolabs) using 10−20 ng of vector DNA and 20−40 ng of insert DNA in a total volume of 20 µL. To this volume, 2 µL of 10× ligase buffer, and 1 µL (2 U) of ligase enzyme were added. The reaction was incubated for 1 hr at room temperature or at overnight at 4 ºC.

2.5 Agarose gel electrophoresis

Nucleic acids were visualised by agarose gel electrophoresis. Agarose gels (1% w/v) were made with biotechnology grade agarose 1 (Amresco LLC) and melted in 0.5× TBE. After the gel had cooled to 50 °C, 1 U/mL of Gel Red™ Nucleic Acid Gel Stain (Gel Red, Biotium) was added.

Gels were submerged in 0.5× TBE and samples were loaded with bromophenol blue loading dye (2.5 g/L bromophenol blue (Sigma-Aldrich), 400 g/L sucrose (Sigma-Aldrich), in diH_2O). Gels were visualised using a Kodak EDAS 290 Electrophoresis Documentation and Analysis System with a DC290 Camera (Eastman Kodak Company).

2.6 DNA Sequencing and sequence analysis

DNA sequences were obtained by PCR amplification of the region of interest from the *P. aeruginosa* PAO1 genome or cloning of the amplicon into pGEM-T (Promega Corporation). Automated DNA sequencing of the PCR amplicon or pGEM-T clones was performed by the Australian Genome Research Facility (University of Queensland, Brisbane, Queensland, Australia) and Macrogen Inc. (Seoul, Korea) using BigDye terminator chemistries. Oligonucleotides for amplifying regions of the *P. aeruginosa* genome were designed with MacVector Primer 3 (Oxford Molecular Group) using sequences obtained from Pseudomonas.com (Winsor et al., 2011). Nucleotide and predicted protein sequences were analysed using MacVector (Oxford Molecular Group).

2.7 Antibodies

The antibodies used in this Thesis were a polyclonal primary antibody raised in rabbit against PAK PilA (α-286) (Whitchurch laboratory collection) and an anti-rabbit IgG-alkaline phosphatase secondary antibody (Sigma-Aldrich).

2.8 PilA protein quantification assays

2.8.1 Enzyme-linked immunosorbent assays (ELISA)

100 µL of overnight cultures (see section 2.3.3) of *P. aeruginosa* strains were spread plated onto agar plates (LBA or BMA as indicated, see section 2.1.12 and 2.1.13) and incubated overnight at 37 °C. Cells were then harvested from the confluent lawns and resuspended in 10 mL coating buffer (section 2.1.1) to an OD_{600} ± 0.01 OD units. 1 mL of this resuspension was used for whole cell Western blot analyses (see section 2.8.2.1 below). 200 µL of the resuspension was dispensed into 8 wells (1 column) of an ELISA plate (MaxiSorb Immunoplate, Nunc). Coating buffer was also added into 2 columns of each ELISA plate as a negative control. Plates were covered with ELISA sealing tape (Excel Scientific Inc.) and incubated at 4 °C overnight. Cells were removed by vigorously tapping the ELISA plate into a beaker, followed by 2 washes of all wells with PBST (200 µL/well). 200 µL of BSA (3% in PBST) was added to all wells, the plate covered with ELISA sealing tape, followed by incubation at 37 °C for 1 hr. The plate was then washed 3 times with PBST (200 µL/well) and 100 µL of PBST dispensed into each well (except for the 1st row of the plate). 200 µL of anti-PAK PilA antisera (1/250 dilution in PBST) was added to each well in the 1st row of the plate. 100 µL was then taken from the first row and serially diluted down all columns in the plate, with 100 µL being discarded from the last row. The plate was then incubated at 37 °C for 1.5 hr after which the plate was washed 3 times with PBST (200 µL/well). 100 µL of secondary antibody (1:5000 dilution in PBST of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma Aldrich)) was then added to all wells and the plate incubated for 1.5 hr at 37 ºC. The plate was then washed again 3 times with PBST (200 µL/well) and 100 µL *p*-nitrophenyl phosphate (1 mg/mL) (Sigma Aldrich) in 1 M Tris-Cl buffer (pH 8.0) added to all wells. Alkaline phosphastase activity was determined by measuring absorbance at 405 nm on a plate reader (Tecan, Austria).

2.8.2 PilA immunoblotting

2.8.2.1 Quantitative whole cell Western sample preparation

1 mL of cells resuspended in coating buffer to an OD_{600} ± 0.01 OD units were obtained as for PilA ELISAs (section 2.8.1). Cells were pelleted by centrifugation (18, 200 *g* for 10 min at room temperature). The pellet was resuspended by vortexing in 200 µL of loading buffer

(section 2.1.6) plus 5 µL 1 M DTT to lyse the cells. The samples were then stored at -20 °C. Cells were thawed on ice and DNA sheared by passing the lysate through a 26 gauge needle 15 times. The samples were heat treated (99 °C, 5 min) and stored on ice until 10 µL of each sample was loaded on an SDS PAGE gel (section 2.8.3).

2.8.2.2 Pili preparation for surface Westerns

Confluent lawn plates were generated as for PilA ELISAs (section 2.8.1). All cells on the plate were harvested and resuspended in a 15 mL tube (BD Falcon®) containing 1.5 mL PBS. Each sample was vortexed for 10 min to shear the fimbriae off the cell surface. Cells were pelleted by centrifugation (18, 200 *g* for 20 min at room temperature), the supernatant taken and transferred to a new 1.5 mL eppendorf tube, and the centrifugation repeated (18, 200 *g* for 20 min at room temperature). If no cell pellet was visible 1 mL of supernatant was transferred to a new 1.5 mL eppendorf tube, otherwise if a cell pellet was visible the centrifugation step was repeated once more (18, 200 *g* for 20 min at room temperature). 100 µL of 1 M $MgCl_2$ was added to the supernatant, the tube vortexed briefly and then incubated at 4 °C overnight. Centrifugation was repeated (18, 200 *g* for 20 min at room temperature) and the pellet containing the precipitated pili was resuspended in 100 µL 1x loading buffer (section 2.1.6) plus 5 µL 1M DTT. Samples were then stored at -20 °C and thawed on ice prior to loading 10 µL of each sample on an SDS PAGE gel (section 2.8.3).

2.8.3 SDS PAGE

Whole cell and surface pili Western samples were separated using SDS PAGE. Samples were loaded into a precast N,N-methylene-bisacrylamide NuPAGE 10 % Bis Tris Mini Gel (Life Technologies Corporation) which was placed in a XCell *Sure Lock*™ Mini Cell (Life Technologies Corporation) with 1x NuPAGE® MES SDS running buffer (section 2.1.7). 5 µL of a Novex pre-stained protein standard (Life Technologies Corporation) was loaded into one well of each gel. Gels were run for 50 min at 150 V.

2.8.2.4 iBlot® dry gel transfer

After SDS PAGE, protein samples were transferred onto an iBlot® mini gel transfer stack containing a PVDF membrane (Life Technologies Corporation) using an iBlot® dry blotting system (Life Technologies Corporation) (iBlot® program P3, 20V, 7 min).

2.8.2.5 Western Breeze® chemiluminescent Western blot immunodetection

After transfer, membranes were probed with a Western Breeze® chemiluminescent Western blot immunodetection kit (Life Technologies Corporation) according to manufacturer's instructions as follows: Membranes were blocked with 10 mL of blocking solution for 30 min on a rotary shaker (1 revolution/sec) at room temperature. The blocking solution was decanted and the membrane washed twice with 20 mL of MQ water for 5 min. The membrane was incubated with 10 mL of primary antibody solution (PAK anti-PilA, diluted 1/2500 in the Primary Antibody Dilutent Solution) on a rotary shaker (1 revolution/sec) at room temperature for 1 hr. The solution was then decanted and the membrane washed four times for 5 min with 20 mL Antibody Wash. After the final wash was decanted, 10 mL of Secondary Antibody Solution (prepared solution of alkaline phosphatase conjugated, anti-rabbit IgG) was added and the membrane incubated for 30 min on a rotary shaker (1 revolution/sec) at room temperature. The membrane was then washed four times for 5 min in 20 mL of Antibody Wash, followed by three 2 min washes with MQ water. The membrane was placed on a sheet of transparency plastic and 2.5 mL of Chemiluminescent Substrate applied evenly across the membrane. After 5 min incubation at room temperature excess Substrate was blotted off the membrane, and another piece of transparency plastic applied to the membrane prior to Chemiluminescent detection on a ChemiDoc XRS Chemiluminescence machine (BioRad).

2.9 Twitching motility assays

2.9.1 Macroscopic twitching motility assays

Twitching motility was assayed macroscopically using a sub-surface stab assay. A small patch of the *P. aeruginosa* strain to be tested was grown on an LB agar plates (containing appropriate antibiotics if necessary). This overnight growth was harvested with a loop and mixed thoroughly on an uninoculated area of the plate. A small amount of these cells were then stab inoculated through a 1% agar plate (section 2.1.21) using a pipette tip. The plates were inoculated at 37 ^{o}C in a humid environment and the zone of interstitial biofilm expansion at the agar and petri dish interface was visualised by flooding the plate with a solution of 20 % methanol and 10 % acetic acid. The longest (a) and shortest (b) diameters of each interstitial biofilm were measured and the surface area calculated using the formula: area = $\pi(a+b/2)^2$.

2.9.2 Microscopic twitching motility assays

Microscopic analysis of twitching motility was assayed on gellan gum-solidified nutrient media or gellan gum-solidified base media (sections 2.1.19 and 2.1.20) coated microscope slides as follows: gellan gum-solidified nutrient media or gellan gum-solidified base media were melted in a microwave and poured into 50 mL tubes (BD® Falcon) which had been pre-heated to 65 °C. To prevent setting gellan gum media was kept at 65 °C, and used on the same day that it was melted. Microscope slides (Hurst Scientific Pty. Ltd.) were flame-sterilised in 95 % ethanol and placed in a 150 mm petri dish (Greiner Bio-One) on a level 65 °C heating platform. 5 mL aliquots of gellan gum-solidified media was poured and spread in an even layer across the surface of four sterilised microscope slides. The petri dish containing the gellan gum-solidified media coated microscope slides was then transferred to a level room temperature platform and the media allowed to fully set before being stored at 4 °C for no more than 1 week. Prior to use in microscopic twitching motility assays each set of gellan gum-solidified media coated microscope slides was dried in a biosafety cabinet and media removed from the edges (~3 mm) of each microscope slide to allow the slide to sit completely level on the microscope stage. A small inoculum of the strain of interest was taken from a fresh overnight LB agar plate culture and applied to the media-coated slide. A 22 x 32 mm or 22 x 40 mm coverslip (0.13-0.16 mm thick) (Menzel Glaser, Germany) was carefully placed onto the solidified media and the slide incubated at 37 ºC for the indicated time. The slide was moved to the stage of an Olympus IX71 inverted research microscope fitted with phase contrast objectives. Image acquisition was performed using an FViewII camera (Olympus) driven by AnalySIS Research Pro software (Soft Imaging Systems, Olympus). Biofilm stitch images were generated using PhotoShop software (Adobe) and Image J software (Schneider et al., 2012) and the longest (a) and shortest (b) diameters of each interstitial biofilm measured to calculate the surface area using the formula: area = $\pi(a+b/2)^2$.

2.9.3 Microscopic twitching motility assay with saturated filter disc

A filter disc diffusion assay was used to examine the twitching motility response of *P. aeruginosa* to chemical gradients. Gellan gum-solidified nutrient media coated microscope slides were generated and prepared for inoculation as in section 2.9.2. A filter disc (6mm, GE Healthcare) was saturated with 75 µL of the chemical of interest and incubated aseptically at room temperature within a 92 mm petri disc for 45 min. The saturated disc was moved to a dry area of the plate and left to dry for 1 hr 45 min. The soaked and dried disc was then applied to

a gellan gum-solidifed media coated microscope slide (prepared as in section 2.9.2) and incubated at room temperature for 1 hr. A small inoculum of the strain of interest from a fresh overnight LB agar plate culture was inoculated on the solidified media at a fixed distance from the filter disc. A 22 x 22 mm coverslip (0.13-0.16 mm thick) (HD Scientific, Australia) was carefully placed onto the solidified media at the edge of the filter disc, and the slide incubated at 37 ^{o}C for the indicated time. The GelGro slide was imaged as described in section 2.9.2. Time lapse image sequences were combined using Image J software (Schneider et al., 2012).

2.10 Intracellular 3', 5'-cyclic adenosine monophosphate quantification assay

Intracellular 3', 5'-cyclic adenosine monophosphate (cAMP) levels were quantitated using a cAMP enzyme immunoassay (EIA) kit (Cayman Chemical Company). All reagents in the kit were prepared according to manufacturer's instructions: EIA buffer (10x) was reconstituted in 90 mL MQ water (stored at 4 ^{o}C for no longer than 2 months); 5 mL of wash buffer was added to 2 L MQ water and 1 mL Tween 20 (stored at 4 ^{o}C for no longer than 2 months); cAMP AChE tracer (500 determinations) was reconstituted in 30 mL EIA buffer (stored at 4 ^{o}C for no longer than 1 month); cAMP EIA antiserum (500 determinations) was reconstituted in 30 mL EIA buffer (stored at 4 ^{o}C for no longer than 1 month); 4 M potassium hydroxide (500 determinations) was reconstituted in 50 mL MQ water and stored at 4 ^{o}C.

Overnight cultures of the strains to be analysed were diluted 1:100 into 2 mL of LB or BM (appropriate antibiotics included if required) and incubated at 37 ^{o}C shaking at 250 r.p.m until OD_{600} = 1.0. 1.5 mL of each culture was pelleted by centrifugation (13, 000 *g* for 3 min at room temperature). The pellet was gently resuspended in 1 mL of 0.9 M ice-cold NaCl. The cells were then washed twice with 1 mL of 0.9 M ice-cold NaCl, with cells kept on ice and centrifugation steps (13, 000 *g*, 3 min) performed at 4 ^{o}C. After the final wash step the pellet was resuspended by vortexing in 100 ul 1 M HCl. The samples were kept on ice for 10 min, and briefly vortexed every 2 min to lyse the cells. The lysed cells were then pelleted by centrifugation at 16, 900 *g* for 5 min at 4 ^{o}C. 90 ul of supernatant was transferred to a new 1.5 mL eppendorf tube and each sample acetylated by addition of 18 ul 4 M KOH, then, in quick succession, addition of 4.5 ul acetic anhydride, followed by 10 sec of vortexing, and subsequent addition of 4.5 ul 4 M KOH, followed by a further 10 sec of vortexing. Acetylated samples

were typically diluted 1:2 in EIA buffer, however this dilution was adjusted to allow sample icAMP values to fall between the 20-80% region of the standard curve.

cAMP standards were prepared according the manufacturer's instructions as follows: The icAMP standard stock solution was reconstituted with 1 mL of EIA buffer (7,500 pmol/mL) and stored at 4 ºC for no more than 6 weeks. For each standard curve 80 ul of stock icAMP standard was diluted to 200 pmol/mL in EIA buffer and used to generate 8 standards by serial dilution (10 pmol/ml, 5 pmol/ml, 2.5 pmol/mL, 1.25 pmol/mL, 0.625 pmol/mL, 0.313 pmol/mL, 0.156 pmol/mL and 0.078 pmol/mL). These standards were acetylated as for each of the samples, however 100 ul of 4 M KOH and 25 ul of acetic anhydride were used.

cAMP AChE tracer, cAMP EIA antiserum and EIA buffer were added to the samples, standards, and controls (blanks, total enzymatic activity of the AChE tracer (TA), non-specific binding of the tracer (NSB), maximum amount of tracer that the antiserum can bind in the absence of free cAMP anylate (B_0)) in the provided 96-well plate according to manufacturer's instructions). The plate was covered with plastic film provided in the kit, and incubated at 4 ºC for 18 hr. The wells were then emptied and washed five times with wash buffer. The plate was developed by addition of 200 ul of Ellman's reagent (reconstituted in 20 mL MQ water for 100 determinations) to all wells and addition of 5 ul AChE tracer to the total activity well. The plate was again covered with plastic film and incubated at room temperature on an orbital shaker for 90-120 min, and the plate read at A_{405nm}.The ratio of absorbance of the standards to that of the B_0 well (%B/B_0) was used to generate a standard curve fitted with linear regression by plotting logit (B/B_0) vs. log icAMP concentration. This standard curve was used to calculate the icAMP concentration based upon the logit B/B_0 of each sample.

2.11 Growth assays

Growth of *P. aeruginosa* strains was followed by recording changes in OD_{595nm} over a 20 hr period at 37ºC, shaking at 250 rpm. To a microtitre plate 75 µL of cells were added at a final concentration of 1 x 10^{-6} cells/mL in 75 µL CAMHB (1x or 1:10x as indicated); CAMHB supplemented with ATP (Sigma-Aldrich) at indicated concentrations; Apyrase at 200 mU/mL (Sigma-Aldrich); BM or BM supplemented with BSA (0.1 % w/v), mucin (0.05 % (w/v)), tryptone (3 % (w/v)) and GlcNAc (50 mM). No cell controls for each media type were also included in each biological replicate. Each sample and control was assayed in five individual wells within each biological replicate.

2.12 Detection of extracellular ATP in colony biofilms

2.12.1 Generation of colony biofilms

1.5 % 30 mL LB agar plates (see section 2.1.12) were poured and allowed to set overnight at room temperature. The following morning the agar plates were flipped into a larger petri dish to expose the smooth underside set against the petri dish base which promotes rapid twitching motility-mediated biofilm expansion (Semmler et al., 1999). 1.5 mL of an overnight culture was pelleted by centrifugation (13,000 *g*, 3 min). The pellet was then gently resuspended and inoculated onto the centre of a flipped 1.5 % 30 mL agar plate. The petri-dish lid was applied and incubated in a humid environment statically for 72 hr at 37 ^{o}C. Cells were harvested from the outer edge which corresponds to the active twitching zone visualised as a "ground-glass edge". Harvested cells were resuspended in 1 mL PBS and used in assays to detect eATP within the extracellular environment of actively expanded *P. aeruginosa* biofilms (2.12.2).

2.12.2 Detection of eATP in the extracellular environment of colony biofilms

eATP was detected in the extracellular environment of cells harvested from surface biofilms (section 2.12.1) using a BacTitre-Glo^{TM} assay (Promega Corportation). From the 1 mL of resuspended cells harvested from surface colony biofilms 500 µL was pelleted by centrifugation (16, 900 *g*, 5 min). 110 µl of supernatant was added into triplicate wells of a white 96-well plate (Greiner Bio-one) and 10 µL used to generate four serial 1:11 dilutions, with each well retaining 100 µL final volume. 100 µL of ATP standards ranging from 0-1 µM were also added to the plate in duplicate. 30 µL of room temperature BacTiter-Glo™ Buffer with precombined BacTiter-Glo™ substrate was added to all wells, mixed and incubated at room temperature for 5 min. Luminescence of all wells was read using an integration time of 250 msec.

2.13 Sessile biofilm assays

2.13.1 Microtitre plate static biofilm assays

The effect of eATP on sessile biofilm formation was assayed using a microtitre plate assay with crystal violet staining. Overnight cultures of *P. aeruginosa* were diluted 1:100 in

CAMHB (1/10) and 75 µL added to wells of a 96-well microtitre plate (Microtest-96, BD) containing 75 µL of eATP at final concentrations ranging from 0-2.8 mM. The plates were covered with Aeraseal™ (Excel Scientific Inc.) and incubated statically at 37 ºC for 24 hr. Plates were then washed three times with PBS and stained with crystal violet (0.2 % (w/v)) for at least 1 hr, statically at room temperature. The crystal violet was then removed and PBS washes were repeated. The crystal violet stain was extracted with 30 % (v/v) acetic acid and the plates incubated for 30 min at room temperature on an orbital shaker. The A_{595} of extracted stain was measured for each of the wells using a plate reader (Tecan, Austria).

2.13.2 Fluoro dish static biofilm assays

The effect of eATP on sessile biofilm development of *P. aeruginosa* was examined by growing biofilms in glass-bottom Fluorodishes (dish 35 mm, glass 10 mm, World Precision Instruments, Inc.). An overnight culture was diluted 1:100 in CAMHB (1/10) and 100 µL added to each dish with 100 µL of either CAMHB (1/10) for the control, or 2 mM ATP, 2 mM ATP with 200 mU/mL Apyrase, or 200 mU/mL Apyrase alone. The dishes were incubated statically at 37 ºC for 24 hr, after which time the media was carefully replenished and incubated for a further 24 hr. After 48 hr the planktonic growth was carefully removed and the biofilm biomass stained with 200 µL of 0.25 µM SYTO-9 green fluorescent nucleic acid stain (Invitrogen Molecular Probes) for 1 hr at room temperature. The stained cells were then washed twice with 200 µL of PBS to remove excess stain. Confocal scanning light microscopy (CLSM) images of stained cells were obtained with a Nikon A1 confocal microscope with Z-series images taken in 0.5 µm slices. Biofilms were volume-rendered using IMARIS® Imaging Software and COMSTAT analyses (Heydorn et al., 2000) performed.

Chapter Three

Extragenic suppressor mutations that restore twitching motility to *fimL* mutants of *Pseudomonas aeruginosa* are associated with elevated intracellular cyclic AMP levels

Publication information and author contributions

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The results presented in this chapter were all generated and analysed by Laura M Nolan, with the following exceptions:

Construction of isogenic *fimL* mutant and isolation of revertant strains – Dr. Scott A. Beatson

CpdA homology modelling – Dr. Peter M. Jones and A/Prof. Anthony M. George

Library preparation and SOLiD sequencing of PAO1*fimL*$_{Rev1}$ – Dr. Jo-Ann Stanton

Analysis of genome PAO1*fimL*$_{Rev1}$ sequence – Larry Croft, Dr. Scott A. Beatson, A/Prof. Cynthia Whitchurch

3.1 Introduction

Pseudomonas aeruginosa is a Gram negative opportunistic pathogen which grows in soil, marine environments and infects many organisms including plants, nematodes, insects and animals (Rahme et al., 2000, Mahajan-Miklos et al., 2000). In humans *P. aeruginosa* causes serious, and often chronic, infections in those with cystic fibrosis and in immunocompromised individuals, such as those with AIDS, patients undergoing chemotherapy and those with severe burns (Lyczak et al., 2000, Tatterson et al., 2001). The success of this organism in various environments is attributed to its broad metabolic versatility (Stover et al., 2000) and its ability to produce many cell-associated and secreted virulence and survival factors (Van Delden and Iglewski, 1998).

In *P. aeruginosa* the expression of these factors is regulated by components of a large number of complex signalling pathways. One signalling molecule associated with regulation of virulence factors is 3', 5'-cyclic adenosine monophosphate (cAMP). In *P. aeruginosa*, cAMP is an allosteric regulator of the transcriptional activator Vfr (virulence factor regulator) (West et al., 1994) which has been shown to regulate, either directly or indirectly, more than 200 genes, including those associated with biogenesis and function of type IV pili (tfp), type III secretion systems (T3SS), flagella-mediated motility, and quorum sensing systems (Beatson et al., 2002a, Albus et al., 1997, Dasgupta et al., 2002, Wolfgang et al., 2003).

Since cAMP is involved in controlling such a wide range of important cellular functions it is imperative that cAMP homeostasis is tightly controlled. In *P. aeruginosa* cAMP is synthesized by two adenylate cyclases, CyaA and CyaB, with the latter contributing the majority of intracellular cAMP (icAMP) (Fulcher et al., 2010, Wolfgang et al., 2003). In *P. aeruginosa* the 3'-5' nucleotide phosphodiesterase CpdA serves to reduce the levels of intracellular cAMP by hydrolyzing the phosphodiester bond to release the biologically inactive 5'-AMP {Fuchs, 2010 #573}. Expression of *cpdA* is positively regulated by Vfr in response to elevated levels of icAMP {Fuchs, 2010 #573}.

The *P. aeruginosa* Chp chemosensory system and FimL have been shown to be involved in the modulation of icAMP levels (Fulcher et al., 2010, Inclan et al., 2011). The Chp chemosensory system shares many components in common with chemosensory systems that regulate flagella rotation in bacteria and in *P. aeruginosa* is thought to regulate the motors that control the extension and retraction of tfp to mediate a form of surface translocation termed twitching motility (Whitchurch et al., 2004, Darzins, 1993, Darzins, 1994, Darzins, 1995).

Control of icAMP levels by the Chp system appears to be mediated by the CheY-like components of the Chp system, PilG and PilH, which are thought to modulate icAMP levels by modulating CyaB activity (Fulcher et al., 2010).

The *P. aeruginosa* protein FimL is homologous to the N-terminal domain of ChpA and is required for normal twitching motility in *P. aeruginosa* strains PAO1 and PA103 (Whitchurch et al., 2005). Mutants of *fimL* have been recently shown to have reduced levels of icAMP compared to isogenic wildtype strains (Fulcher et al., 2010, Inclan et al., 2011). It has been suggested that regulation of icAMP levels by FimL may occur through post-translational modulation of CyaB activity (Inclan et al., 2011).

We have previously reported that revertants of *fimL* mutants frequently arise that have regained near wildtype twitching motility presumably due to the acquisition of an extragenic suppressor mutation(s) (Whitchurch et al., 2005). Twitching motility revertants were readily detectable as flares of twitching cells erupting from the edges of surface or interstitial colonies of *fimL* mutants (Whitchurch et al., 2005). One PAO1Δ*fimL* revertant was examined in more detail and was found to have significantly increased icAMP levels compared to the parent *fimL* mutant (Whitchurch et al., 2005).

In this study we have characterised another five independently isolated *fimL* twitching motility revertants and have determined that all had increased icAMP levels compared to the parent *fimL* mutant. Whole genome sequencing of one of these strains revealed that it had acquired a loss-of-function mutation in *cpdA* that accounts for the elevated icAMP levels and restoration of twitching motility. Characterisation of the other four *fimL* revertants indicates that there is at least one other site of suppressor mutation that can cause phenotypic reversion in *fimL* mutants and that is involved in modulation of icAMP levels of *P. aeruginosa*.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and media

The strains used in this study and their relevant characteristics are shown in Table 3-1. *E. coli* strain DH5α was used in all genetic manipulations and in the preparation of DNA sequencing templates, and *E. coli* S17-1 was used as the donor strain in bacterial conjugation for allelic exchange mutagenesis.

P. aeruginosa and *E. coli* were cultured on Luria-Bertani (Sambrook, 1989) (LB) broth solidified with agar at 1.5% or 1% (for twitching motility stab assays) and grown overnight at 37 ˚C. Cultures were grown in either cation-adjusted Mueller Hinton broth (CAMHB) or LB broth for *P. aeruginosa* or LB broth for *E. coli* and incubated overnight at 37˚C, with shaking at 250 rpm. Light microscopy was performed with nutrient media (4 g/L tryptone, 2 g/L, yeast extract, 2 g/L NaCl) solidified with 8 g/L GelGro (ICN). Antibiotic concentrations used for selection of *E. coli* were 100 µg/mL ampicillin, 12.5 µg/mL tetracycline and 50 µg/mL kanamycin and for *P. aeruginosa* were 250 µg/mL carbenicillin and 200 µg/mL tetracycline.

3.2.2 Recombinant DNA techniques

The preparation of plasmid DNA (Qiagen), restriction endonuclease digestion (New England Biolabs) and ligation reactions (Promega and New England Biolabs) were carried out using standard protocols (Sambrook, 1989). The preparation of *E. coli* competent cells and transformations were performed as previously described (Sambrook, 1989). *P. aeruginosa* competent cells were prepared by $MgCl_2$ treatment and transformed as previously described (Mattick et al., 1987). *P. aeruginosa* cells were prepared by sucrose treatment for electroporation and electroporated as previously described (Choi et al., 2006) with the following modifications: Briefly, overnight 10 ml CAMHB cultures of *P. aeruginosa* were harvested by centrifugation at 3,000 *g* for 10 min at 4°C. The cell pellet was washed 4 times in 1 ml of ice-cold 300 mM sucrose, and finally resuspended in 200 µl of ice-cold 300 mM sucrose. 300 ng of plasmid DNA was added to 80-100 µl of electrocompetent cells, incubated at room temperature for 5 min, and then transferred to a 1 mm gap-width electroporation cuvette. After applying a pulse (settings: 25 µF; 200 Ω; 2.5 kV on a BTX 600 Electro Cell Manipulator; Harvard Apparatus Inc.) the cells were added to 1 ml of CAMHB with 0.2 % glucose and incubated at 37 °C, shaking, for 1 h and then plated onto LB agar containing appropriate antibiotic selection.

Particular care was taken to ensure that *fimL* revertants were not inadvertently included in PAO1*fimL*::Tn*5*-Tc transformations. Vector controls and CpdA alleles were transformed using the same preparation of competent cells and at least 10 independent colonies of PAO1*fimL*::Tn*5*-Tc transformants were chosen for further phenotypic characterisations including confirmation that the vector control transformants had the expected abrogated twitching motility phenotype.

Table 3-1. Strains and plasmids used in chapter 3

Strains	**Relevant characteristic(s)**	**Source or reference**
E. coli		
DH5α	*recA endA1 gyrA96 hsd*R17 *thi-1 supE44 relA1* φ80 d*lacZΔM15*	Invitrogen
S17-1	*thi pro hsdR recA chr::RP4-2*	(Simon et al., 1983)
P. aeruginosa		
PAO1	Wildtype *P. aeruginosa* strain ATCC 15692	American Type Culture Collection
PAO1*pilA*	*PAO1 with pilA* inactivated by allelic displacement with a tellurite resistance cassette; Tel^R	(Klausen et al., 2003b)
PAO1293-31E6	PAO1293 with mTn*5*-Tc insertion in *fimL;* Tc^R	(Whitchurch et al., 2005)
PAO1*fimL*::mTn*5*-Tc	PAO1 with same mTn*5*-Tc insertion in *fimL* as PAO1293-31E6; Tc^R	This study
PAO1$fimL_{Rev1}$	Independent twitching motility revertant isolated from PAO1*fimL*:: mTn*5*-Tc; Tc^R	This study
PAO1$fimL_{Rev2}$	Independent twitching motility revertant isolated from PAO1*fimL*:: mTn*5*-Tc; Tc^R	This study
PAO1$fimL_{Rev3}$	Independent twitching motility revertant isolated from PAO1*fimL*:: mTn*5*-Tc; Tc^R	This study
PAO1$fimL_{Rev4}$	Independent twitching motility revertant isolated from PAO1*fimL*:: mTn*5*-Tc; Tc^R	This study
PAO1$fimL_{Rev5}$	Independent twitching motility revertant isolated from PAO1*fimL*:: mTn*5*-Tc; Tc^R	This study
Plasmids		
pUCPSK	*P. aeruginosa-E. coli* shuttle vector; Ap^R	(Watson et al., 1996a)
pGEM-T	*E. coli* cloning vector; Ap^R	Promega
pOK12	*E. coli* cloning vector; Km^R	(Vieira and Messing, 1991)
pRIC380	*P. aeruginosa* suicide vector; Ap^R	(Alm and Mattick, 1996)
pSB62.4	Marker rescue clone of PAO1293-31E6 genomic DNA containing *fimL*::mTn*5*-Tc insertion site; Ap^R, Tc^R	(Whitchurch et al., 2005)
pSB172.10	3.1 Kb *Pst*I insert from pSB62.4 cloned into pOK12; Km^R, Tc^R	This study
pSB172.1	3.1 Kb *Spe*I insert from pSB172.10 cloned into pRIC380; Ap^R, Tc^R	This study
pUCPCpdA	*cpdA* amplified from wildtype PAO1 and cloned into pUCPSK with *Plac*; Ap^R	This study
pUCPCpdAL187R	*cpdA* amplified from PAO1$fimL_{Rev1}$ and cloned into pUCPSK with *Plac*; Ap^R	This study
pUCPCpdAR75G	*cpdA* amplified from PAO1$fimL_{Rev2}$ and cloned into pUCPSK with *Plac*; Ap^R	This study

3.2.3 Phenotypic assays

Twitching motility was assayed using a modification of the sub-surface stab assay described previously (Semmler et al., 1999). Briefly, the *P. aeruginosa* strain to be tested was stab inoculated through a 1% agar plate and after overnight growth at 37ºC the zone of interstitial biofilm expansion at the agar and petri dish interface was visualised by flooding the plate with a solution of 20% methanol and 10% acetic acid. The longest (a) and shortest (b) diameters of each interstitial biofilm were measured and the surface area calculated using the formula: area = abπ.

Microscopic analysis of twitching motility mediated interstitial biofilm expansion on GelGro solidified nutrient media was assayed using a modification of the slide assay described previously (Semmler et al., 1999). Briefly, 5 mL of molten Gelgro nutrient media was poured across four slides on a level surface and allowed to set. The slide was dried briefly and spotted with a small inoculum of the strain of interest taken from a fresh overnight plate culture. A 22 x 40 mm coverslip (0.13-0.16 mm thick) was carefully placed onto the solidified media and incubated at 37ºC for 5 h. The GelGro slide was moved to the stage of an Olympus IX71 inverted research microscope fitted with phase contrast objectives. Images acquisition was performed using a FViewII camera (Olympus) driven by AnalySIS Research Pro software (Soft Imaging Systems, Olympus).

Intracellular cAMP (icAMP) assays were performed as described previously (Inclan et al., 2011). Briefly, strains were sub-cultured 1/100 from an overnight CAMHB culture into LB and incubated at 37 ºC with shaking until OD_{600}=1.0. 1.5 mL of cells were harvested by centrifugation at 13,000 g for 2 min at 4ºC and washed twice with ice-cold 0.9 M NaCl. Pellets were resuspended in 100 µL 0.1 N HCl and incubated on ice for 10 min with occasional vortexing to lyse the bacteria. Lysates were centrifuged at 13,000g for 5 min at 4ºC to remove cellular debris, and the supernatant used to measure intracellular cAMP with an ELISA-based assay (Cayman Chemical Company) as per manufacturer's instructions.

3.2.4 Construction of isogenic *fimL* mutant and isolation of revertant strains

A *fimL* allelic exchange mutant in PAO1 was constructed by using the sucrose counter-selection system described previously (Alm and Mattick, 1996, Schweizer, 1992). A 3.1 Kb *Pst*I fragment containing the *fimL*::mTn*5*-Tc allele from PAO2913-31E6 was subcloned from the marker rescue clone pSB62.4 into the vector pOK12. The resultant clone pSB172.10 was

then digested with *Spe*I and the *fimL*::mTn*5*-Tc allele cloned into the suicide vector pRIC380 to produce pSB172.1. This vector carries the genes *sacBR,* which promote sensitivity to sucrose, and *oriT* which enables conjugal transfer. pSB172.1 was transformed into the *E. coli* donor strain S17-1 in preparation for mating with *P. aeruginosa* PAO1. Following conjugation, the transconjugants were plated onto 5% sucrose medium containing tetracycline to select for colonies in which the plasmid had excised while leaving the homologously recombined *fimL*::mTn*5*-Tc allele in the chromosome. Mutants were genotypically confirmed by Southern blot analysis.

3.2.5 Sequencing and sequence analysis

PAO1*fimL*$_{Rev1}$ genomic DNA was sequenced using an Applied Biosystems SOLiD System 2.0 sequencer at the High-throughput DNA Sequencing Unit, University of Otago, New Zealand. The 2 x 25 bp mate-pair format was utilised for sequencing with insert sizes between 1.5 and 2.5 Kb. Approximately 34 million individual sequence tags were mapped, with an average depth of 131-fold coverage, to the reference genome of *P. aeruginosa* PAO1 (NC_002516.2) using the SOLiD System Analysis Pipeline Tool (corona lite). Fourteen high quality SNPs were identified in PAO1*fimL*$_{Rev1}$ relative to the *P. aeruginosa* PAO1 reference genome including three that were located in intergenic regions. In total, non-synonymous substitutions were detected in seven protein-coding genes: PA0159, W35C [183697T>C]; PA0400, A125R [4423850C>G]; PA1029, M1V [1116213G>C]; PA1459, G34A [1589438G>C]; PA2400/PvdJ, P819A [2669175G>C]; PA4341, E158D [4869855T>G]; and PA4969/CpdA, L187R [5578940A>C].

The nucleotide sequences of the coding and upstream promoter regions of *cpdA, cyaA, cyaB, pilG, pilH* and *vfr* were obtained by PCR amplification of the region of interest from the *P. aeruginosa* PAO1 genome and cloning of the amplicon into pGEM-T (or pUCPSK in the case of *vfr*). Automated DNA sequencing of the pGEM-T clones was performed by the Australian Genome Research Facility (University of Queensland, Brisbane, Queensland, Australia) and Macrogen Inc. (Seoul, Korea) using BigDye terminator chemistries. *cpdA* was amplified using the forward primer 5'-CATCGGGAACGGGCTAATG-3' and the reverse primer 5-'GTAGACCCGCACTTCCAGCC- 3'; *cyaA* was amplified using the forward primer 5'-CTGAGCGGACGGAAAGTC-3', and the reverse primer 5'-CAGCGAGCAGGGTAATAC-3'; *cyaB* was amplified using the forward primer 5'-GCATCGGTCTCTTCTTGTTC-3' and the reverse primer 5'-GTTTCGGCGGAGGAGTTC-

3'; *pilG* and *pilH* were amplified using the forward primer 5'-TCCGGGCATTATGGATAGAG-3' and the reverse primer 5'-AACCGCAGAGGTCCATGAT-3'; *vfr* was amplified using the forward primer 5'-GCCGGTACCCTTGACCACGAAGTGC-3' (*Kpn*I site underlined) and the reverse primer 5'-CCTAAGCTTGTTCTTCCAGGAGCGTGG-3' (*Hind*III site underlined). Nucleotide and predicted protein sequences were analysed using MacVector (Oxford Molecular Group).

3.2.6 Protein homology modelling

Primary amino acid alignments were carried out using ClustalW software (Thompson et al., 1994). A homology model of wildtype CpdA from *P. aeruginosa* was generated using the comparative alignment of Rv0805 from *Mycobacterium tuberculosis* and CdpA and the high-resolution (1.8 Å) structure of Rv0805 (PDB: 3IB8) from *M. tuberculosis* as the template to build the CpdA 3-dimensional structure with Modeller ((Sali and Blundell, 1993); available at: http://www.salilab.org/modeller/).

3.3 Results

3.3.1 *fimL* revertants have increased intracellular cAMP levels

In order to further investigate the mechanism(s) of phenotypic reversion of *fimL* mutants we isolated five independent twitching motility revertants of the PAO1 *fimL* mutant PAO1*fimL*::mTn*5*-Tc. These *fimL* revertant strains are herein designated PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$, and PAO1*fimL*$_{Rev5}$.

The twitching motility phenotypes of the five revertants were examined via the sub-surface stab assay in which wildtype twitching motility results in rapid expansion of the interstitial biofilm that forms at the agar-petri dish interface. In this assay non-twitching mutants (such as *pilA* mutants) show no zone of biofilm expansion (Figures 3-1A, B). As expected, the *fimL* mutant PAO1*fimL*::mTn*5*-Tc displayed severely abrogated twitching motility producing only a small zone of interstitial biofilm expansion via this assay (Figures 3-1A, B) as described previously for a *fimL* deletion mutant of PAO1 (Whitchurch et al., 2005). Each of the *fimL* revertants demonstrated significantly greater twitching motility than the parental PAO1*fimL*::mTn*5*-Tc, although none had attained complete restoration to wildtype PAO1 levels (Figures 3-1A, B).

Light microscopy was used to examine the twitching motility mediated biofilm expansion that occurs at the interstitial interface of a glass coverslip and a pad of nutrient media solidified with gellan gum. Under these conditions, wildtype *P. aeruginosa* interstitial biofilms actively expand via tfp-mediated twitching motility and produce a characteristic micromorphological pattern that is comprised of rafts of cells at the leading edge of the biofilm, behind which forms an intricate lattice-like network of cells (Figure 3-1C; (Semmler et al., 1999)). Non-twitching mutants, such as *pilA* mutants, demonstrate no differentiation of the biofilm edge (Figure 3-1C; (Semmler et al., 1999)) and *fimL* mutants form large rafts of cells at the leading edge but do not produce the intricate lattice-like network (Figure 3-1C; (Whitchurch et al., 2005)). Microscopic examination of interstitial biofilms produced by the PAO1*fimL*::mTn*5*-Tc *fimL* revertant strains revealed obvious differences in the micromorphological patterns of the interstitial biofilms. The interstitial biofilms formed by PAO1*fimL*$_{Rev5}$ were most similar to those formed by wildtype PAO1, except that the leading edge rafts were routinely smaller than wildtype (Figure 3-1C). The micromorphological patterns of the interstitial biofilms formed by PAO1*fimL*$_{Rev2}$ routinely displayed wide leading edge rafts and thicker trails immediately behind the rafts (Figure 3-1C). Interestingly, the interstitial biofilms of PAO1*fimL*$_{Rev1}$ and PAO1*fimL*$_{Rev3}$ had similar leading edge rafts to wildtype but lacked the ability to form the intricate lattice-like network of trails (Figure 3-1C). Instead, the cells were loosely distributed in the region behind the leading edge rafts (Figure 3-1C). Finally, the interstitial biofilms formed by PAO1*fimL*$_{Rev4}$ appeared very similar to the parental PAO1*fimL*::mTn*5*-Tc and were comprised of extremely large, wide rafts and the cells in the regions behind the rafts were densely packed and lacked any kind of lattice-like network arrangement. (Figure 3-1C).

We have previously determined that a revertant of PAO1Δ*fimL* showed increased levels of icAMP (Whitchurch et al., 2005). To determine if elevated icAMP is a phenotype common to all *fimL* revertants, we assayed icAMP levels for each of the five *fimL* revertants obtained in this study as well as the parent PAO1*fimL*::mTn*5*-Tc and wildtype PAO1 strains (Figure 3-1D). We found that under the conditions of our assay PAO1*fimL*::mTn*5*-Tc has reduced icAMP levels relative to wildtype PAO1 (Figure 3-1D), as has been described previously (Inclan et al., 2011). Interestingly, each of the five *fimL* revertants showed significantly elevated levels of icAMP relative to their parent PAO1*fimL*::mTn*5*-Tc strain, although the levels of intracellular cAMP varied widely (Figure 3-1D). Strains PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev3}$, and PAO1*fimL*$_{Rev5}$ had icAMP levels that were 10-20 fold higher than wildtype PAO1, whereas PAO1*fimL*$_{Rev2}$

had icAMP levels that were only 2-fold higher than PAO1 and PAO1*fimL*$_{Rev4}$ had wildtype icAMP levels (Figure 3-1D). These observations suggest that extragenic suppressor mutations that lead to elevated icAMP levels are able to restore, at least in part, twitching motility to the PAO1*fimL*::mTn*5*-Tc mutant.

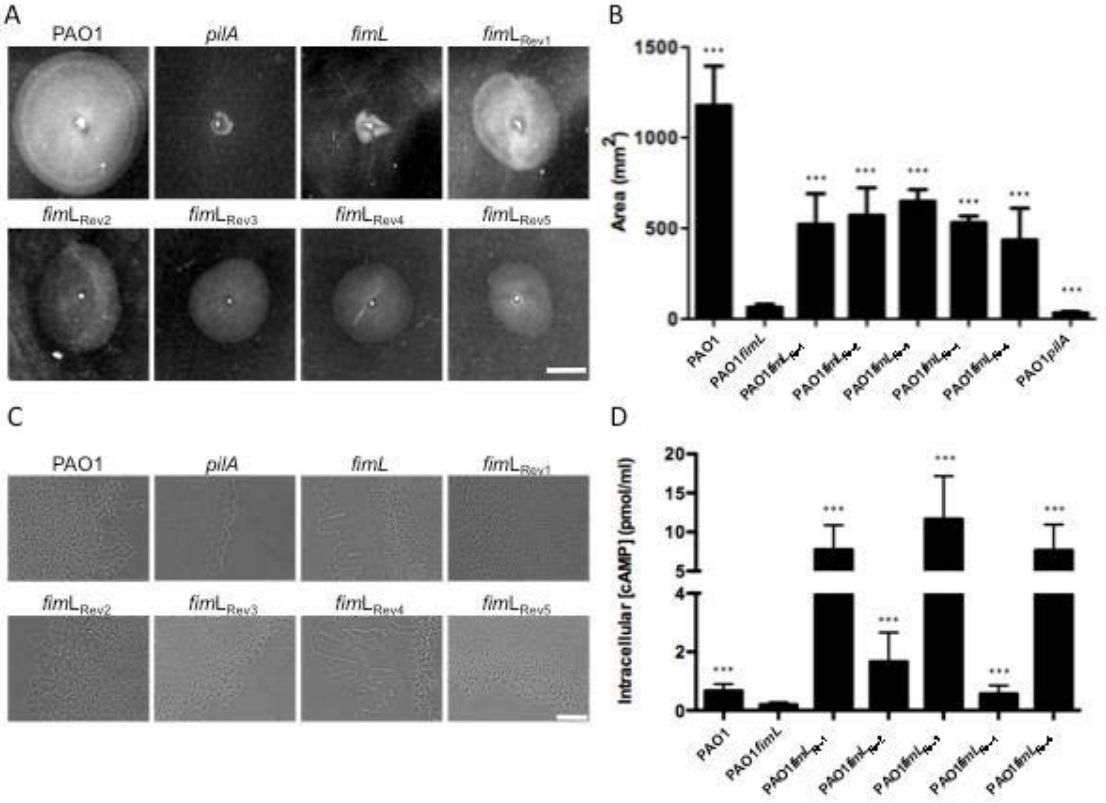

Figure 3-1. Phenotypic reversion of *fimL* mutants

(A, B) Sub-surface twitching motility mediated interstitial biofilm expansion at agar/plastic interface after 24 h incubation at 37°C. (A) Scanned images of interstitial biofilms of PAO1, PAO1*pilA*, PAO1*fimL*::mTn*5*-Tc, PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$. Bar represents 1 cm. (B) Areas of interstitial biofilms were measured and presented as the mean ± SD from three independent experiments performed in triplicate. (*** $p < 0.0001$, Mann-Whitney U test compared to PAO1*fimL*::mTn*5*-Tc).

(C) Light microscopy images of typical biofilm expansion at the interstitial surface between glass coverslip and Gelgro solidified nutrient media by PAO1, PAO1*pilA*, PAO1*fimL*::mTn*5*-Tc, PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$. Strains were incubated at 37 °C for 5 h before imaging. Bar represents 100 μm.

(D) Intracellular cAMP concentrations of PAO1, PAO1*fimL*::mTn*5*-Tc, PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$. Data are presented as the mean ± SD for four independent experiments performed in triplicate. (*** $p < 0.0001$; ** $p < 0.005$ Mann-Whitney U test compared with PAO1*fimL*:: mTn*5*-Tc).

3.3.2 *cpdA* is one site of extragenic suppressor mutation of *fimL*

To identify the site of the extragenic suppressor mutation in *fimL* revertants we first sequenced the genome of PAO1*fimL*$_{Rev1}$ and compared this to the available genome sequence of PAO1 (Winsor et al., 2011, Stover et al., 2000). Seven single nucleotide polymorphisms (SNPs) causing non-synonymous amino acid substitutions were identified in the genome sequence of PAO1*fimL*$_{Rev1}$. One of these SNPs was located in *cpdA,* which encodes a cAMP phosphodiesterase responsible for cAMP degradation in *P. aeruginosa* {Fuchs, 2010 #573}. Given the observed elevated icAMP levels in all of our *fimL* revertants we considered the possibility that *cpdA* may be the site of extragenic suppressor mutation in PAO1*fimL*$_{Rev1}$ that is responsible for the observed phenotypic reversion of twitching motility and icAMP levels. This is also consistent with the observations of Inclan *et al* (2011), who found that deletion of *cpdA* restores twitching motility and T3SS function to a PAO1 *fimL* mutant (Inclan et al., 2011).

The SNP in *cpdA* of PAO1*fimL*$_{Rev1}$ was confirmed by PCR amplification of *cpdA* from PAO1*fimL*$_{Rev1}$ and sequence analysis of the cloned amplicon. The SNP in *cpdA* results in a leucine to arginine amino acid substitution at position 187 (L187R). To determine if this SNP in *cpdA* is responsible for the observed increased icAMP levels and restoration of twitching motility in PAO1*fimL*$_{Rev1}$, the plasmid pUCPCpdA (containing wildtype *cpdA*) was transformed into PAO1, PAO1*fimL*::mTn*5*-Tc and PAO1*fimL*$_{Rev1.}$ Consistent with *cpdA* encoding a cAMP phosphodiesterase, introduction of pUCPCpdA significantly reduced icAMP levels in both wildtype PAO1 and PAO1*fimL*$_{Rev1}$ (Figure 3-2B). Interestingly, the presence of pUCPCpdA reduced twitching motility of PAO1*fimL*$_{Rev1}$ to levels similar to those of the parental PAO1*fimL*::TcR whereas the reduction in twitching motility was not as severe when pUCPCpdA was present in PAO1 (Figure 3-2A). As introduction of the wildtype CpdA allele to PAO1*fimL*$_{Rev1}$ restored the severely abrogated twitching motility phenotype and icAMP to parental PAO1*fimL*::mTn*5*-Tc levels, this indicates that the L187R SNP in *cpdA* is likely to be the site of suppressor mutation in PAO1*fimL*$_{Rev1}$.

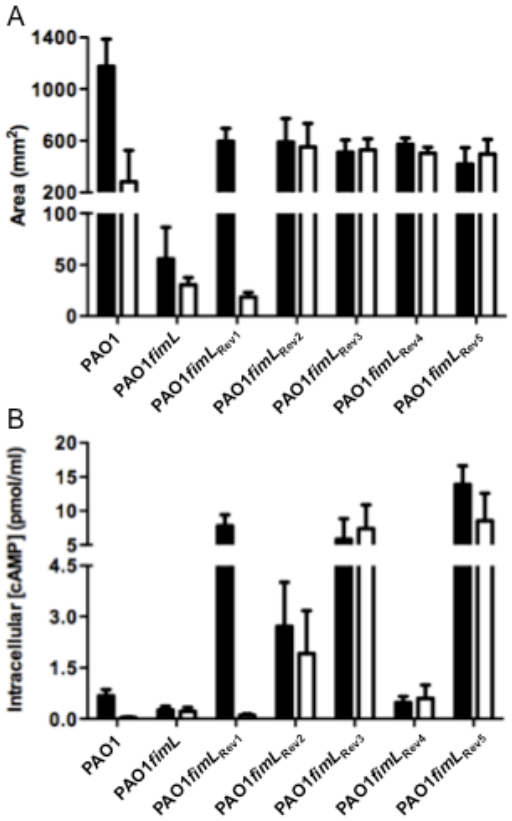

Figure 3-2. CpdA restores *fimL* phenotypes to PAO1*fimL*$_{Rev1}$

(A) Sub-surface twitching motility mediated interstitial biofilm expansion at the agar/plastic interface. Areas of interstitial biofilms of PAO1, PAO1*fimL*::mTn*5*-Tc, PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$ transformed with pUCPSK (solid bars) and pUCPCpdA (open bars) were measured after 24 h incubation at 37°C. Data are presented as the mean ±SD for three independent experiments performed in triplicate.

(B) Intracellular cAMP concentrations of PAO1, PAO1*fimL*::mTn*5*-Tc, PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$ transformed with pUCPSK (solid bars) and pUCPCpdA (open bars). Data are presented as the mean ±SD for four independent experiments performed in triplicate.

After determining that the mechanism of reversion in PAO1*fimL*$_{Rev1}$ is likely to be via acquisition of an extragenic suppressor mutation in *cpdA* that results in elevated icAMP levels and restoration of twitching motility, we wanted to determine if *cpdA* is also the site of suppressor mutation in the other *fimL* revertants (PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$, and PAO1*fimL*$_{Rev5}$). Interestingly, introduction of pUCPCpdA did not restore the abrogated twitching motility to any of these revertants, nor did it reduce the levels of icAMP to the same extent as when it was introduced into PAO1 or PAO1*fimL*$_{Rev1}$. These observations

suggest that *cpdA* may not be the site of suppressor mutation in these revertants and furthermore, suggest that the suppressor mutation in these strains is causing elevation of icAMP levels by a mechanism(s) that is less affected by the activity of exogenous CpdA expressed from pUCPCpdA.

We also sequenced the coding and upstream promoter regions of *cpdA* from these four *fimL* revertant strains. Sequence analysis revealed that three of the *fimL* revertants (PAO1*fimL*$_{Rev3,}$ PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$) have wildtype *cpdA* sequences confirming that the site of extragenic suppressor mutation has occurred elsewhere in these strains. Interestingly, PAO1*fimL*$_{Rev2}$ was found to have acquired a SNP in *cpdA,* which is predicted to result in an arginine to glycine amino acid change at position 75 (CpdAR75G). However, the observation that introduction of wildtype *cpdA* did not complement the revertant twitching motility and icAMP phenotypes back to parental *fimL* mutant levels suggests that this may not be the primary site of suppressor mutation in this strain or that the CpdAR75G allele is dominant negative over the introduced wildtype CpdA allele.

3.3.3 CpdA alleles from *fimL* revertants have reduced activities *in vivo*

Our results suggest that *fimL* mutants are able to restore twitching motility by acquiring extragenic suppressor mutations that lead to elevated icAMP levels, and that this increase is generated via at least two different mechanisms. In PAO1*fimL*$_{Rev1}$ and PAO1*fimL*$_{Rev2}$ we have identified two different SNPs in *cpdA,* which appear to affect CpdA activity, at least one of which (L187R) is likely to be responsible for phenotypic reversion in PAO1*fimL*$_{Rev1}$. To further investigate the effect of these SNPs on CpdA activity we cloned each of the *cpdA* genes from PAO1*fimL*$_{Rev1}$ (pUCPCpdAL187R) and PAO1*fimL*$_{Rev2}$ (pUCPCpdAR75G) and assayed the *in vivo* activity of each allele by measuring twitching motility and icAMP levels in PAO1 harbouring clones expressing wildtype and mutant CpdA alleles (Figure 3-3). These assays show that whilst pUCPCpdA significantly reduced both twitching motility and icAMP levels, pUCPCpdAL187R did not influence either twitching motility or icAMP levels suggesting that the L187R mutation leads to a loss-of-enzyme function *in vivo*. PAO1 containing pUPCpdAR75G demonstrated a mild reduction in both twitching motility and icAMP levels, which suggests that the R75G mutation leads to an enzyme with attenuated, but not absent, phosphodiesterase activity. Furthermore, as introduction of the CpdAR75G allele did not result in elevation of icAMP levels, these observations indicate that the CpdAR75G allele is not dominant negative over the wildtype CpdA allele present in PAO1.

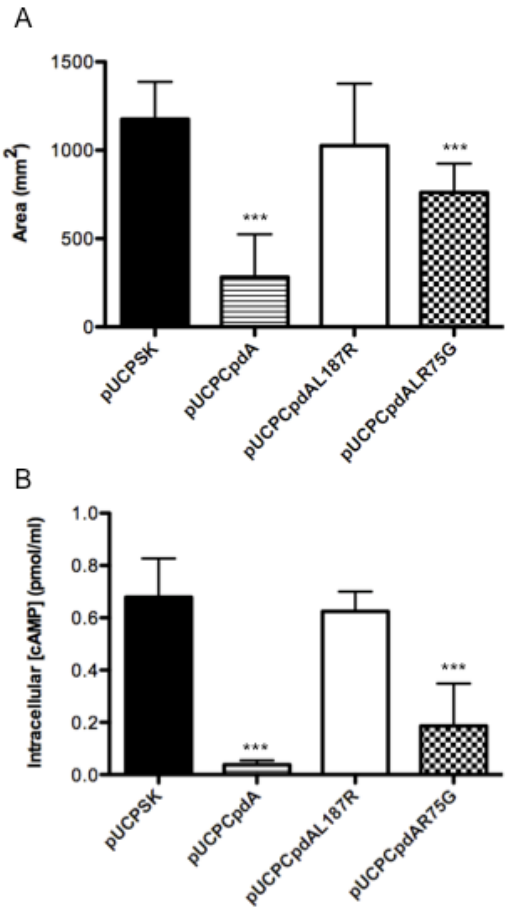

Figure 3-3. *In vivo* activities of CpdA alleles

(A) Sub-surface twitching motility mediated interstitial biofilm expansion at the agar/plastic interface. Areas of interstitial biofilms of PAO1 transformed with pUCPSK, pUCPCpdA, pUCPCpdAL187R and pUCPCpdAR75G were measured after incubation at 37 °C for 24 h. The data is presented as the mean ± SD from three independent experiments performed in triplicate. (*** $p < 0.0001$, Mann-Whitney U test compared with PAO1 transformed with pUCPSK).

(B) Intracellular cAMP concentrations of PAO1 transformed with pUCPSK pUCPCpdA, pUCPCpdAL187R and pUCPCpdAR75G. Data are presented as the mean ± SD for four independent experiments performed in triplicate. (*** $p < 0.0001$, Mann-Whitney U test compared with PAO1 transformed with pUCPSK).

To further investigate how the L187R and R75G substitutions are likely to affect CpdA activity we first generated a homology model of *P. aeruginosa* CpdA. Information on the structure and catalytic mechanism of *Escherichia coli* CpdA has been previously deduced by generating a homology model based on the pig purple acid phosphatase structural template (Richter, 2002). We decided that a similar analysis might yield insights into the effects of the

amino acid substitutions on the activity of the CpdA alleles from PAO1*fimL*$_{Rev1}$ and PAO1*fimL*$_{Rev2}$. The primary sequences of CpdA homologs from *E. coli, Enterobacter aerogenes* and *Mycobacterium tuberculosis* were aligned with CpdA from *P. aeruginosa* using ClustalW (Thompson et al., 1994) then adjusted manually to allow for minor poor matches caused by the *E. aerogenes* GpdQ sequence (Figure 3-4A). Marked on the alignment are the five highly conserved and histidine-rich metal ion binding sites and the leucine187 or arginine75 that are substituted with arginine or glycine in PAO1*fimL*$_{Rev1}$ and PAO1*fimL*$_{Rev2}$, respectively. From this alignment it is clear that leucine187 and arginine75 are highly conserved in all of these CpdA homologs, suggesting that substitutions at these positions may affect the ability of the enzyme to utilise cAMP as a substrate.

The CpdA homolog in *M. tuberculosis* is the cyclic nucleotide phosphodiesterase Rv0805 and in *E. aerogenes* is the glycerophosphodiesterase GpdQ. Crystal structures of both Rv0805 and GpdQ have been solved (Shenoy et al., 2007, Podobnik et al., 2009, Jackson et al., 2007). Although the *E. aerogenes* esterase does not hydrolyze cAMP it does belong to the same metallo-phosphoesterase family of enzymes that hydrolyses the 3'-5' phosphodiester bond of glycerophoesters (Jackson et al., 2007). Thus, GpdQ contains the same α/β barrel metallophosphoesterase fold as Rv0805 from *M. tuberculosis*. Despite their different substrate specificities, the core and homodimer interfacial regions of GpdQ and Rv0805 are strikingly similar, with only a 2.1 Å r.m.s deviation between the dimers of the two structures (Podobnik et al., 2009).

A homology model of *P. aeruginosa* CpdA was generated using the comparative alignment of Rv0805 and CpdA (Figure 3-4A). The structure of *M. tuberculosis* Rv0805 (PDB: 3IB8) was then used as the template to build the CpdA 3-dimensional structure with Modeller (Sali and Blundell, 1993). The rendered homology model of the complete homodimer of wildtype CpdA from *P. aeruginosa* is depicted in Figure 3-4B.

We then explored the predicted effects of the *cpdA* SNPs present in PAO1*fimL*$_{Rev1}$ (L187R) and PAO1*fimL*$_{Rev2}$ (R75G). The highly conserved L187 occurs at the C-terminus of α-helix 6, and is buried in the hydrophobic core of the protein (Figure 3-4C). The L187R amino acid substitution, in placing the large and hydrophilic arginine side chain in this buried location, would likely destabilise the structural integrity of the surrounding region. This includes secondary structural elements, α5, α6, β8, and β9, which provide anchor points for loops involved in formation of the substrate-binding pocket (Figure 3-4B,C). Thus, while L187 is somewhat remote from the active site, its mutation could affect function via its influence on

the structure and/or dynamics of these important secondary structural elements. In addition, the hydrophobic pocket in which L187 resides also includes β-strand 9 and the loop joining it to β-strand 8, which form a significant part of the dimer interface, interacting with β-strand 11' from the opposite protomer in the homodimer (Figure 3-4C). Thus, the L187R substitution could also influence enzyme function by affecting the dimer interface, the dimer being the active form of the enzyme.

R75 occurs in α-helix 2 (Figure 3-4B) and may be involved in stabilizing the loop at the N-terminus of α-helix 1 through a salt bridge to D28 and a hydrogen bond to the backbone carbonyl oxygen of E27 (Figure 3-4D). This loop contains R31, which is in close proximity to the phosphate group of the bound cAMP, and therefore may be involved in catalysis (Figure 3-4D). The R75G substitution could therefore destabilize the geometry of the active site and effect substrate binding and/or catalysis at the active site.

3.3.4 Extragenic suppressor mutations of *fimL* are not in *cyaA*, *cyaB*, *vfr*, *pilG* or *pilH*

Our analyses have revealed that PAO1*fimL*$_{Rev1}$ and PAO1*fimL*$_{Rev2}$ have acquired SNPs in *cpdA* that lead to either loss-of-function in the CpdAL187R allele from PAO1*fimL*$_{Rev1}$ or reduced activity in the CpdAR75G allele from PAO1*fimL*$_{Rev2}$. The SNP present in PAO1*fimL*$_{Rev1}$ appears to fully account for the phenotypic reversion of this strain as introduction of the wildtype CpdA allele restores the parental *fimL* mutant twitching motility and icAMP phenotypes. Interestingly, the parental *fimL* phenotypes were not restored to any of the remaining revertants, including PAO1*fimL*$_{Rev2,}$ by introduction of pUCPCpdA indicating that at least one other site of extragenic suppressor mutation exists in these strains. As all revertants have elevated icAMP levels, the site(s) of extragenic suppressor mutation in these strains likely occurs in a gene encoding a factor involved in controlling intracellular cAMP levels. In an attempt to identify these alternate sites of extragenic suppressor mutation we cloned and sequenced the coding genes and upstream promoter regions of *cyaA*, *cyaB*, *vfr*, *pilG* and *pilH* from our PAO1 strain and *fimL* revertants PAO1*fimL*$_{Rev2}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$, and PAO1*fimL*$_{Rev5}$. No mutations were identified in any of these regions compared to the wildtype *P. aeruginosa* PAO1 reference sequence (Winsor et al., 2011, Stover et al., 2000). Therefore the sites of extragenic suppressor mutation in these *fimL* revertants must occur within other components that are involved in modulation of icAMP levels.

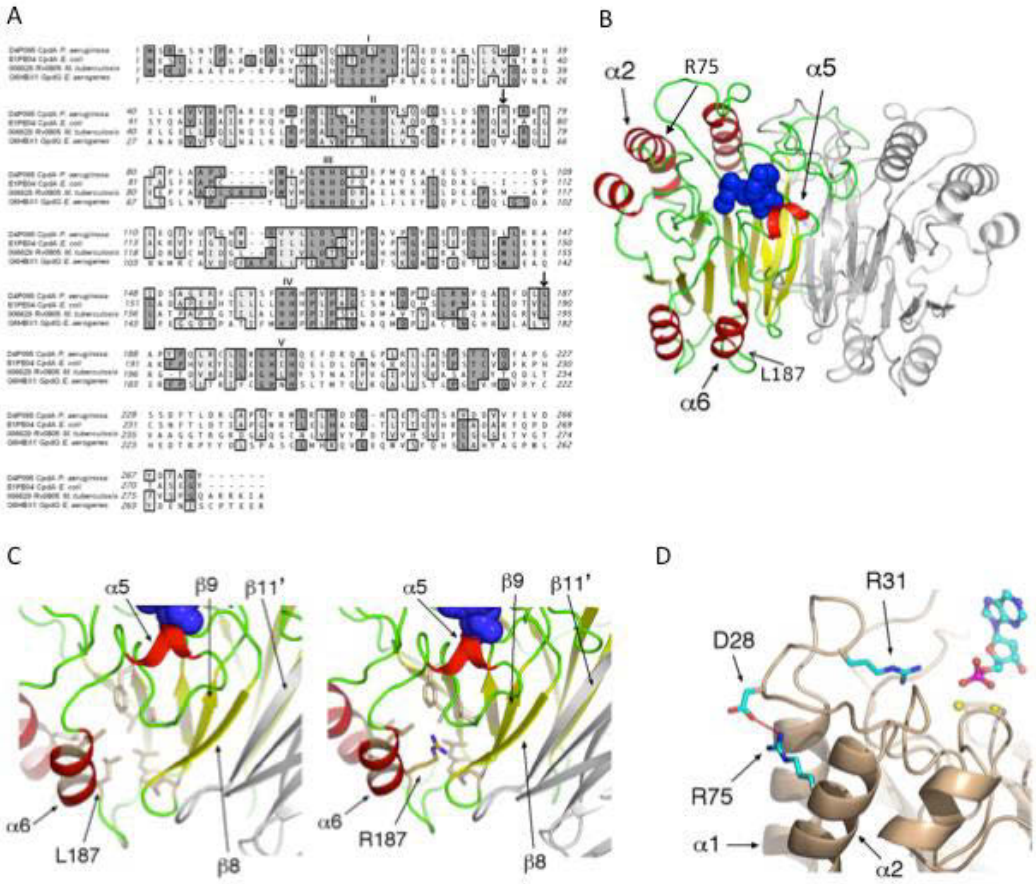

Figure 3-4. Homology modeling of CpdA alleles

(A) ClustalW alignment of *P. aeruginosa* CpdA and homologs. ClustalW alignment of primary amino acid sequences from (top to bottom): 3'-5'-cAMP phosphodiesterase from *P. aeruginosa* (CpdA Uniprot ID: D4P095); 3'-5'-cAMP phosphodiesterase from *E. coli* (CpdA Uniprot ID: E1PE04); 3'-5'-cAMP phosphodiesterase from *M. tuberculosis* (lcc Uniprot ID: 006629); and glycerophosphodiesterase from *E. aerogenes* (phosphohydrolase Uniprot ID: Q6XBH1). The alignment was manually adjusted to fit minor mismatches. The boxed residues indicate identity (dark grey) or similarity (light grey). The five conserved, histidine-rich regions that make up the metal ion binding sites are numbered in Roman numerals. The conserved L187 and R75 are arrowed. The final thirty-two residues for the *M. tuberculosis* phosphodiesterase (006629) are deleted as there is not an equivalent C-terminal sequence in the other three proteins.

(B) Homology model of CpdA from *P. aeruginosa* using the Rv0805 resolved structure (PDB: 3IB8) from *M. tuberculosis* as the template. The homodimer is shown with one protomer coloured and the other in greyscale. Helices are in red, strands in yellow, and loops in green. 5'-cAMP was co-crystallized in the active site and is shown as space-filled blue spheres. The α-strands 4, 5 and 6 are indicated as are the L187 and R75 residues.

(C) *P. aeruginosa* CpdA homology model showing the amino acid substitution L187R. Model depicts a close-up section of the CpdA dimer with one protomer in colour and the other in

greyscale. Left panel shows the conserved L187 residue and relevant secondary structural elements indicated by arrows and right panel shows the substituted R187 residue and relevant secondary structural elements indicated by arrows.

(D) Model depicts a close-up section of one of the CpdA protomers. The residues D28, R31, R75 and their positively-charged side-chains are indicated, as are relevant secondary structural elements.

3.4 Discussion

In this study we have characterised five independent *fimL* twitching motility revertants and determined that each revertant has increased icAMP levels compared to the parental *fimL* mutant. Interestingly, the icAMP levels differed between the revertants, with three of the five displaying extremely high levels of icAMP. Furthermore, whilst macroscopic sub-surface stab assays demonstrated that each revertant had restored twitching motility-mediated interstitial biofilm expansion to almost wildtype levels, microscopic examination revealed differences in the micromorphological patterning of the expanding biofilms. Sequencing of the whole genome of PAO1*fimL*$_{Rev1}$, identified a number of SNPs, including one in *cpdA* which encodes a cAMP phosphodiesterase. We demonstrated that this SNP (CpdAL187R) results in a loss-of-function mutation of CpdA, and is responsible for the observed increase in icAMP levels and restoration of twitching motility in PAO1*fimL*$_{Rev1}$. We also determined that a second revertant, PAO1*fimL*$_{Rev2}$, possesses a different SNP in *cpdA* (CpdAR75G) which likely results in reduced activity of CpdA. However, it is not clear if this SNP accounts for the increased icAMP levels and restoration of twitching motility in PAO1*fimL*$_{Rev2}$ as introduction of wildtype CpdA to this revertant did not restore the parental *fimL* mutant phenotypes. These observations suggested that either the SNP in *cpdA* is not the primary site of mutation responsible for the phenotypic reversion or, alternatively, that the CpdAR75G allele has a dominant negative influence over the introduced wildtype CpdA allele. However, we found that when the CpdAR75G allele was introduced into a wildtype *cpdA* background, the levels of icAMP were reduced rather than elevated. This indicates that the CpdAR75G allele possesses some cAMP phosphodiesterase activity and that this allele is not dominant negative over the wildtype allele. It is probable, therefore, that the primary site of suppressor mutation occurs elsewhere in the genome of PAO1*fimL*$_{Rev2}$.

Interestingly, the three remaining revertants (PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev4}$ and PAO1*fimL*$_{Rev5}$) were found to have wildtype *cpdA* sequences indicating that the suppressor

mutation has occurred elsewhere in the genomes of these strains. The observed differences in the twitching motility and icAMP phenotypes of the five revertants are also consistent with the possibility that there are at least two mechanisms of reversion. PAO1*fimL*$_{Rev1}$, PAO1*fimL*$_{Rev3}$, PAO1*fimL*$_{Rev5}$ each have very high levels of icAMP and near wildtype twitching motility when examined microscopically, yet only PAO1*fimL*$_{Rev1}$ has acquired a SNP in *cpdA*. Furthermore, PAO1*fimL*$_{Rev2}$ has 2-fold higher icAMP levels relative to PAO1 and slightly aberrant twitching motility whereas PAO1*fimL*$_{Rev4}$ has wildtype icAMP levels but very aberrant twitching motility when examined microscopically.

Sequencing did not reveal any mutations in the coding and upstream promoter regions of *cyaA*, *cyaB*, *vfr*, *pilG* and *pilH* in the *fimL* revertants indicating that the site(s) of extragenic suppressor mutation must occur within another gene encoding a factor that is involved in control of icAMP levels in *P. aeruginosa*. The Chp chemosensory system which has been shown to be involved in controlling icAMP levels and twitching motility (Fulcher et al., 2010, Darzins, 1993, Darzins, 1994, Darzins, 1995, Whitchurch et al., 2004), encompasses an approximately 15 Kb region. It is possible that the alternate site(s) of extragenic suppressor mutation is present within this region, however, it is also possible that there are additional components involved in controlling icAMP that are yet to be identified.

In this study we have investigated the mechanism of twitching motility reversion in five independent *P. aeruginosa fimL* mutants. This revealed that increasing intracellular cAMP levels is a general mechanism of twitching motility reversion in *fimL* mutants and that this increase can occur via at least two different mechanisms. cAMP is an important second messenger signal associated with virulence factor regulation in *P. aerginosa*. Allosteric regulation by cAMP of the transcriptional activator, Vfr (West et al., 1994) controls the expression of a large range of virulence-associated genes (Beatson et al., 2002a, Albus et al., 1997, Dasgupta et al., 2002, Wolfgang et al., 2003) and it is therefore imperative that icAMP homeostasis is tightly controlled. Regulation of the adenylate cyclases CyaA and CyaB and the phosphodiesterase CpdA which synthesise and breakdown cAMP, respectively, (Wolfgang et al., 2003, Fulcher et al., 2010, Fuchs et al., 2010a) is mediated by a number of complex, intersecting pathways (Fulcher et al., 2010, Inclan et al., 2011), which is still not fully understood. Identification of the sites of extragenic suppressor mutation in the *fimL* mutant revertants that do not contain SNPs in *cpdA* will further increase our understanding of how icAMP levels and twitching motility are regulated to ultimately contribute to the overall virulence of *P. aeruginosa*.

Chapter Four

Extracellular ATP inhibits twitching motility in *Pseudomonas aeruginosa*

All experiments and analysis of results presented in this chapter have been performed by Laura Nolan.

This chapter is in preparation for submission to BMC Microbiology. The only changes made to the manuscript text have been in conversion to a Thesis format.

Movie files are included within the results presented in this chapter as referenced in the text (see Appendix 1 for Movie files).

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Dervilla McGowan was responsible for generation of the PAK*chpC* mutant and Jennifer Sargent was responsible for generation of the PAK*pilH* mutant used in this Chapter.

4.1 Abstract

Background

Pseudomonas aeruginosa is an opportunistic pathogen that exploits damaged epithelia to cause infection. Type IV pili (tfp) are polarly located filamentous structures which are the major adhesins for attachment of *P. aeruginosa* to epithelial cells. The extension and retraction of tfp powers a mode of surface translocation termed twitching motility that is involved in biofilm development and also mediates the active expansion of biofilms across surfaces. Extracellular adenosine triphosphate (eATP) is a key "danger" signalling molecule that is released by damaged epithelial cells to alert the immune system to the potential presence of pathogens. As *P. aeruginosa* has a propensity for infecting damaged epithelial tissues we have explored the influence of eATP on tfp biogenesis and twitching motility of *P. aeruginosa*.

Results

In this study we have found that eATP inhibits *P. aeruginosa* twitching motility at levels that are not inhibitory to growth. We have determined that eATP does not inhibit expression of the tfp major subunit, PilA, but reduces the levels of surface assembled tfp. Additionally, our results demonstrate that the active twitching zone of expanding *P. aeruginosa* biofilms contain large quantities of eATP which may serve as a signalling molecule to coordinate cell movements in the expanding biofilm.

Conclusions

Endogenous eATP produced by *P. aeruginosa* serves as a signalling molecule to coordinate complex multicellular behaviours of this pathogen. Given the propensity for *P. aeruginosa* to infect damaged epithelial tissue, our observations suggest that eATP released by damaged cells may provide a cue to reduce twitching motility of *P. aeruginosa* in order to establish infection at the site of damage. Furthermore, eATP produced by *P. aeruginosa* biofilms and by damaged epithelial cells may play a role in *P. aeruginosa* pathogenesis by inducing inflammatory damage and fibrosis. Our findings have significant implications in the development and pathogenesis of *P. aeruginosa* biofilm infections.

Keywords: 3',5'-adenosine triphosphate, ATP, twitching motility, Type IV pili, tfp.

4.2 Background

Pseudomonas aeruginosa is a Gram negative bacterium found throughout the environment and is an opportunistic pathogen of a wide variety of eukaryotic hosts (Mahajan-Miklos et al., 2000, Rahme et al., 2000). *P. aeruginosa* causes acute and chronic infections in immunocompromised humans and is the major contributor to the morbidity and mortality of individuals with cystic fibrosis (Lyczak et al., 2000, Tatterson et al., 2001). *P. aeruginosa* establishes infections at sites of epithelial tissue damage such as is encountered in patients with severe burns, mechanical ventilation, or corneal damage due to contact lens use (Ramphal and Pyle, 1983, Kazmierczak et al., 2001). *P. aeruginosa* can establish chronic infections which are associated with the formation of complex, surface-associated communities termed biofilms (Costerton et al., 1999).

Type IV pili (tfp) are the major adhesins that promote attachment to host epithelial cells. These polar, filamentous surface structures also mediate a form of surface translocation termed twitching motility that occurs through the extension, surface binding and retraction of tfp (Whitchurch, 2006). The active expansion of *P. aeruginosa* biofilms is a complex, multicellular, collective behaviour that is mediated by twitching motility (Semmler et al., 1999, Gloag et al., 2013). A number of host derived signals including mucin, serum albumin, oligopeptides, phosphatidylcholine (PC), lactoferrin and low levels of available iron have been shown to stimulate twitching motility by *P. aeruginosa* (Barker et al., 2004, Hammond et al., 2010, Huang et al., 2003, Patriquin et al., 2007) . Interestingly, mucin, serum albumin, lactoferrin, and low iron have also been shown to inhibit the formation of sessile biofilms (Hammond et al., 2010, Huang et al., 2003, Singh et al., 2002, Patriquin et al., 2007). It has been suggested that the effects of these host-derived compounds may provide a protective advantage to the host by inhibiting the ability of *P. aeruginosa* to form resistant, sessile biofilms, and thus giving the immune system a better chance of clearing the infection (Singh et al., 2002).

Another host-derived signal, extracellular 3',5'-adenosine triphosphate (eATP) is rapidly released in high levels by stressed or injured epithelial cells to alert the host to the presence of invading pathogens, resulting in the recruitment of immune system factors to clear the infection (Di Virgilio, 2007, Burnstock, 2007). As *P. aeruginosa* displays a propensity for infecting damaged epithelial tissues (Ramphal and Pyle, 1983) it is therefore likely to encounter high levels of eATP at the site of damaged epithelium where it initiates infection. In this study

we have explored the possibility that eATP may be a host-derived molecule that affects twitching motility in *P. aeruginosa*.

4.3 Results

4.3.1 Extracellular ATP inhibits twitching motility

To determine if eATP influences *P. aeruginosa* twitching motility, we utilised our interstitial biofilm expansion assay to examine the effect of incorporating eATP into the solidified nutrient media. In this assay *P. aeruginosa* cells are inoculated at the interface between a glass coverslip and a microscope slide coated in nutrient media that has been solidified with gellan gum. Twitching motility mediates rapid, active biofilm expansion at the interstitial interface. The resultant biofilm is imaged using phase-contrast microscopy and quantitated using image analysis tools. To determine if eATP affects *P. aeruginosa* PAK twitching motility, varying concentrations of eATP (0 mM-15 mM) were incorporated into the nutrient media. After 16 hrs incubation at 37 ^{o}C the resulting biofilms were imaged (Figure 4-1A) and the surface areas covered by the biofilms calculated (Figure 4-1B). These analyses revealed that eATP concentrations of 5 mM and above dramatically reduced twitching motility-mediated biofilm expansion to levels equivalent to that of PAK*pilA*::TcR (Figure 4-1B) which does not produce tfp and is unable to twitch (Watson et al., 1996c, Semmler et al., 1999). Furthermore, microscopic examination of the interstitial biofilms (Figure 4-1A) shows that at eATP concentrations of 7.5mM and above, the resultant biofilms resemble those of the non-twitching PAK*pilA*::Tc^{R} strain as they no longer form the leading-edge rafts and lattice-like trail network which are characteristic micromorphological features of wild-type *P. aeruginosa* interstitial biofilms in this assay (Semmler et al., 1999, Gloag et al., 2013) (Figure 4-1A). Interestingly in the presence of 2.5 mM and 5 mM eATP the interstitial biofilms had very large leading edge rafts and had lost the ability to self-organise into the characteristic refined trail network (Figure 4-1A).

We determined if the reduction in interstitial biofilm expansion might be a consequence of reduced growth in the presence of eATP. The planktonic growth of *P. aeruginosa* PAK in nutrient media supplemented with eATP at concentrations of 0-15 mM was measured by recording OD_{595nm} over a 20 hr period (Figure 4-1C). These assays show that up to 10 mM eATP does not affect growth, while at 12.5mM and 15 mM ATP the growth rate was slightly reduced. These observations indicate that addition of exogenous eATP at concentrations

between 2.5 mM and 10 mM specifically inhibits twitching motility-mediated biofilm expansion of *P. aeruginosa* while growth remains unaffected.

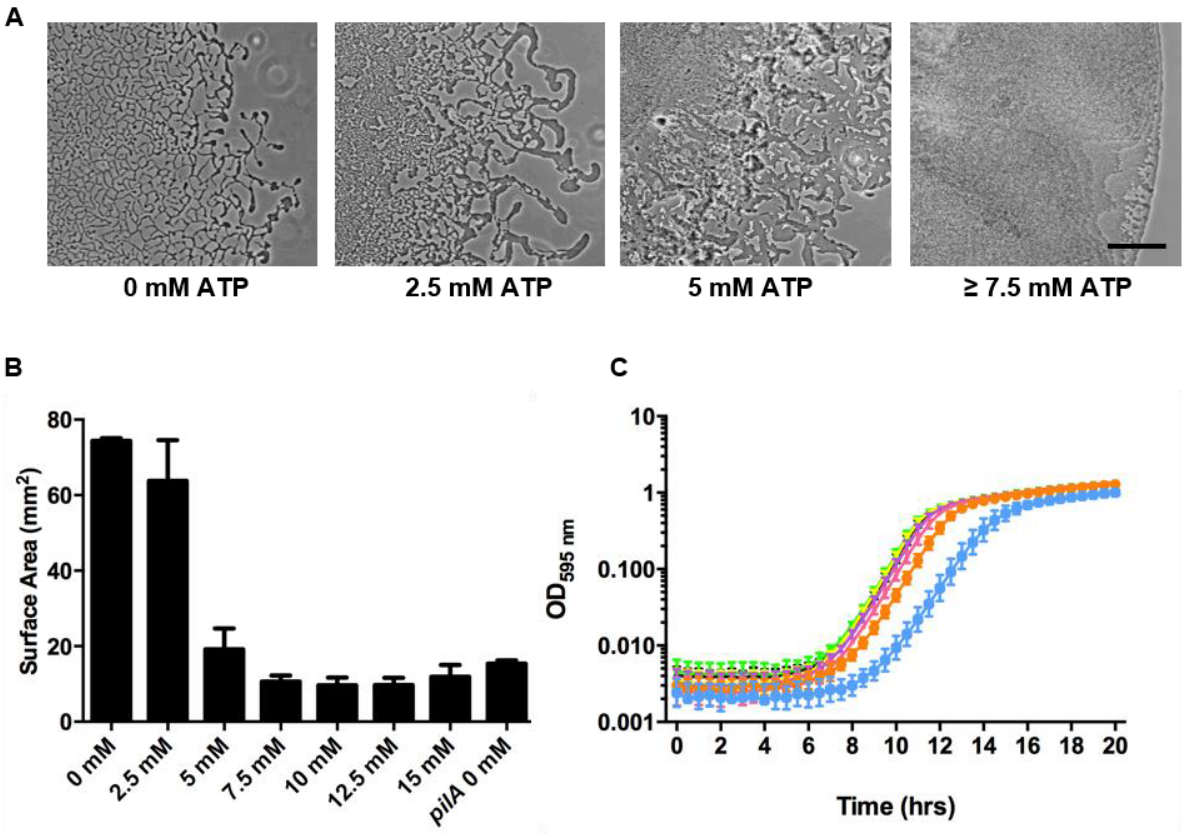


Figure 4-1. Extracellular ATP specifically affects twitching motility

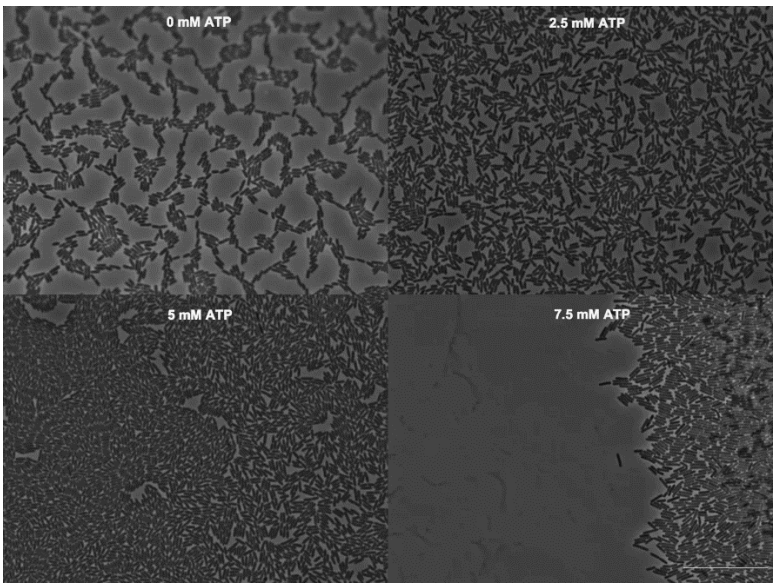
(A) Representative light microscopy images of the twitching edges formed by *P. aeruginosa* PAK at the interstitial space between a microscope slide coated in solidified nutrient media supplemented with a range of eATP concentrations and a coverslip after incubation at 37℃ for 16 hrs. Scale 50 µm.

(B) Surface areas of the twitching motility zones formed by *P. aeruginosa* PAK and PAK*pilA* at the interstitial space between a microscope slide coated in solidified nutrient media supplemented with a range of eATP concentrations and a coverslip after incubation at 37℃ for 16 hrs. Mean ± SD from three independent experiments run in duplicate are presented.

(C) Growth rate of *P. aeruginosa* PAK at 37℃ over 20 hrs in media supplemented with 0 mM ATP (black), 2.5 mM ATP (green), 5 mM ATP (yellow), 7.5 mM ATP (magenta), 10 mM ATP (pink), 12.5 mM ATP (orange) or 15 mM ATP (blue). Mean ± SD from three independent experiments each consisting of five technical replicates is presented.

To further understand the influence of addition of eATP to twitching motility-mediated biofilm expansion, we performed high resolution time-lapse imaging of expanding biofilms in

the presence of 0 mM, 2.5 mM, 5 mM and 7.5 mM eATP (Movie 4-1). In the absence of eATP, cells within the trails behind the leading-edge rafts, maintain a lattice-like network, which is characteristic of twitching motility-mediated interstitial biofilm expansion (Semmler et al., 1999, Gloag et al., 2013). However, at eATP concentrations of 2.5 mM and 5 mM, cells within the trails are no longer able to maintain this lattice-like network. While most of the cells are still moving in the presence of 2.5 mM and 5 mM eATP, cells have lost the ability to coordinate their movement and instead appear to be moving randomly. At an eATP concentration of 7.5 mM cells do not appear to be moving at all, and instead have formed a biofilm edge which is similar to that of a non-twitching PAK*pilA*::Tc^R strain (Semmler et al., 1999). These results suggest that eATP concentrations of 2.5 mM and greater cause a loss of coordinated movement, which affects the ability of *P. aeruginosa* cells to effectively undergo twitching motility-mediated biofilm expansion.



Movie 4-1. Exogenous ATP affects twitching motility of *P. aeruginosa*

Time-lapse phase microscopy of *P. aeruginosa* PAK twitching motility-mediated biofilm expansion at the interstitial space between a solidified nutrient media-coated microscope slide containing 0 mM, 2.5 mM, 5 mM or 7.5 mM ATP and a coverslip at 37 ℃. Scale bar is 20 μm. Original movie 15 mins, played back at 50x (see Appendix 1 for Movie file).

4.3.2 Extracellular ATP decreases surface tfp levels

Our observations have indicated that 10 mM eATP causes twitching motility to cease but does not affect growth. One mechanism via which this may occur is through modulation

of tfp production. To investigate this, levels of surface assembled tfp of wild-type PAK cultured on agar containing 0 mM or 10 mM eATP was assessed by ELISA of whole cells and Western analysis of whole cells and sheared tfp using antisera against the major pilin subunit PilA (Figure 4-2A, B). These analyses indicate that levels of surface assembled tfp are reduced in the presence of 10 mM eATP (Figure 4-2A, B). Western analysis of whole cells using anti-PilA antisera indicates that the levels of cell-associated pilin are not altered by 10 mM eATP (Figure 4-2B). These observations suggest that eATP does not affect tfp pilin expression but rather is likely to be reducing tfp assembly or increasing tfp retraction resulting in the observed reduction of surface-assembled tfp levels.

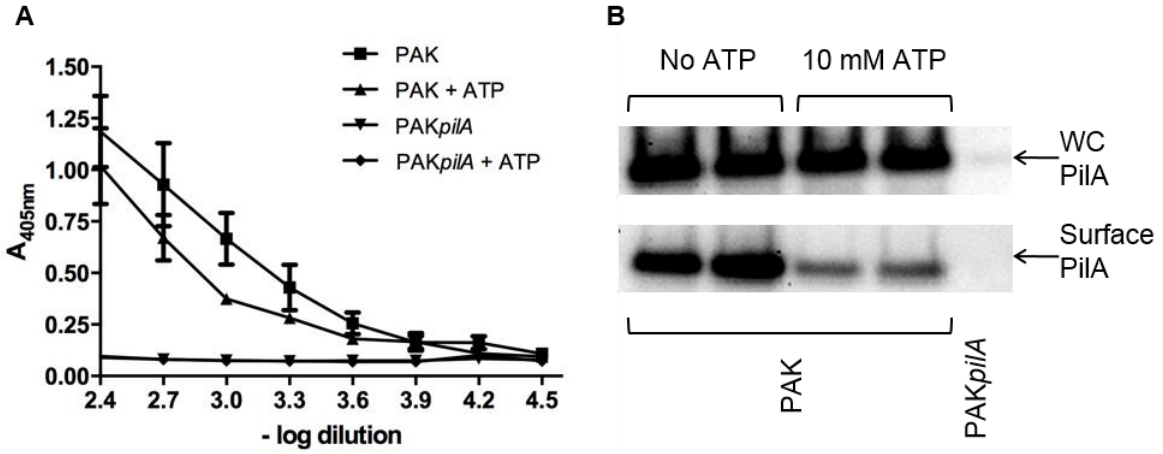


Figure 4-2. Extracellular ATP decreases surface PilA levels

(A) ELISA of whole cells from *P. aeruginosa* strains PAK and PAK*pilA* obtained from overnight (20 hr) confluent lawns grown at 37℃ on LB agar plates containing 0 mM or 10 mM ATP. Surface pilin were detected with anti-PilA serum. Equal amounts of cells were used in each ELISA. The graphed data is presented as the mean ± SD and is representative of results obtained from four independent experiments run in triplicate.

(B) Immunoblot of pilin detected in whole cell (WC) preparations (top panel) and in sheared surface pili preparations (bottom panel) of strains PAK (lanes 1-4) and PAK*pilA* (lane 5) obtained from overnight (20 hr) confluent lawns grown at 37 ℃ on LB agar plates containing 0 mM or 10 mM ATP. Equal amounts of cells were used in each assay. The results shown in the immunoblots are representative of results obtained in four independent experiments run in duplicate.

4.3.3 The active twitching motility zone contains endogenous eATP

Our time-lapse microscopy of *P. aeruginosa* twitching motility in the presence of exogenous eATP indicates that addition of eATP at 2.5-5 mM into the solidified nutrient media

appears to confuse cell movement while an eATP concentration of 7.5 mM causes cell movement to cease (Movie 4-1). A possible explanation for this phenomenon is that a gradient of eATP within the active migration zone acts as a directional signal for cells to enable them to differentiate which direction to travel to ensure that overall motility is directed toward virgin territory and away from the mature biofilm. At higher concentrations, eATP may also be acting as a cue to indicate that a cell is no longer located at the leading edge and that movement should cease. Interestingly, Semmler et al. (1999) noted that bacterial cells in the mature regions of the biofilm appear to settle, which would be consistent with higher eATP concentrations in this region.

It has been previously reported that exponentially growing strains of *P. aeruginosa* secrete eATP (Hironaka et al., 2013, Mempin et al., 2013). To explore the possibility that *P. aeruginosa* produces endogenous eATP in the actively migrating zone at the edge of the expanding biofilm, we resuspended cells from the actively migrating edge of a colony biofilm and measured the concentration of ATP in the cell-free supernatant. These assays indicate that the active zone of twitching motility at the edge of expanding *P. aeruginosa* PAK colony biofilms contains about 3 mM eATP. Our assay is not sufficiently sensitive to determine if there is a gradient of eATP within this zone. However, given that addition of 2.5-10 mM eATP into the nutrient media appears to inhibit coordinated directional movement and ultimately causing the cells to cease movement, this would be consistent with these levels of exogenous eATP confounding an endogenous eATP gradient within the actively migrating zone.

4.3.4 eATP gradients inhibit twitching motility in approaching biofilms

We have observed that if *P. aeruginosa* is inoculated twice in neighbouring locations on the same media-coated microscope slide that twitching motility at the proximal edges of each adjacent biofilm is inhibited, resulting in a significantly decreased rate of expansion of the proximal edges compared to the distal edges (Figure 4-3). This suggests that an endogenously produced extracellular signal(s) is able to diffuse through the media to repress twitching motility mediated expansion of the neighbouring biofilms. Given our observations that *P. aeruginosa* biofilms contain endogenously produced eATP we hypothesised that *P. aeruginosa* biofilms may establish a diffused gradient of eATP beyond the colony edge that is sufficient to inhibit twitching motility mediated expansion of approaching biofilms.

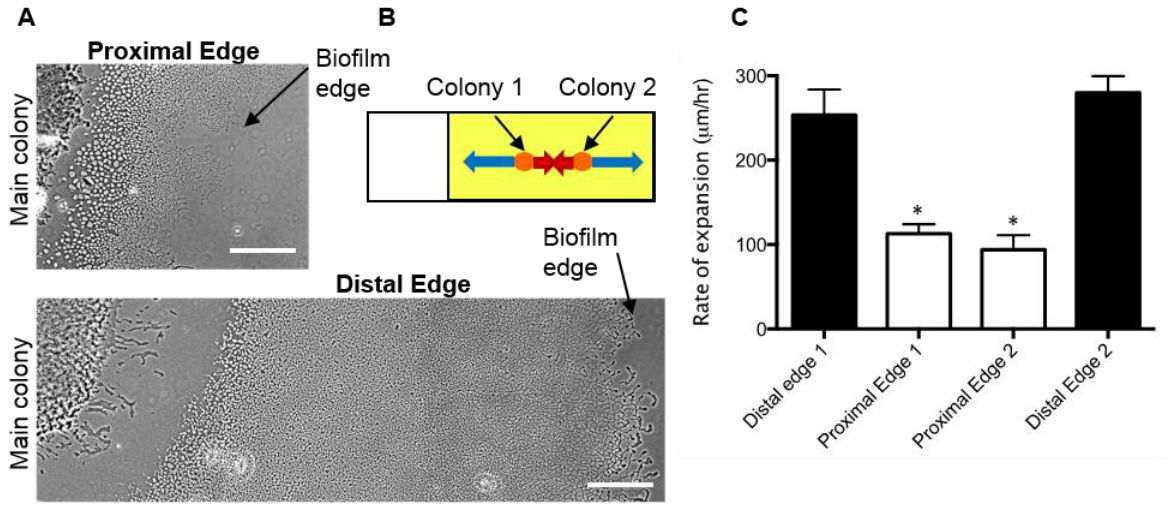


Figure 4-3. Self-produced extracellular signals inhibit twitching motility-mediated expansion of *P. aeruginosa* biofilms

Inoculation of *P. aeruginosa* PAK at two adjacent locations on a solidified media-coated microscope slide results in twitching motility-mediated expansion of two neighbouring biofilms at the interstitial space between the media and coverslip.

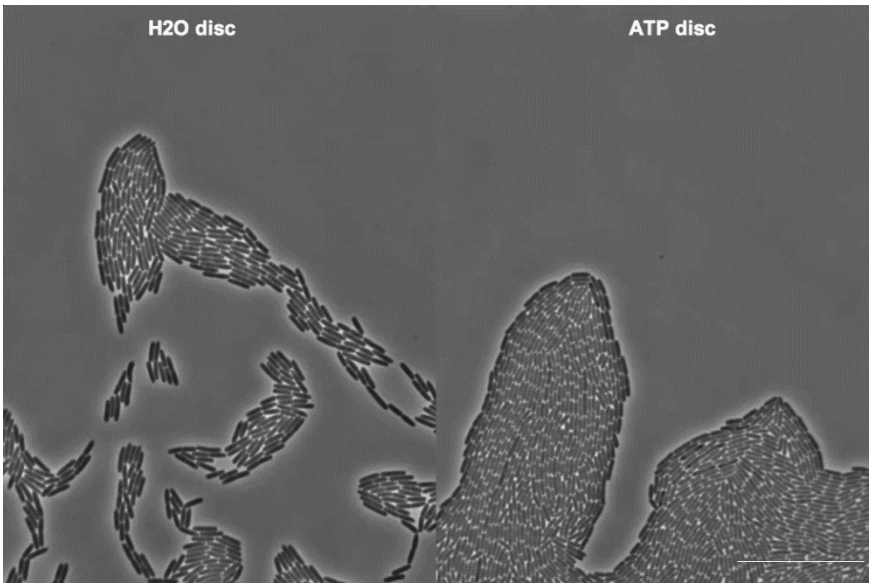
(A) Representative light microscopy images of the twitching motility zones formed at the proximal and distal edges of two neighbouring biofilms after incubation at 37℃ for 6 hrs. Scale is 200 µm. Images are representative of the twitching motility zones formed in three independent experiments.

(B) Assay setup: Two *P. aeruginosa* PAK colonies inoculated on a nutrient media-coated microscope slide expand to form two neighbouring interstitial biofilms at the proximal and distal edges of the inoculation points.

(C) The rate of expansion via twitching motility away from the main colony at the proximal and distal edges of two neighbouring interstitial biofilms after incubation at 37℃ for 6 hrs. Mean ±SD is presented from three independent experiments (two-tailed Student's t-test, *$p<0.05$).

To explore the possibility that gradients of eATP inhibit twitching motility of approaching biofilms we established an eATP gradient on media coated slides using ATP-saturated filter discs. Filter discs saturated in H_2O were also used to control for the possible influence of increased water content of the medium surrounding the filter discs. *P. aeruginosa* PAK was then inoculated at a fixed distance from the saturated filter discs and a coverslip applied at the edge of the filter disc to establish the interstitial biofilm as per our standard protocol. Time-lapse microscopy was used to examine the effect of the eATP or H_2O-saturated discs on twitching motility over time (Movie 4-2). This movie demonstrates that while cells

continue to flow through the trail network to expand the biofilm towards the H_2O-saturated disc, in the presence of an eATP-saturated disc the cells essentially stop twitching. The wild-type *P. aeruginosa* strains PAO1, PA14 and PA103 were also inhibited by gradients of eATP (Figure 4-4). This indicates that inhibition of twitching motility by eATP is conserved across *P. aeruginosa* strains.



Movie 4-2. Extracellular ATP gradients affect twitching motility of *P. aeruginosa*

Time-lapse phase microscopy of *P. aeruginosa* PAK twitching motility-mediated biofilm expansion at the interstitial space between a solidified nutrient media-coated microscope slide and a coverslip in the presence of an ATP- or H_2O-saturated disc after 4 hrs of incubation at 37 ℃. Scale bar is 20 μm. Original movie 30 mins, played back at 90x (see Appendix 1 for Movie file).

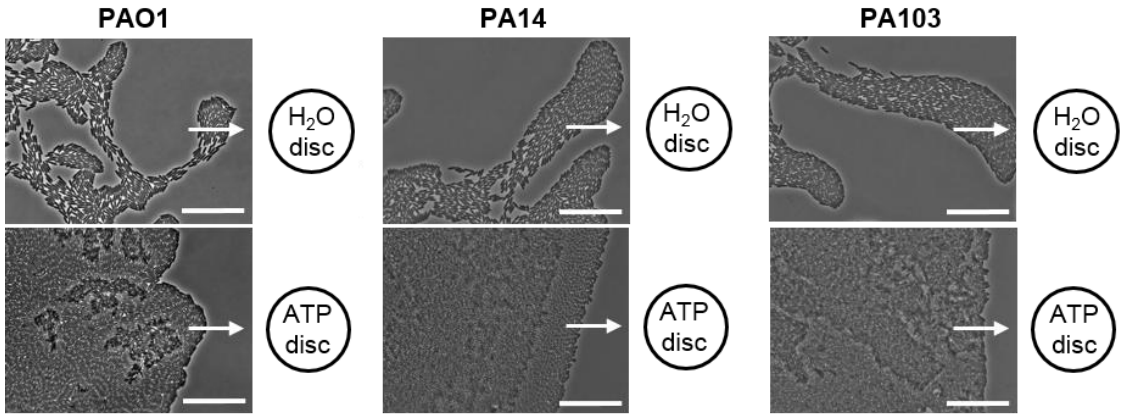


Figure 4-4. Extracellular ATP inhibits twitching motility of a range of wild-type *P. aeruginosa* strains

Phase microscopy of twitching motility-mediated biofilm expansion of *P. aeruginosa* PAO1, PA14 and PA103 in the presence of a H_2O-saturated disc (upper images) or ATP-saturated disc (lower images) at the interstitial space between a solidified media-coated microscope slide and coverslip after incubation for 15 hrs at 37℃. Scale bar is 50 μm and the arrows indicate the direction of expansion towards the disc. Images are representative of three independent experiments.

Our observations suggest that *P. aeruginosa* has the capacity to modulate twitching motility in response to a gradient of eATP concentrations. *P. aeruginosa* twitching motility is regulated by a putative chemosensory system, the Chp system (Whitchurch et al., 2004, Darzins, 1993, Darzins, 1994, Darzins, 1995) which is homologous to the Che chemosensory system which controls swimming motility of *Escherichia coli* in response to environmental stimuli (Baker et al., 2006, Wadhams and Armitage, 2004). In *P. aeruginosa* the core signalling components of this system include a putative histidine kinase encoded by *chpA* (Whitchurch et al., 2004). ChpA is predicted to be coupled to a methyl-accepting chemotaxis protein (MCP) receptor, PilJ, by one of two CheW adaptor protein homologues, PilI and ChpC (Whitchurch et al., 2004). This complex is predicted to sense a currently unknown environmental signal which results in PilJ undergoing a conformational change, causing ChpA to be autophosphorylated, with the resulting phosphates being transferred to two CheY-like response regulators, PilG and PilH (Darzins, 1993, Darzins, 1994). These phosphorylated CheY-homologs are thought to interact with the tfp motor complex to mediate tfp extension and retraction (Whitchurch et al., 2004, Guzzo et al., 2009, Bertrand et al., 2010). Adaptation to the environmental signal(s) is predicted to be mediated through methylation of PilJ by the competing activities of the methyltransferase PilK and the methylestrase ChpB (Darzins, 1995,

Whitchurch et al., 2004). The Chp system gene cluster also encodes the genes *chpD* and *chpE* which appear to be part of the operon encoding the Chp system but these are not homologous to components of other chemosensory systems and their function have not as yet been determined (Whitchurch et al., 2004).

We explored the possibility that the Chp system may be involved in control of twitching motility in response to eATP by assaying mutants of the system that retained near wild-type levels of twitching motility to determine if any of these had lost the ability to respond to eATP. It was not possible to examine mutants of the core components PilG, PilH, PilI, PilJ and ChpA as these are severely defective in twitching motility (Whitchurch et al., 2004, Darzins, 1993, Darzins, 1994). However, PilK, and ChpB are predicted to provide the adaptation mechanism that enables response to a chemical gradient, therefore we predicted that if eATP is indeed sensed by this system it is likely that mutants of *pilK* and *chpB* will show defective responses.

The effects of eATP on the twitching motility of isogenic PAK mutants of the Chp system (*chpB*, *chpC*, *chpD*, *chpE* and *pilK*) were examined in our interstitial biofilm expansion assay in the presence of 7.5 mM eATP incorporated into the media and in our filter disc gradient assay. These assays revealed that all strains showed similar responses to wild-type PAK to both a constant concentration of eATP and an eATP gradient (Figure 4-5). This suggests that the Chp chemosensory system is not responsible for control of twitching motility in response to eATP. However, we cannot rule out the possibility that the core components of the Chp system (PilG, PilH, PilI, PilJ and ChpA) are involved.

Twitching motility is modulated by levels of intracellular cAMP (icAMP) (Nolan et al., 2012, Inclan et al., 2011, Fulcher et al., 2010) which are controlled by the adenylate cyclases CyaA and CyaB that catalyse the conversion of ATP to cAMP, and the phosphodiesterase CpdA which degrades cAMP (Wolfgang et al., 2003, Fuchs et al., 2010a). It is possible that eATP is modulating intracellular icAMP through these enzymes. We therefore examined the effect of eATP on the twitching motility of isogenic PAK mutants of the icAMP synthesis/degradation system (*cyaA*, *cyaB*, *cyaAB* and *cpdA*) in our interstitial biofilm expansion assay in the presence of 7.5 mM eATP incorporated into the media and in our filter disc gradient assay. These assays revealed that all strains showed similar responses to wild-type PAK to both a constant concentration of eATP and an eATP gradient (Figure 4-5). This suggests that the icAMP synthesis/degradation system is not responsible for control of twitching motility in response to eATP.

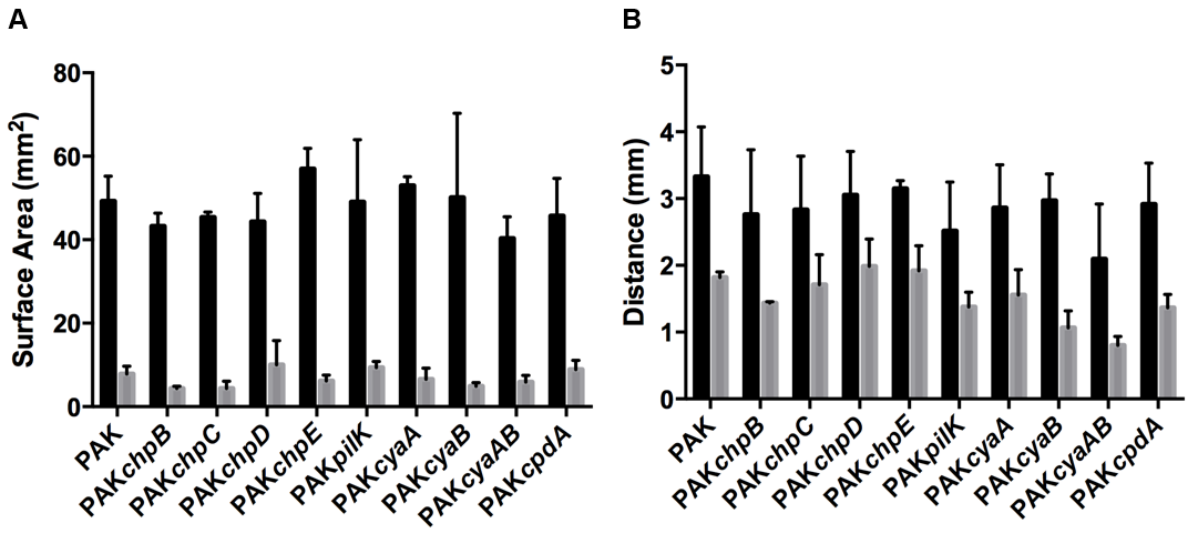


Figure 4-5. The twitching motility response of a range of mutants to eATP

The twitching motility response of PAK, PAK*chpB*, PAK*chpC,* PAK*chpD,* PAK*chpE*, PAK*pilK,* PAK*cyaA,* PAK*cyaB*, PAK*cyaAB* and PAK*cpdA* to eATP at the interstitial space between a solidified nutrient media-coated microscope slide and a coverslip after incubation for 15 hrs at 37℃. (A) Surface area of twitching motility-mediated biofilms formed in the absence of eATP (black bars) a constant concentration of 7.5 mM eATP (grey bars); (B) biofilm expansion towards a H_2O-saturated disc (black bars) or ATP-saturated disc (grey bars). The data are represented as the mean ± SEM for three independent experiments.

4.3.5 Effects of other nucleotides on twitching motility

To determine whether the observed inhibition of twitching motility was specific for eATP or whether other nucleotides had equivalent effects, gradients of a range of nucleotides were generated using our filter disc gradient assay. Adenosine monophosphate (AMP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), and uridine triphosphate (UTP), did not have any effect on twitching motility (Figure 4-6). The intracellular second messenger signals bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) and 3',5'-cyclic adenosine monophosphate (cAMP), which are involved in controlling a transition between motile and sessile modes of life (Simm et al., 2004) and in tfp assembly and function (Fulcher et al., 2010, Nolan et al., 2012, Inclan et al., 2011), respectively, also had no effect on twitching motility (Figure 4-6). Interestingly, deoxyadenosine triphosphate (dATP) and cytidine triphosphate (CTP) had similar inhibitory effects as eATP on twitching motility of the approaching biofilms (Figure 4-6).

To determine if the observed inhibition of twitching motility by eATP required hydrolysis of the molecule, we examined the effect of the non-hydrolysable ATP analogue, adenosine-5'-(β,γ-imido) triphosphate (AMP-PNP) in our filter disc gradient assay. We found that the non-hydrolysable ATP analogue did not affect twitching motility indicating that the observed inhibition of eATP on twitching motility requires hydrolysis of the molecule (Figure 4-6). This suggests that the twitching motility response of *P. aeruginosa* to eATP is more likely to be mediated by a metabolic process as opposed to a signal transduction system.

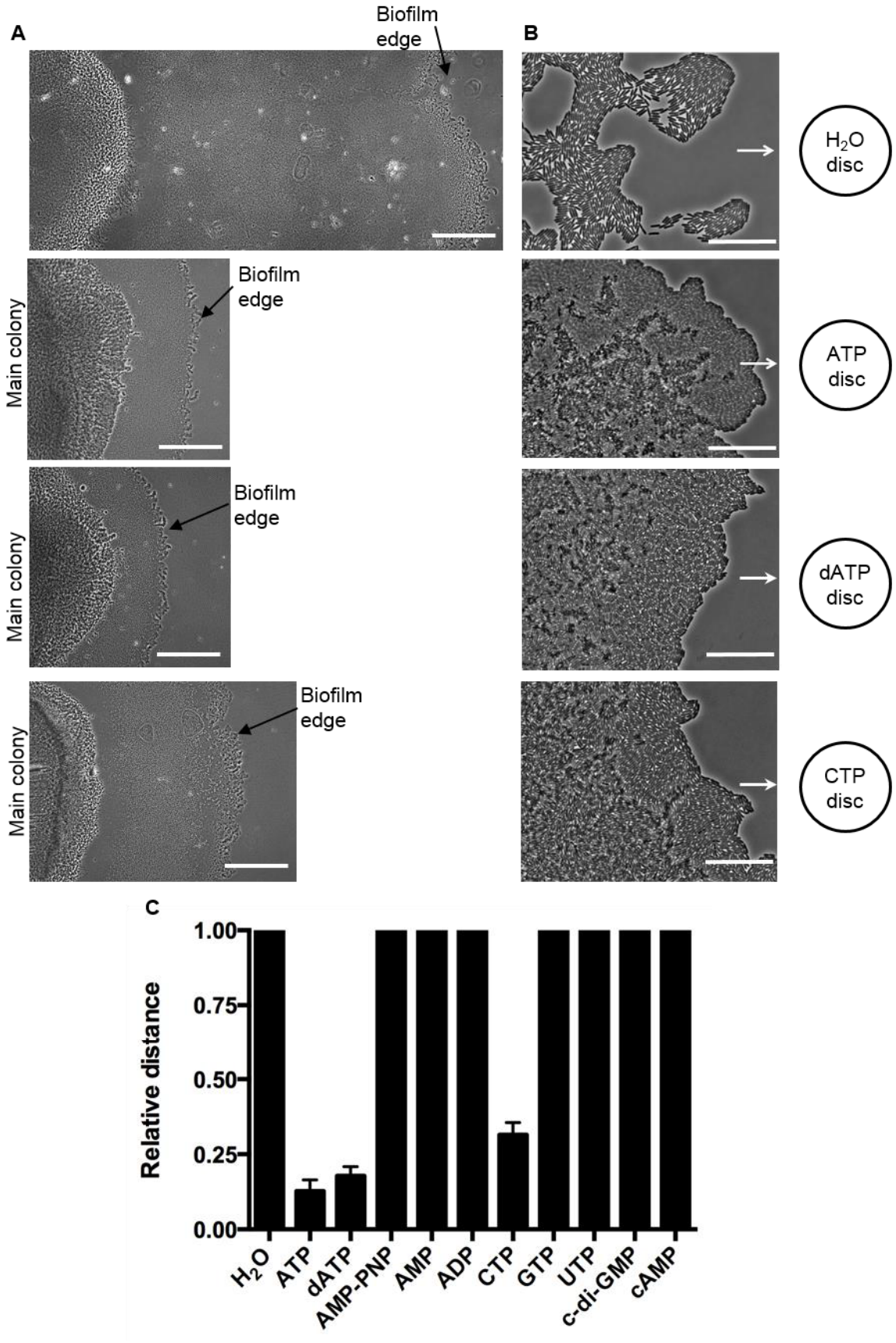

A
Biofilm edge
Main colony
Biofilm edge
Main colony
Biofilm edge
Main colony
Biofilm edge
B
H_2O disc
ATP disc
dATP disc
CTP disc
C
Relative distance
1.00
0.75
0.50
0.25
0.00
H_2O
ATP
dATP
AMP-PNP
AMP
ADP
CTP
GTP
UTP
c-di-GMP
cAMP

Figure 4-6. The effect of a range of extracellular nucleotide signals on twitching motility-mediated expansion of *P. aeruginosa* biofilms

Phase microscopy of biofilms formed by twitching motility-mediated biofilm expansion of *P. aeruginosa* PAK in the presence of a range of nucleotides at the interstitial space between a solidified media-coated microscope slide and coverslip after incubation for 15 hrs at 37 ℃. (A) Low magnification images of expansion of the biofilm away from the main colony towards a H_2O, ATP, dATP or CTP-saturated disc (scale bar is 500 μm) and (B) high magnification images of the expanded biofilm edge closest to the disc (scale bar is 50 μm and the arrows indicate the direction of expansion towards the disc). Images are representative of four independent experiments.

(C) Relative expansion distances of *P. aeruginosa* PAK towards a range of saturated discs at the interstitial space between a solidified media-coated microscope slide and coverslip after incubation for 15 hrs at 37 ℃. A value of 1 indicates that *P. aeruginosa* expanded up to the edge of the saturated disc. The data are represented as the mean ±SD from four independent experiments.

4.4 Discussion

In this study we investigated the effect of eATP on twitching motility by *P. aeruginosa*. Our observations have revealed that eATP is inhibitory to twitching motility at concentrations that are not inhibitory to growth. We found that the presence of eATP results in a reduction in surface assembled tfp levels and that inhibition of twitching motility occurs when eATP is either added at a uniform concentration into the solidified nutrient media or when encountered as a gradient from a source external to the biofilm.

Twitching motility mediated biofilm expansion by *P. aeruginosa* is a complex, multicellular behaviour (Semmler et al., 1999, Gloag et al., 2013) that can lead to the emergence of intricate network patterns of trails that are a characteristic feature of *P. aeruginosa* interstitial biofilms that form under the conditions of our assay. We have recently determined that extracellular DNA and furrow-mediated stigmergy are involved in this self-organisation of the expanding biofilm (Gloag et al., 2013). However, it remains unclear how individual bacterial cells are able to determine in which direction to travel to enable overall expansion of the biofilm into new territories. We have shown previously that homoserine lactone quorum sensing is not involved in this process (Beatson et al., 2002b). Our observations in this study indicate that eATP is an important extracellular signalling molecule that coordinates twitching motility-mediated biofilm expansion of *P. aeruginosa*. Time-lapse

microscopy suggests that exogenous eATP at concentrations of 2.5-5 mM may be confounding bacteria within the biofilm so that they were unable to maintain directional movement and that higher eATP concentrations signal the cells to cease movement. We also found that the edge of actively expanding *P. aeruginosa* colony biofilms contain endogenously produced eATP at about 3 mM. Taken together, these observations suggest that a gradient of eATP in the zone of active twitching motility may serve as a signal to direct bacterial traffic toward the virgin territory (low eATP) and away from the older regions of the biofilm (high eATP). High levels of eATP then indicate to the cells that they are no longer near the leading edge and to cease movement.

These observations suggest that a self-produced eATP gradient is sensed by the bacterial cells within the biofilm to direct movement away from regions of high eATP. We examined the possibility that the Chp chemosensory system may be responsible for eATP gradient detection but did not find any evidence that this system is involved. However, as we were unable to examine the core components of this system we cannot completely rule out the possibility. In fact, it is entirely plausible that eATP is the central signalling molecule sensed by the Chp chemosensory system to coordinate active twitching motility-mediated expansion of the biofilm. Indeed, the observed defects in twitching motility and tfp biogenesis by mutants of these core Chp system components (Whitchurch et al., 2004, Darzins, 1993, Darzins, 1994) are consistent with an inability to detect and respond to eATP.

Interestingly, we determined that ATP hydrolysis appears to be necessary for the inhibition of twitching motility as the non-hydrolysable ATP analogue, AMP-PNP had no effect on *P. aeruginosa* twitching motility. A recent report of eATP release by *E. coli* and *Salmonella* sp. in exponential growth phase suggests that this release enhances bacterial survival within stationary phase (Mempin et al., 2013). Mempin et al. (2013) also suggest that eATP needs to be hydrolysed or degraded at the cell surface, which is consistent with our observation that eATP needs to be hydrolysed in order to inhibit *P. aeruginosa* twitching motility. It is plausible that phosphate and/or adenosine, products of ATP hydrolysis, are sensed by the cell to control twitching motility. It would therefore be interesting to investigate this possibility in future work. As twitching motility is modulated by icAMP levels and icAMP is generated by the enzymatic conversion of ATP to cAMP we assessed whether the icAMP synthesis/degradation system might be responsible for eATP inhibition of twitching motility. However mutants of this pathway showed no defects in the twitching motility response to eATP. Our observation that CTP and dATP also inhibit *P. aeruginosa* twitching motility

suggests that the enzyme which hydrolyses eATP is also able to use these nucleotides as substrates. It is however surprising that we did not see a similar repression of twitching motility in the presence of UTP and GTP, as one would also expect that an enzyme which have activity towards ATP and CTP would also hydrolyse these substrates. One possible explanation could be that all four substrates effect twitching motility but GTP and UTP exert an effect at a different concentration to that which was tested in our assay (100 mM). It would be interesting to investigate such concentration-dependent effects of ATP, CTP, GTP and UTP on *P. aeruginosa* twitching motility in future work. Interestingly, cAMP and c-di-GMP, two intracellular second messengers which are involved in controlling twitching motility (Simm et al., 2004, Inclan et al., 2011), did not have any effect on twitching motility when provided exogenously.

P. aeruginosa is an opportunistic pathogen that exploits damaged mucosa to cause infection (Ramphal and Pyle, 1983, Kazmierczak et al., 2001). Interestingly, damage to epithelial cells results in a rapid increase in eATP levels, which results in the recruitment of host immune system factors via the P2X and P2Y receptors (Virgilio, 2007). Thus it is likely that *P. aeruginosa* is exposed to eATP at the site of epithelial damage. As the cytoplasmic concentration of ATP in mammalian cells is of the order of 5-10 mM, injury causing acute plasma membrane damage is expected to result in the release of eATP concentrations in this range at the injury site (Di Virgilio, 1998). Our observations indicate that eATP above 5 mM is sufficient to inhibit twitching motility by *P. aeruginosa*. This cessation of twitching motility in response to high concentrations of eATP may lead to initiation of infection at the site of epithelial injury. Subsequent cytotoxic damage caused by *P. aeruginosa* may lead to further release of eATP from the damaged epithelial cells. Interestingly, exposure of mammalian cells to mM concentrations of eATP can lead to necrotic or apoptotic cell death (Di Virgilio, 1998). Our observations indicate that the leading edge of *P. aeurginosa* biofilms produce eATP in this cytotoxic range which suggests that eATP produced by *P. aeruginosa* biofilms may be a virulence factor in its own right. Furthermore, chronic *P. aeruginosa* infection of cystic fibrosis patients is associated with significant inflammatory damage and fibrosis (Pukhalsky et al., 1999) and eATP has recently been associated with pulmonary inflammation and fibrosis via the $P2X_7$ receptor in a murine model of lung fibrosis (Riteau et al., 2010). Thus it is conceivable that high levels of eATP produced by *P. aeruginosa* biofilms and through epithelial cell damage contribute to the pathogenesis of chronic *P. aeruginosa* infection.

4.4.1 Conclusions

This study has shown that eATP produced by *P. aeruginosa* biofilms functions as an extracellular signalling molecule that coordinates cell movements during active biofilm expansion. As *P. aeruginosa* is an opportunistic pathogen that infects damaged epithelial tissues it is conceivable that the presence of host-derived eATP at sites of epithelial cell damage is exploited by *P. aeruginosa* to detect potential infection sites. We propose that eATP is an inter-kingdom signalling molecule that contributes to the complex host-pathogen interplay during *P. aeruginosa* infection.

4.5 Methods

4.5.1 Bacterial strains, plasmids and media

The *P. aeruginosa* strains used in this study are listed in Table 1. *P. aeruginosa* strains were cultured on Luria-Bertani (LB) (Sambrook, 1989) broth solidified with agar at 1.5% and grown overnight at 37℃. Planktonic cultures were grown in cation-adjusted Mueller Hinton broth (CAMHB) at 37℃, with shaking at 250 rpm. Twitching motility assays were performed with nutrient media (4 g/L tryptone, 2 g/L, yeast extract, 2 g/L NaCl) solidified with 8 g/L GelGro (ICN). ATP (Sigma Aldrich, St Louis, MO) was added at a final concentration of 1.6-15 mM (as indicated) in GelGro-solidified nutrient media (0.4% tryptone, 0.2% yeast extract, 0.2% NaCl, 0.1% $MgSO_4.7H_2O$ and 0.8% GelGro).

4.5.2 Twitching motility assays

Twitching motility-mediated interstitial biofilm expansion on GelGro-solidified nutrient media was assayed using the solidified nutrient media coated microscope slide assay described previously (Nolan et al., 2012). Interstitial biofilms were examined with phase-contrast microscopy and analysed using ImageJ (Schneider et al., 2012).

4.5.3 Filter disc diffusion assays

Filter discs (Whatman 6 mm, GE Healthcare) were soaked with sterile H_2O or 100 mM of the nucleotides adenosine triphosphate (ATP), deoxyadenosine triphosphate (dATP), adenosine monophosphate (AMP), adenosine monophosphate (ADP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), and adenosine-5'-(β,γ-

imido)triphosphate (AMP-PNP), 2.9 mM 3',5'-cyclic diguanylic acid (c-di-GMP) and 30 mM 3',5'-cyclic adenosine monophosphate (cAMP) (the concentrations used for both c-di-GMP and cAMP were just below the saturation limit) were and applied to a solidified nutrient media coated microscope slide as follows. 75 µL of the test solution was allowed to fully soak into a filter disc. Each disc was then dried for 2 hrs and applied to a dried solidified nutrient media coated microscope slide and a gradient allowed to establish for 1 hr, after which the slide was inoculated with the strain of interest, a coverslip applied and incubated statically at 37^{o} C for the indicated time. In the mutant screening assays each saturated disc was soaked in 30 mM ATP or sterile H_2O and used as described above.

Table 1. List of strains used in this Chapter

Strain	Relevant characteristics	Source or reference
PAO1	Wild-type *P. aeruginosa* strain ATCC 15692	American Type Culture Collection
PAK	Wild-type *P. aeruginosa* strain	D. Bradley, Memorial University of Newfoundlands, St John's, Canada
PA14	Wild-type *P. aeruginosa* strain	(Rahme et al., 1995)
PA103	Wild-type *P. aeruginosa* strain	(Liu, 1966)
PAK*pilA*:Tc^R	*pilA* inactivated by allelic displacement with a tetracycline resistance cassette (Tet^R)	(Watson et al., 1996c)
PAK*chpB*:Tc^R	*chpB* inactivated by allelic displacement with a tetracycline resistance cassette (Tet^R)	(Whitchurch et al., 2004)
PAK*chpC*:Tc^R	In frame deletion of *chpC* in wildtype strain PAK	Dervilla McGown, unpublished
PAK*chpD*:Tc^R	*chpD* inactivated by allelic displacement with a tetracycline resistance cassette (Tet^R)	(Whitchurch et al., 2004)
PAK*chpE*:Tc^R	*chpE* inactivated by allelic displacement with a tetracycline resistance cassette (Tet^R)	(Whitchurch et al., 2004)
PAK*pilK*:Tc^R	*pilK* inactivated by allelic displacement with a tetracycline resistance cassette (Tet^R)	Jennifer Sargent, unpublished
PAK*cyaA*	In frame deletion of *cyaA*	(Wolfgang et al., 2003)
PAK*cyaB*	In frame deletion of *cyaB*	(Wolfgang et al., 2003)
PAK*cyaAcyaB*	In frame deletions of *cyaA* and *cyaB*	(Wolfgang et al., 2003)
PAK*cpdA*	In frame deletion of *cpdA*	(Fuchs et al., 2010a)

4.5.4 Growth assays

Growth of *P. aeruginosa* was followed by recording changes in OD_{595nm} over a 20 hr period. Cells were grown in microtitre plates, and incubated at 37°C, shaking at 250 rpm. CAMHB or CAMHB supplemented with ATP at indicated concentrations was used in growth assays.

4.5.5 Quantification of eATP in *P. aeruginosa* biofilms

eATP was detected within *P. aeruginosa* surface colony biofilms as follows. 30 mL LB 1.5 % agar plates were poured and allowed to set overnight at room temperature. The following morning the agar plates were flipped into a larger petri dish to expose the smooth underside set against the petri dish base which promotes rapid twitching motility-mediated biofilm expansion (Semmler et al., 1999). 1.5 mL of an overnight *P. aeruginosa* PAK culture was pelleted by centrifugation (13,000 *g*, 3 min). The pellet was then gently resuspended and inoculated onto the centre of a flipped 1.5% 30 mL agar plate. The petri-dish lid was applied and incubated in a humid environment statically for 72 hrs at 37 ºC. Cells were harvested from the outer, active twitching edge visualised as a "ground-glass edge". Harvested cells were resuspended in 1 mL PBS and the eATP detected using a BacTitre-Glo™ assay (Promega Corportation). 500 µL of resuspended cells were pelleted by centrifugation (16, 900 *g*, 5 min). 110 µl of supernatant was removed and added into triplicate wells of a white 96-well plate (Greiner Bio-one) and 10 µL used to generate four serial 1:11 dilutions, with each well retaining 100 µL final volume. 100 µL of ATP standards ranging from 0-1 µM were also added to the plate in duplicate. 30 µL of room temperature BacTiter-Glo™ Buffer with precombined BacTiter-Glo™ substrate was added to all wells, mixed and incubated at room temperature for 5 min. Luminescence of all wells was read using an integration time of 250 ms.

4.5.6 PilA immunoblotting

Preparation of sheared surface tfp and cell-associated pilin samples was performed as described previously (Whitchurch et al., 2004) with cells being harvested from plates grown at 37ºC on LB agar containing 0 mM or 10 mM ATP for 20 hrs. Samples were displayed on a 10 % Bis-Tris Mini Gel (Life Technologies Corporation) and transferred onto an iBlot® mini gel transfer stack containing a PVDF membrane (Life Technologies Corporation). Membranes were probed with a Western Breeze® chemiluminescent western blot immunodetection kit (Life Technologies Corporation) according to manufacturer's instructions. A 1:5000 dilution of primary anti-PilA antibody was used.

4.5.7 PilA ELISA

Enzyme-linked immunosorbent assays (ELISAs) to determine the amount of surface tfp of cells grown in the presence and absence of ATP were performed as described previously

(Whitchurch et al., 2004) with cells being harvested from plates grown at 37 ºC on LB agar containing 0 mM or 10 mM ATP for 20 hrs.

Chapter 5
Extracellular ATP stimulates *Pseudomonas aeruginosa* sessile biofilm formation

5.1 Introduction

In addition to the role of twitching motility in mediating active biofilm expansion, this form of surface translocation is also involved in formation of *P. aeruginosa* biofilms within a fully-hydrated environment. Within this setting *P. aeruginosa* biofilms form on surfaces by the aggregation of cells into microcolonies, followed by the development and maturation of these microcolonies into sessile, mushroom-shaped structures (Stoodley et al., 2002). Bacteria within sessile biofilms are often more resistant to antibiotics and host immune system factors than cells within a planktonic state (Høiby et al., 2010a). The formation of sessile biofilms is also associated with the chronic nature of *P. aeruginosa* infection (Costerton, 1999). Twitching motility is involved in both the initial stages of microcolony formation (O'Toole and Kolter, 1998) and in the formation of the mushroom cap structures in mature biofilms (Klausen et al., 2003a, Klausen et al., 2003b, Barken et al., 2008). Interestingly, a number of host-derived signals have been shown to have an inverse effect on twitching motility-mediated active biofilm expansion and sessile biofilm formation. These include iron limitation, bovine serum albumin (BSA), adult human serum (AHS), as well a component of AHS, lactoferrin, which have been shown to stimulate active biofilm expansion and inhibit sessile biofilm formation. It has been suggested that the inverse effects of some of these host-derived signals on twitching motility and sessile biofilm formation may provide a protective advantage to the host by inhibiting the ability of *P. aeruginosa* to form resistant, sessile biofilms, thus giving the immune system a better chance of clearing the infection (Singh et al., 2002).

The role of another host-derived signal, extracellular 3',5'-adenosine triphosphate (eATP), in providing a protective advantage for a eukaryotic host has been studied in detail. The rapid release of high levels of eATP by stressed or injured epithelial cells acts to alert the host to the presence of invading pathogens, resulting in the recruitment of immune system factors which can work on clearing the infection (Virgilio, 2007). Interestingly, *P. aeruginosa* is only able to infect damaged epithelial cells (Ramphal and Pyle, 1983) and thus is likely to encounter eATP within an infection setting. In Chapter 4 of this Thesis, the effect of eATP on *P. aeruginosa* twitching motility-mediated active biofilm expansion was characterised. The results presented in Chapter 4 suggest that host-derived eATP could provide a potential protective advantage for the bacterium by inhibiting twitching motility. Furthermore, these results suggest that endogenously-produced bacterial eATP coordinates twitching motility-mediated biofilm expansion, and additionally may act as a potential virulence factor to increase

local epithelial cell damage and promote further *P. aeruginosa* infection. eATP has been shown to stimulate sessile biofilm formation in *E. coli*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and *Staphylococcus aureus* (Xi and Wu, 2010). As a range of host-derived signals have been shown to have an inverse effect on sessile biofilm formation and active expansion (Hammond et al., 2010, Huang et al., 2003, Singh et al., 2002, Patriquin et al., 2007), and given our demonstration of eATP-mediated twitching motility inhibition, we predicted that eATP would stimulate *P. aeruginosa* sessile biofilm formation. In this Thesis Chapter the effect of eATP on *P. aeruginosa* sessile biofilm formation under static conditions is investigated.

5.2 Materials and Methods

5.2.1 Bacterial strains, plasmids and media

P. aeruginosa wildtype strains PAO1 (ATCC 15692) and PAK (D. Bradley, Memorial University of Newfoundlands, St John's, Canada) were used in this Chapter. *P. aeruginosa* was cultured on LB broth solidified with agar at 1.5% and grown overnight at 37 ˚C (see section 2.1.12). *P. aeruginosa* cultures were grown in nutrient media (CAMHB), diluted if necessary (as indicated) and incubated overnight at 37 ˚C, with shaking at 250 rpm (see section 2.1.18 and 2.3.3).

5.2.2 Microtitre static biofilm assay

The effect of eATP on sessile biofilm formation was assayed using a microtitre plate assay with crystal violet staining. Overnight cultures of *P. aeruginosa* PAO1 and PAK were diluted 1:100 in nutrient media (CAMHB diluted 1 in 10) and added to wells of a 96-well microtitre plate (Microtest-96, Becton Dickinson) with eATP concentrations ranging from 0-2.8 mM and 10 mM, to a total volume of 150 µL. The plates were covered with Aeraseal^{TM} film and incubated statically at 37 ˚C for 24 hrs. Plates were then washed with PBS and stained with crystal violet (0.2 %) for at least 1 hr, statically at room temperature. Crystal violet was then removed and PBS washes were repeated. The crystal violet stain was extracted with 30 % acetic acid and the plates incubated for 30 mins at room temperature on an orbital shaker. The A_{595} of extracted crystal violet stain was then measured for each of the wells.

5.2.3 Static sessile biofilm formation assay

The effect of eATP on *P. aeruginosa* sessile biofilm development was studied in more detail by growing biofilms in coverslip-bottomed Fluorodishes (dish 35 mm, glass 10 mm, World Precision Instruments, Inc.). An overnight culture was diluted 1:100 in nutrient media (CAMHB diluted 1 in 10) and 100 µL added to each dish with 100 µL of either nutrient media (CAMHB diluted 1 in 10) for the control, or nutrient media supplemented with 2 mM ATP, 2 mM ATP with 200 mU/mL apyrase, or 200 mU/mL apyrase alone. The dishes were incubated statically at 37℃ for 24 hrs, after which time the media was replenished and incubated for a further 24 hrs. After 48 hrs the planktonic growth was carefully removed and the biofilm biomass stained with 200 µL of 0.25 µM SYTO-9 green fluorescent nucleic acid stain (Invitrogen Molecular Probes) for 1 hr at room temperature. After removal of excess stain by PBS washing, confocal laser scanning microscopy (CLSM) images were obtained with a Nikon A1 confocal microscope with Z-series images taken in 0.5 µm slices. Biofilms were volume-rendered using IMARIS® Imaging Software and COMSTAT analyses (Heydorn et al., 2000) performed.

5.2.4 Growth assays

Growth of *P. aeruginosa* was followed by recording changes in OD_{595nm} over a 20 hr period as described in section 2.11. PAO1 cells were grown in nutrient media (CAMHB diluted 1 in 10) for the control, or nutrient media (CAMHB diluted 1 in 10) supplemented with 2 mM ATP, 2 mM ATP with 200 mU/mL apyrase, or 200 mU/mL apyrase alone.

5.3 Results

5.3.1 Extracellular ATP stimulates *P. aeruginosa* sessile biofilm formation

In Chapter 4 of this Thesis, we report that eATP concentrations of greater than 2.5 mM inhibit twitching motility, and concentrations up to 10 mM had no effect on the growth of *P. aeruginosa*. Additionally, we demonstrate that 10 mM of eATP decreases surface tfp levels. Based upon these results we tested the effect of eATP at a concentration of 10 mM on the formation of *P. aeruginosa* PAK sessile biofilms within a microtitre plate assay. Biofilms were grown statically at 37 ˚C for 24 hr, after which the resulting biofilm biomass was stained with crystal violet, and the $OD_{595\ nm}$ recorded (Figure 5-1). These assays demonstrated that at this concentration eATP did not have a significant effect on sessile biofilm formation. As Xi et al.

(2010) reported stimulation of sessile biofilm formation of *E. coli*, *A. baumannii*, *S. maltophilia* and *S. aureus* at an eATP concentration of 400 µM we repeated the microtitre plate assay in a range of eATP concentrations (0-2.8 mM) closer to this value. Additionally, to determine if the effect of eATP on sessile biofilm formation was strain specific we used both strain PAK and strain PAO1 in these assays (Figure 5-2). These results demonstrate that for PAK and PAO1, biofilm biomass is significantly increased at eATP concentrations from 0.2-2 mM eATP for PAO1 and 1.2-2 mM eATP for PAK, with the largest biomass occurring at 2 mM eATP for PAO1 and at 1.2 mM eATP for PAK. A sharp decrease in biomass is observed for eATP concentrations above 2 mM for both PAO1 and PAK. Interestingly, for PAO1, eATP concentrations of 2.4 and 2.8 mM restored biomass levels to the same level as the control, while in PAK these eATP concentrations decreased biomass to a level significantly less than the control.

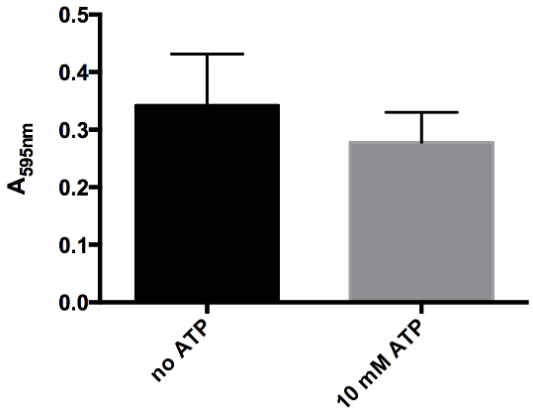


Figure 5-1. 10 mM of eATP does not significantly affect *P. aeruginosa* sessile biofilm formation.

P. aeruginosa PAK extracted crystal violet A_{595nm} readings for biofilms grown statically for 24 hrs at 37℃ within a microtitre plate in nutrient media with and without the addition of 10 mM eATP. The results are presented as the mean ± SD for three independent experiments performed in triplicate. A two-tailed Student's t-test did not report any significant difference between the test and the control ($p = 0.1128$).

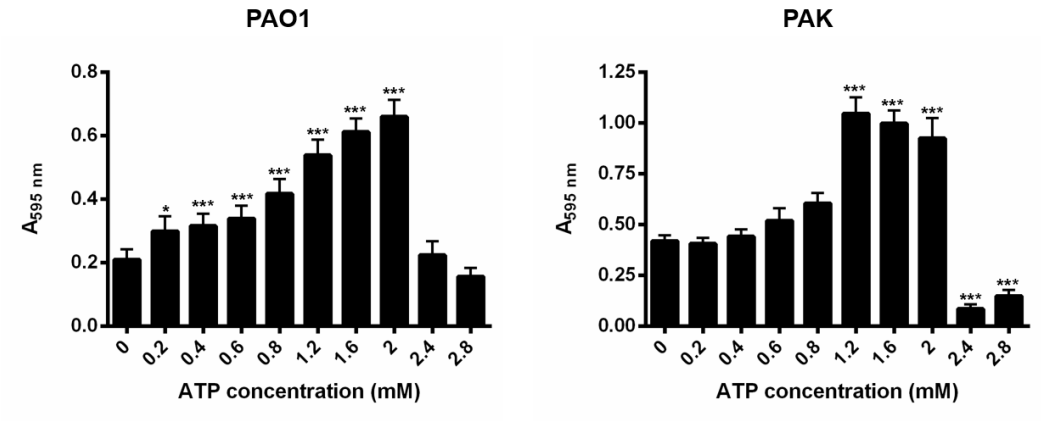


Figure 5-2. The effect of a range of eATP concentrations on *P. aeruginosa* PAO1 and PAK sessile biofilm formation.

P. aeruginosa PAO1 and PAK extracted crystal violet A_{595nm} readings for biofilms grown statically for 24 hrs at 37℃ within a microtitre plate in nutrient media supplemented with a range of eATP concentrations (0-2.8 mM). The results are presented as the mean ± SEM for six independent experiments each with five technical replicates (two-tailed Student's t-test $^{***}p< 0.0001$; $^{*}p < 0.01$ compared to 0 mM eATP for the parent wildtype strain).

The concentration which provided the greatest stimulation of sessile biofilm formation in a microtitre plate assay was used in sessile biofilm assays performed in a coverslip-bottomed Fluorodish, which allowed more detailed examination of the effects of eATP on biofilm morphology using CLSM. Under the assay conditions PAK did not form strong, robust biofilms (Figure 5-3) and thus strain PAO1 was used in all subsequent sessile biofilm assays.

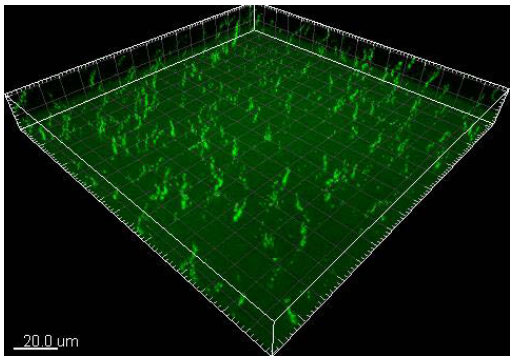


Figure 5-3. The formation of *P. aeruginosa* PAK sessile biofilms in a coverslip-bottomed Fluorodish.

P. aeruginosa PAK biofilms grown statically in nutrient media for 48 hrs at 37℃ in a glass-bottomed Fluorodish, stained with SYTO-9 and imaged using CLSM. Image is volumed rendered and is representative of the results obtained from three independent experiments performed in duplicate. Scale bar represents 20 µm.

The effect of eATP, eATP in combination with the ATP hydrolysing enzyme apyrase, and apyrase alone on PAO1 sessile biofilm formation was examined by SYTO-9 staining and CLSM and quantitated using COMSTAT analyses (Figure 5-4). These results demonstrate that sessile biofilms formed in the presence of eATP appear thicker and flatter compared to the control (Figure 5-4A). This is reflected in the COMSTAT analyses which show that eATP significantly increased biofilm biomass, and decreased roughness (Figure 5-4B). When eATP was added in combination with apyrase, biofilm biomass levels and roughness were restored to a level similar to that of an untreated biofilm (Figure 5-4A, B). Interestingly, treatment with apyrase alone significantly reduced biofilm biomass, and increased roughness (Figure 5-4A, B) compared to untreated biofilms, which indicated that endogenous eATP contributes to sessile biofilm development in *P. aeruginosa*.

To determine if these results were due to growth-related effects, growth assays of *P. aeruginosa* PAO1 in the presence of nutrient media, and nutrient media supplemented with 2 mM ATP, 2 mM ATP with 200 mU/ml apyrase or 200 mU/ml of apyrase alone (Figure 5-5) were performed. While a longer lag phase was observed for cells in the presence of eATP and eATP with apyrase, the rates of exponential growth and the final biomass levels were no different to those for the nutrient media control. These results demonstrate that exogenously added eATP stimulates *P. aeruginosa* sessile biofilm formation. Additionally, these results show that endogenously produced bacterial eATP also contributes to *P. aeruginosa* sessile biofilm formation.

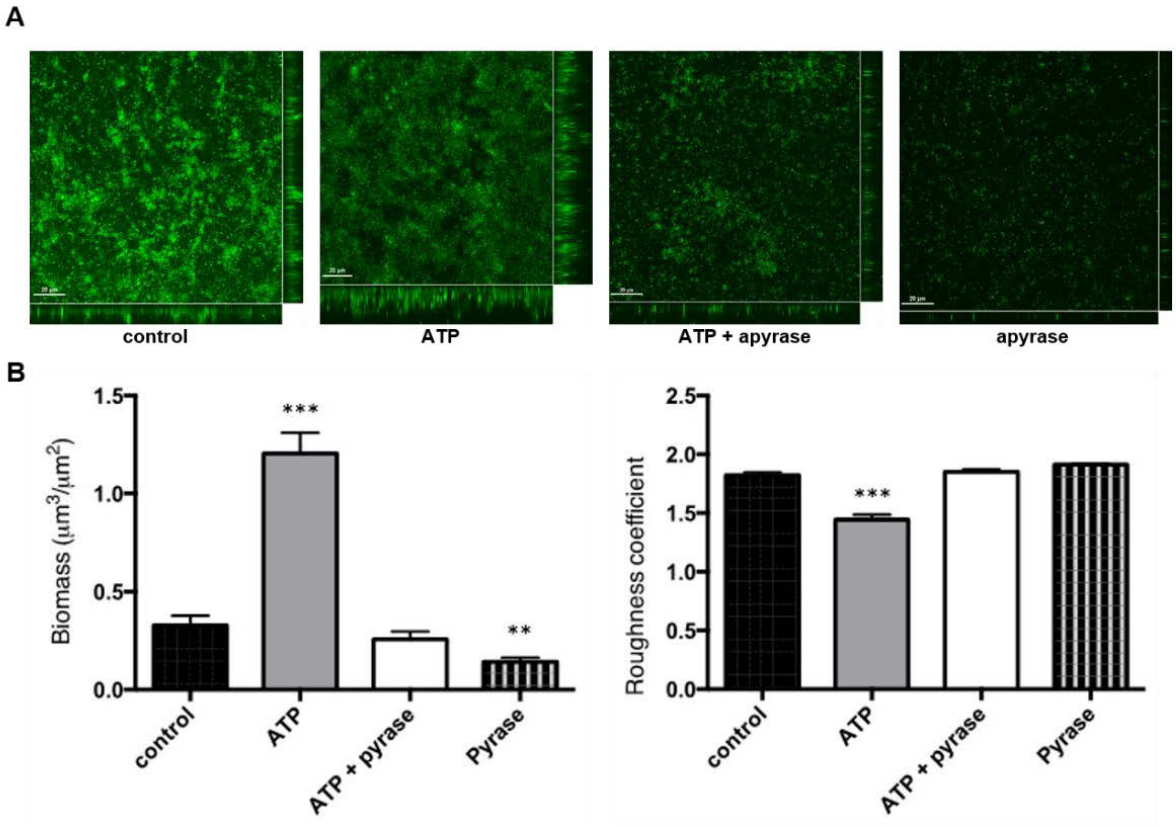


Figure 5-4. The effect of eATP and apyrase on *P. aeruginosa* sessile biofilm formation under static conditions

(A) *P. aeruginosa* PAO1 sessile biofilms grown statically for 48 hrs at 37℃ in coverslip-bottomed Fluorodishes, stained with SYTO-9 and imaged using CLSM. Cells grown in nutrient media (control), or nutrient media supplemented with 2 mM ATP, 2 mM ATP + 200 mU/ml apyrase, or 200 mU/ml apyrase. Images are presented in slice view and are representative of the results obtained from three independent experiments performed in duplicate. Scale bar represents 20 µm.

(B) COMSTAT analyses of *P. aeruginosa* PAO1 sessile biofilms grown statically in nutrient media (control) or nutrient media supplemented with 2 mM ATP, 2 mM ATP + 200 mU/ml apyrase or 200 mU/ml apyrase for 48 hrs at 37℃ in glass-bottomed Fluorodishes. The biofilm biomass and roughness coefficient results are presented as the mean ± SEM for three independent experiments in duplicate (two-tailed Student's t-test *** $p<0.0001$ and ** $p<0.005$ compared to the control).

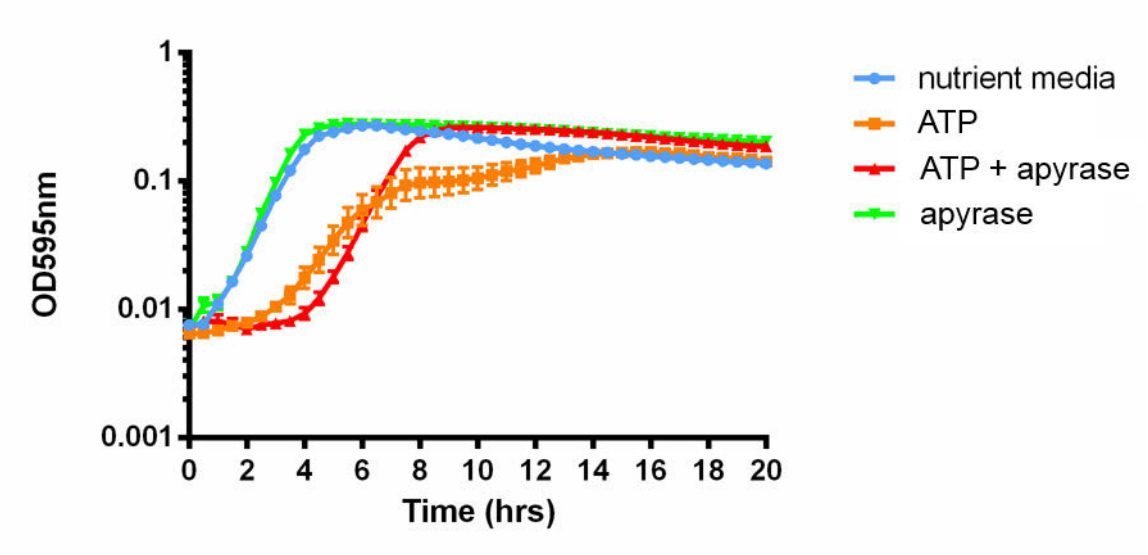


Figure 5-5. The effect of eATP and apyrase on growth of *P. aeruginosa*.

Growth rate of *P. aeruginosa* PAO1 at 37℃ over 20 hrs in nutrient media or nutrient media supplemented with 2 mM ATP, 2 mM ATP + 200 mU/mL apyrase or 200 mU/mL apyrase. Mean ± SD from three independent experiments each consisting of five technical replicates is presented.

5.4 Discussion

In this Chapter the effect of eATP on *P. aeruginosa* sessile biofilm formation under static conditions was investigated. Exogenously added eATP was found to stimulate biofilm formation in strains PAK and PAO1. Interestingly, our results demonstrate that endogenously produced eATP is also important in *P. aeruginosa* sessile biofilm formation. While, due to time constraints, we have not been able to fully understand the exact mechanism of eATP-mediated stimulation of sessile biofilm formation, these results provide a very interesting follow up to the work presented in Chapter 4. Together the results from both Chapters demonstrate that eATP is an important host-derived signal which mediates a transition between motile and sessile modes of life that is likely to provide a significant advantage for *P. aeruginosa* within an infection setting. These results also suggest that endogenously produced bacterial eATP acts as an extracellular signalling molecule to coordinate *P. aeruginosa* multicellular behaviours.

The results from the microtitre plate assays performed in this Chapter demonstrated that *P. aeruginosa* sessile biofilm formation is stimulated at eATP concentrations from 0.8-2 mM, with the greatest stimulation of biofilm biomass occurring at 1.2 mM eATP for PAK and at 2 mM eATP for PAO1. Interestingly, these assays also revealed a sharp decrease in the

biofilm biomass at eATP concentrations greater than 2 mM for both PAO1 and PAK. While 2.4 mM and 2.8 mM eATP reduced PAO1 biofilm biomass to a level similar to the control, these same concentrations significantly decreased PAK biofilm biomass compared to the control. It is unlikely that these effects are growth related, as eATP concentrations of less than 12.5 mM were shown to not have any effect on growth of *P. aeruginosa* (Chapter 4). We have also demonstrated in Chapter 4 that within a semi-hydrated environment, eATP concentrations of 2.5 mM or greater inhibit *P. aeruginosa* twitching motility-mediated biofilm expansion. Given the involvement of twitching motility in sessile biofilm formation (Klausen et al., 2003a, Klausen et al., 2003b, Barken et al., 2008, O'Toole and Kolter, 1998) one possibility is that the sharp decrease observed in sessile biofilm formation is due to complete inhibition of twitching motility.

The differences in the eATP concentration range that inhibits twitching motility (> 2.5 mM) in a semi-hydrated environment, compared to the range that may reduce sessile biofilm biomass in a hydrated setting (> 2 mM) could occur for a number of reasons. One important consideration is that within a semi-hydrated setting *P. aeruginosa* twitching motility-mediated biofilm expansion involves coordinated movement of the entire population, whereas twitching motility-mediated development of sessile biofilms within a fully-hydrated environment involves small subpopulations of cells/single cells (Klausen et al., 2003a, Klausen et al., 2003b, Barken et al., 2008, Semmler et al., 1999, Gloag et al., 2013). Thus eATP may affect *P. aeruginosa* twitching motility at different concentration ranges depending upon whether the population is undergoing twitching motility as a collective multicellular community or as small groups and single cells. It could also be possible that different MCPs are involved in sensing and responding to eATP to direct twitching motility at these different concentration ranges. Indeed, varying responses to the same extracellular signal have been demonstrated for the MCPs CtpH and CtpL which sense inorganic phosphate at high and low concentrations, respectively, to modulate *P. aeruginosa* swimming motility (Wu et al., 2000). *P. aeruginosa* possesses 26 MCPs (Kato et al., 2008). It is possible that one or more of these additional MCPs are involved in sensing eATP, or a product of eATP hydrolysis to modulate twitching motility.

Flagella-mediated motility is involved in sessile biofilm formation (O'Toole and Kolter, 1998, Klausen et al., 2003b, Barken et al., 2008), but not active biofilm expansion under the semi-hydrated conditions of our assays in Chapter 4. Although we have not examined the effect of eATP on flagella-mediated swimming motility due to project time constraints, it is possible that the observed decrease in biofilm biomass at eATP concentrations greater than 2 mM is due

to eATP-mediated inhibition of both twitching motility and/or swimming motility. It is also possible that alternatively, or in addition to these motility-related effects, the availability of eATP within a microtitre plate and a semi-solid media coated microscope slide is different, and thus the concentrations of eATP which appear to inhibit twitching motility are different in these two conditions.

The coverslip-bottomed Fluorodish assays confirm that addition of exogenous eATP at a concentration of 2 mM stimulates sessile biofilm formation of PAO1. This is reflected in the significant increase in biofilm biomass compared to the control (Figure 5-4B). Interestingly, the roughness coefficient is also significantly decreased for PAO1 biofilms formed in the presence of eATP (Figure 5-4B). This decrease in roughness appears to be consistent with the formation of a flatter, more uniformly distributed biofilm, which has less microcolonies than the untreated biofilm (Figure 5-4A). As we have discussed above, the sharp reduction in sessile biofilm biomass observed in our microtitre plate assays could be related to inhibition of twitching motility, and possibly also swimming motility. While time constraints have not allowed us to investigate the effect of these lower eATP concentrations on twitching and swimming motility during sessile biofilm formation these would be important future experiments to perform as they may allow us to gain a better understanding of the mechanism of eATP stimulation of sessile biofilm formation. The involvement of twitching and swimming motilities in the formation of flat biofilms, which appear similar to those formed in the presence of eATP, has been investigated previously (Klausen et al., 2003b). Klausen et al. (2003b) reported that formation of flat biofilms with citrate, benzoate or casamino acids as a carbon source within a flow chamber, was initiated by surface attachment, which did not require twitching or swimming motility, followed by clonal growth and subsequent twitching motility-mediated spreading of the cells across the substratum. This suggests that twitching motility is an important component in the ability of *P. aeruginosa* to form flat biofilms under the conditions of the assays conducted by Klausen et al. (2003b). It is possible that the formation of flatter biofilms in the presence of 2 mM eATP also involves twitching motility, however it is important to note that our assays were done in different media under static conditions. Therefore, in addition to assaying the effect of lower concentrations (< 2.5 mM) of eATP on twitching motility, it will also be necessary to repeat these experiments under flow conditions in the presence and absence of eATP, and to consider varying the kind of media used in future experiments. It would also be informative to investigate the effect of eATP on surface piliation levels at this lower concentration range, and correlate these results with the amount of sessile

biofilm formed under these conditions. Overall, these future experiments may be able to provide insight into how a range of eATP concentrations is stimulating twitching motility-mediated biofilm expansion and stimulating sessile biofilm formation.

Our results also suggest that endogenously produced bacterial eATP is important in sessile biofilm formation, as addition of apyrase, which hydrolyses ATP, results in a decrease in biofilm biomass (Figure 5-4). As discussed in Chapter 4, the production of large amounts of eATP by *P. aeruginosa* within actively expanding biofilms appears to direct *P. aeruginosa* twitching motility-mediated biofilm expansion and additionally, may allow potential modulation of host immune system function. Taken together the results presented within both Chapters 4 and 5 suggest that eATP is an important extracellular signalling molecule which coordinates *P. aeruginosa* multicellular behaviours. Xi et al. (2010) have shown that addition of apyrase also decreases *E. coli* sessile biofilm biomass. While the levels of eATP were not quantitated within *E. coli* biofilms, this result suggests that endogenously produced bacterial eATP may also be an important extracellular signal for coordinating complex behaviour in *E. coli*.

Given the potential importance of endogenously produced bacterial eATP, it would be interesting to investigate the mechanism of endogenous eATP release in future work. A remarkable phenomenon has been observed in both *P. aeruginosa* interstitial and sessile biofilms, as well as broth cultures, whereby rod-shaped cells rapidly transition into spherical cells, which then explode (C. B. Whitchurch, personal communication). This process, termed explosive cell lysis, releases large amounts of extracellular DNA (eDNA) and presumably all other intercellular contents including ATP (C. B. Whitchurch, personal communication). Thus this may be one mechanism of eATP release within *P. aeruginosa* interstitial biofilms. *P. aeruginosa* and other organisms have been reported to release eATP within the exponential phase of planktonic growth via secretion (Hironaka et al., 2013, Mempin et al., 2013). Hironaka et al. (2013) observed very few dead cells in *P. aeruginosa* cultures in exponential growth by live/dead stain, and used this result to conclude that eATP was most likely to be released via secretion, as opposed to lysis. While a live/dead stain is able to permeate dead cells with compromised membranes, it would not stain dead cells which had lysed through explosive cell lysis, as this process completely obliterates the cell membrane (C. B. Whitchurch, personal communication). Thus, while it is possible that endogenous eATP is released by secretion, it is also possible that this eATP is released via explosive cell lysis within both planktonic growth and interstitial biofilms.

In this Chapter we also observed that unlike PAO1, PAK did not form robust biofilms within our coverslip-bottomed Fluorodish assay. The importance of the exopolysaccharides Psl and Pel within the EPS component of *P. aeruginosa* sessile biofilms appears to vary amongst strains. Psl is required for PAO1 cell adherence to the biofilm substratum, and acts as a scaffold to hold biofilm cells together within the matrix and maintain biofilm structure (Ma et al., 2009, Matsukawa and Greenberg, 2004). While Pel is involved in both the attachment and maturation stages of PAK sessile biofilm formation (Vasseur et al., 2005), Pel is not required for either the attachment or development stages of PAO1 sessile biofilms (Colvin et al., 2011). It is therefore possible that differences in PAO1 and PAK exopolysaccharide expression contribute to the differences in the ability of these strains to form sessile biofilms under the conditions of our assay. It would be interesting to investigate these differences by staining PAK and PAO1 sessile biofilms with a Psl-specific MOA or HHA lectin stain (Ma et al., 2007).

The results presented in this Chapter, alongside those presented in Chapter 4, demonstrate that eATP is an important host-derived signal which is involved in the transition of *P. aeruginosa* between motile and sessile modes of life. Interestingly, unlike previously identified host-derived signals, which inhibit *P. aeruginosa* sessile biofilm formation and stimulate active expansion (Hammond et al., 2010, Huang et al., 2003, Singh et al., 2002, Patriquin et al., 2007), eATP has the opposite effect. While a stimulation of twitching motility-mediated active expansion, and inhibition of sessile biofilm formation, would be most advantageous to the host, the inverse effect is likely to be more advantageous to the bacterium. Thus our results suggest that the ability of *P. aeruginosa* to stimulate sessile biofilm formation and inhibit twitching motility in response to eATP, could provide a significant advantage to the bacterium within an infection setting. The results of both Chapters 4 and 5 also demonstrate that endogenously produced bacterial eATP is important in twitching motility-mediated biofilm expansion and in sessile biofilm formation, suggesting that eATP is an important extracellular signalling molecule which is involved in coordinating complex multicellular behaviours of *P. aeruginosa*. Future experiments will further uncover the mechanism of eATP-mediated stimulation of sessile biofilm formation as well as the role of endogenously produced bacterial eATP in *P. aeruginosa* pathogenesis, providing us with a better understanding of the biofilm response of *P. aeruginosa* to this important host-derived signal.

Chapter Six
ChpC controls twitching motility-mediated expansion of *Pseudomonas aeruginosa* biofilms in response to albumin, mucin and oligopeptides

All experiments and analysis of results presented in this chapter have been performed by Laura Nolan.

Movie files are included within the results presented in this chapter as referenced in the text (see Appendix 1 for Movie files).

Acknowledgements:

Dervilla McGown was responsible for generation of the PAK*chpC* mutant and pUCPChpC complementation construct.

Phillip Quin generated the Matlab code to display the Morphometrics cell tracking output.

6.1 Introduction

The biogenesis, assembly and twitching motility function of tfp is regulated by a number of complex systems including a putative chemosensory system, the Chp system (Whitchurch, 2006). This system is encoded by the *pilGHIJK-chpABC* gene cluster (Darzins, 1993, Darzins, 1994, Darzins, 1995, Whitchurch et al., 2004), which is homologous to the *E. coli* Che chemosensory system (Whitchurch, 2006). In *E. coli* this system is involved in regulating flagella-mediated chemotaxis in response to environmental conditions (Baker et al., 2006). The core signalling components of the Chp system in *P. aeruginosa* include a putative CheA histidine kinase homolog, ChpA (Whitchurch et al., 2004), which appears to be coupled to the MCP PilJ (Darzins, 1994), via the CheW-like adapter protein, PilI (Darzins, 1994, Whitchurch et al., 2004). Another CheW-homolog, ChpC is thought to link additional MCPs to ChpA (Whitchurch et al., 2004). Alterations in currently unknown environmental signals are thought to cause a conformational change in PilJ, resulting in ChpA autophosphorylation and phosphate transfer to two CheY-like proteins, PilG and PilH (Darzins, 1994, Darzins, 1993). PilG-P and PilH-P are thought to interact with the tfp motor complex composed of PilZ, FimX, and the ATPases PilB, PilT and PilU to control tfp extension and retraction (Bertrand et al., 2010, Guzzo et al., 2009). The combined action of the CheR-like methyltransferase PilK and the CheB-like methylesterase ChpB is thought to control PilJ methylation levels, allowing for adaption to changes in environmental conditions (Darzins, 1995, Whitchurch et al., 2004). Unlike the Che system however, where alterations in attractant and/or repellent levels are specifically linked to either an increase or decrease in CheY-P levels and subsequent modulation of flagella rotation, it is unknown how changes in environmental cues are translated into altered PilG-P and PilH-P levels within the Chp system of *P. aeruginosa* to affect tfp function.

It has previously been reported that *P. aeruginosa* twitching motility is stimulated by the host-derived signals mucin, serum albumin, and oligopeptides, in the form of tryptone (Huang et al., 2003). While FimX appears to have some role in the observed twitching motility response to mucin and tryptone, the stimulatory response to these signals is not completely abolished in a *fimX* mutant suggesting that there are additional regulators involved (Huang et al., 2003).

A common side chain of mucin is *N*-acetylglucosamine (GlcNAc) (Linden et al., 2008). Interestingly, microarray analyses of CF-sputum grown *P. aeruginosa* showed that many genes involved in GlcNAc catabolism are upregulated, which suggests that GlcNAc may be present

within the CF lung environment (Palmer et al., 2005). Additionally, GlcNAc is present in peptidoglycan within bacterial cell walls, which is shed in large amounts from the surface of Gram-positive species (Doyle et al., 1988, Mauck et al., 1971). CF lung infections are polymicrobial in nature, and include both Gram-positive and Gram-negative bacterial species (Sibley et al., 2006). Therefore within a CF lung infection setting *P. aeruginosa* is likely to encounter GlcNAc which is derived from the host, as well as from other bacterial species within the population. Additionally, GlcNAc has recently been shown to stimulate *P. aeruginosa* production of the virulence factors, 2-heptyl-3-hydroxy-4-quinolone (PQS), pyocyanin and elastase via PA0601 which is the sensor component of a two component sensor/regulator pair (Korgaonkar et al., 2012).

Given that mucin has been shown to stimulate *P. aeruginosa* twitching motility (Huang et al., 2003), we predicted that GlcNAc may also affect twitching motility. In the current study we explored this possibility and also investigated the involvement of the Chp system in the twitching motility response to the host-derived signals serum albumin, mucin and oliopeptides.

6.2 Materials and Methods

6.2.1 Bacterial strains, plasmids and media

The strains used in this study and their relevant characteristics are listed in Table 6-1.

P. aeruginosa was cultured on LB broth solidified with agar at 1.5% or 1% (for twitching motility stab assays) and grown overnight at 37˚C (see section 2.1.12 and 2.1.21). *P. aeruginosa* cultures were grown in either CAMHB or LB broth and incubated overnight at 37˚C, with shaking at 250 rpm (see section 2.1.12 and 2.1.18). Light microscopy was performed with nutrient media (4 g/L tryptone, 2 g/L, yeast extract, 2 g/L NaCl) solidified with 8 g/L gellan gum (see section 2.1.19).

6.2.2 Phenotypic assays

Microscopic analysis of twitching motility-mediated interstitial biofilm expansion on gellan gum-solidified nutrient media was assayed using the slide assay described in section 2.9.2. Twitching motility was also examined using a subsurface stab assay as described in section 2.9.1. Intercellular cAMP assays were conducted as described in section 2.10 using cells grown in base media and base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%).

Table 6-1. Strains and plasmid used in this Chapter and their relevant characteristics.

Strain or plasmid	Relevant characteristic	Source or reference
PAK	Wildtype *P. aeruginosa* strain	D. Bradley, Memorial University of Newfoundlands, St John's, Canada
PA14	Wildtype *P. aeruginosa* strain	(Rahme et al., 1995)
PAK*chpC*	In frame deletion of *chpC* in wildtype strain PAK	Dervilla McGown, unpublished
PA14*chpC*	In frame deletion of *chpC* in wildtype strain PA14	Dervilla McGown, unpublished
PA14*fimX*	MAR2xT7 transposon insertion in PA14_65540	(Liberati et al., 2006)
PA14_07820	MAR2xT7 transposon insertion in PA14_07820	(Liberati et al., 2006)
PA14_07840	MAR2xT7 transposon insertion in PA14_07820	(Liberati et al., 2006)
pUCPSK	*P. aeruginosa-E. coli* shuttle vector; Ap^R	(Watson et al., 1996a)
pUCPChpC	*chpC* amplified from PAK and cloned into pUCPSK with *Plac*; Ap^R	Dervilla McGown, unpublished

6.2.3 Time lapse microscopy and cell-tracking analysis

An Olympus IX71 wide-field inverted microscope fitted with an FViewII camera, environmental control chamber (Solent Scientific Ltd) and phase contrast optics was used for imaging of *P. aeruginosa* interstitial biofilms. High magnification time lapse images of wildtype and PAK*chpC* expanding at the interstitial space between a gellan-gum solidified base media or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%)-coated microscope slide and a coverslip were taken 4 hrs after incubation at 37 ºC, with 1 frame/2secs for a total of 300 frames. The high magnification time series images were used for identification and tracking of individual cell movements. Due to limitations in computing power we were only able to track cells across a total of 50 frames. To maximise the information gained we generated a time series which consisted of every second frame of the first 100 frames for a time series (i.e. a total of 50 frames with each frame separated by a 4 sec interval). Sophisticated computer vision methods in the form of Morphometrics (A Matlab-run program written by Tristan Ursell and KC Huang, Stanford University, U.S.A, unpublished) were used to assign cell IDs to all cells appearing in the time series, with x and y coordinates also being assigned to each individual cell ID across these 50 frames. Individual bacterial cells were identified in

each frame by assigning contour curves using a Laplacian edge filter. These regions were further decomposed into unbranched segments to allow separation of individual rod-shaped bacteria. These designated x and y coordinates were used to track the movement of cells across multiple frames to generate a tracking map which was displayed in a Matlab-run GUI (Matlab code written by Phillip Quin, University of Technology Sydney, Australia, unpublished). Manual exclusion of incorrectly tracked cells was performed by inspection of the Morphometrics-generated cell tracking output map in the Matlab-run GUI. The x and y coordinates of all cells appearing in the first frame were outputted for the first and all subsequent frames that each cell appeared in. The Cartesian coordinates of each cell across these frames were used to calculate the distance a cell travelled between frames using the following equation for Euclidean distance (d):

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

where (x_1, y_1) and (x_2, y_2) are the Cartesian coordinates for one cell in frame 1 and frame 2, respectively. The sum of the distance travelled across all frames was used to calculate the total distance a cell travelled. The average speed was calculated by dividing the average speed by the total time a cell was tracked (1 frame = 4 sec). The net distance travelled for individual cells in the data set was calculated by using the Cartesian coordinates for each cell in frame 1 and the final frame the cell appeared in (n) (x_1, y_1) and (x_n, y_n) and the equation for Euclidean distance (above). The net distance over time was calculated by dividing the net distance a cell travelled by the total time the cell was tracked for.

6.2.4 Growth experiments

Growth of *P. aeruginosa* strains was followed by recording changes in OD_{595nm} over a 20 hr period (see section 2.11). Cells were grown in microtitre plates, and incubated at 37ºC, shaking at 250 rpm. Base media supplemented with GlcNAc (50 mM), BSA (3%), mucin (0.05%) and tryptone (3%) was used in growth assays.

6.2.5 PilA immunoblotting

Detection of cell-associated pilin was performed as described in section 2.8.2 with cells being harvested from plates grown at 37ºC for 20 h on base media (0.5% yeast extract, 0.05% NaCl, 1.5% agar) or base media supplemented with BSA (0.1%), mucin (0.05%), or tryptone (3%).

6.2.6 ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed as described in section 2.8.1 with cells being harvested from plates grown at 37 ^{o}C for 20 hr on base media (0.5% yeast extract, 0.05% NaCl, 1.5% agar) or base media supplemented with BSA (0.1%), mucin (0.05%), or tryptone (3%). ELISAs of cells harvested from tryptone plates were treated with 100 Kunitz units/mL DNaseI (D5025, Sigma Aldrich) for 1 hr statically at 37 ^{o}C, then washed three times with PBS, prior to use in the assay.

6.3 Results

6.3.1 ChpC is involved in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone

BSA, mucin and tryptone have previously been shown to stimulate *P. aeruginosa* twitching motility with the effect of mucin and tryptone being mediated to some extent by FimX (Huang et al., 2003). However, as some stimulation of a *fimX* mutant is still observed, this suggests that other components must also be involved in mediating the twitching motility response to these host-derived signals. As the Chp system is predicted to regulate twitching motility in response to environmental signals, we were interested in investigating the role of this system in the twitching motility response to BSA and in contributing to the putative FimX-independent response to mucin and tryptone. In order to test the role of any component in a twitching motility assay, a mutant must still possess some twitching motility ability. While PilI is thought to link the putative MCP PilJ to the putative histidine kinase ChpA and when mutated is non-twitching, ChpC is thought to link other MCPs and ChpA and when mutated, possesses near to wildtype twitching motility levels (Whitchurch et al., 2004). To investigate the role of ChpC in stimulating twitching motility, subsurface stab assays with *P. aeruginosa* wildtype PAK and PAK*chpC* strains containing the pUCPSK vector control, or the pUCPChpC complementation vector were conducted in base media agar or base media agar supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%). Twitching motility of PAK*chpC* containing the pUCPSK vector control on BSA, mucin and tryptone was significantly reduced compared to twitching motility of wildtype PAK containing the pUCPSK vector control on the same kind of media (Figure 6-1). Twitching motility levels of PAK*chpC* on BSA, mucin and tryptone were restored to the same level as PAK containing the pUCPSK vector control by

pUCPChpC complementation (Figure 6-1). These results demonstrate that ChpC is involved in the twitching motility response to BSA, mucin and tryptone.

To determine whether the stimulation of PAK and PAK*chpC* twitching motility on BSA, mucin and tryptone was due to an increase in growth rate, growth assays of these strains were conducted in base media, or base media supplemented with BSA, mucin and tryptone. This revealed that BSA, mucin and tryptone did not have any significant effect on growth of PAK or PAK*chpC* (Figure 6-2).

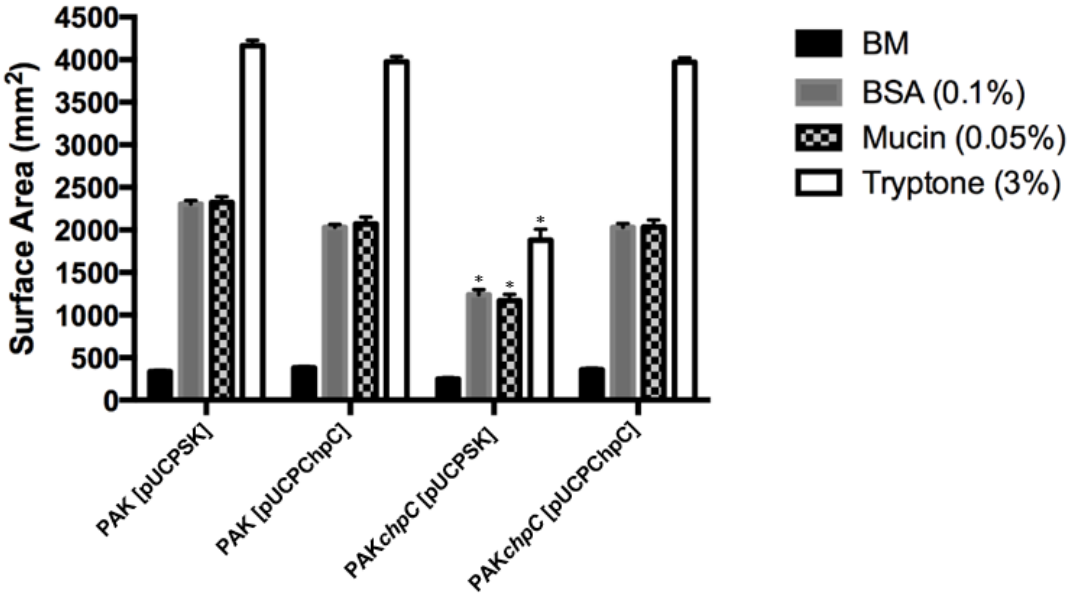


Figure 6-1. ChpC is involved in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone.

Subsurface twitching motility-mediated biofilm expansion of PAK and PAK*chpC* containing pUCPSK or pUCPChpC at the agar/plastic interstitial space after 48 hrs at 37℃ in base media (BM), or base media supplemented with BSA (0.1%), mucin (0.05%) or tryptone (3%). The mean surface area ± SD for three independent experiments performed in triplicate is shown ($^{*}p<0.0001$, Paired Student's two-tailed t-test for PAK*chpC* containing pUCPSK compared to PAK containing pUCPSK on BSA, mucin and tryptone. No significant difference was calculated with a Paired Student's two-tailed t-test for PAK*chpC* expressing pUCPChpC compared to PAK expressing pUCPSK, p = 0.9686 for BSA, p = 0.7576 for mucin and p = 0.9256 for tryptone).

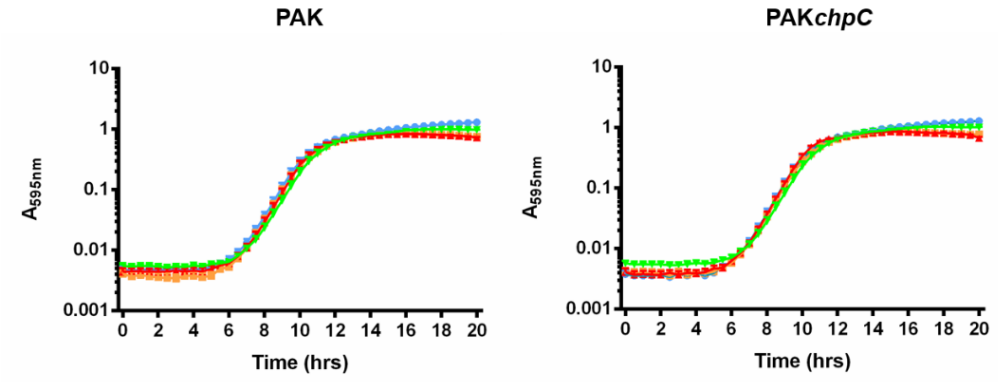


Figure 6-2. Growth rates of *P. aeruginosa* in base media, BSA, mucin and tryptone.

Growth rate of *P. aeruginosa* PAK and PAK*chpC* at 37℃ over 20 hrs in base media (green) or base media supplemented with BSA (0.1%) (red), mucin (0.05%) (orange) and tryptone (3%) (blue). Mean ± SEM from three independent experiments each consisting of five technical replicates is presented.

6.3.2 GlcNAc stimulates twitching motility of *P. aeruginosa* PA14

As GlcNAc is a common sidechain of mucin we were interested in investigating the effect of this signal on *P. aeruginosa* twitching motility. The effect of GlcNAc on *P. aeruginosa* twitching motility was investigated in a subsurface twitching motility stab assay. Initially these assays were conducted with strain PA14 in agar supplemented with 1 mM GlcNAc, which was the concentration used to stimulate *P. aeruginosa* virulence factor production (Korgaonkar et al., 2012). This concentration, and concentrations up to 20 mM GlcNAc did not have any effect on twitching motility (Figure 6-3A), however stimulation of twitching motility was observed at GlcNAc concentrations of 20 mM and 50 mM, with the greatest stimulation occurring in media supplemented with 50 mM GlcNAc (Figure 6-3A). Growth assays with strain PA14 in media supplemented with 50 mM GlcNAc confirmed that the observed stimulation of twitching motility was not simply due to an increased growth rate, as cells grew at the same rate in base media as in base media supplemented with 50 mM GlcNAc (Figure 6-3B). Interestingly, the effect of GlcNAc on twitching motility was strain specific, as a GlcNAc concentration of 50 mM did not have any significant effect on twitching motility of strain PAK (Figure 6-3C).

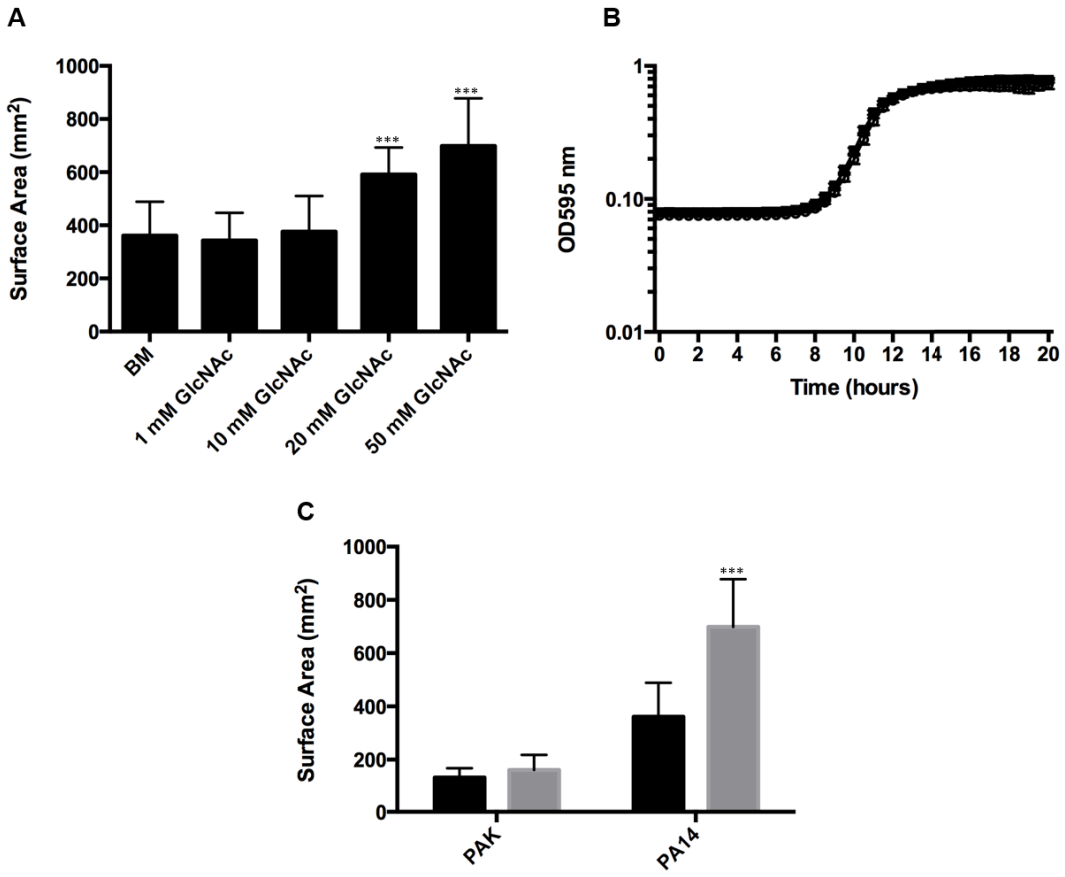


Figure 6-3. The effect of GlcNAc on *P. aeruginosa* twitching motility.

(A) Subsurface twitching motility-mediated biofilm expansion of PA14 at the agar/plastic interstitial space after 48 hrs at 37 ℃ in base media (BM) or base media supplemented with 1 mM, 10 mM, 20 mM and 50 mM GlcNAc. The mean surface area ± SD for at least three independent experiments performed in triplicate is presented ($^{***}p < 0.0001$, Paired Student's two-tailed t-test compared to base media).

(B) Growth rate of *P. aeruginosa* PA14 at 37℃ over 20 hrs in base media (open circle) or base media supplemented with 50 mM GlcNAc (closed square). Mean ± SD from three independent experiments each consisting of five technical replicates is presented.

(C) Subsurface twitching motility-mediated biofilm expansion of PAK and PA14 at the agar/plastic interstitial space after 48 hrs at 37℃ in base media (black bars) or base media supplemented with 50 mM GlcNAc (grey bars). The mean surface area ± SD for three independent experiments performed in triplicate is presented. ($^{***}p < 0.0001$, Paired Student's two-tailed t-test for GlcNAc compared to base media for PA14; no significant different was calculated for GlcNAc compared to base media for PAK with a Paired Student's two-tailed t-test ($p = 0.5547$)).

As ChpC was shown to be involved in the twitching motility of *P. aeruginosa* to mucin, we were interested in testing the role of ChpC in the twitching motility response to GlcNAc. Subsurface twitching motility stab assays revealed that twitching motility of PA14*chpC* was stimulated to a similar extent as wildtype by GlcNAc (Figure 6-4).

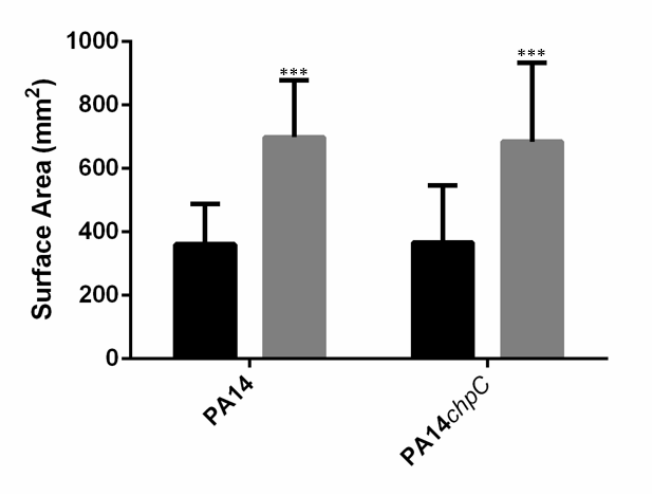


Figure 6-4. ChpC is not involved in the twitching motility response of *P. aeruginosa* to GlcNAc.

Subsurface twitching motility-mediated biofilm expansion of PA14 and PA14*chpC* at the agar/plastic interstitial space after 48 hrs at 37℃ in base media (black bars), or base media supplemented with GlcNAc (50 mM) (grey bars). The mean surface area ± SD for three independent experiments performed in triplicate is shown. (*** $p < 0.0001$, Paired Student's two-tailed t-test compared PAK or PAK*chpC* on base media).

We then examined the role of FimX and the putative two component sensor/regulator pair PA14_07820/ PA14_07840, which is involved in sensing GlcNAc to stimulate virulence factor production (Korgaonkar et al., 2012). Subsurface stab assays of PA14*fimX*, *PA14_07820* and *PA14_07840* in base media and base media supplemented with 50 mM GlcNAc revealed that twitching motility of all mutants was stimulated by GlcNAc (Figure 6-5A, B).

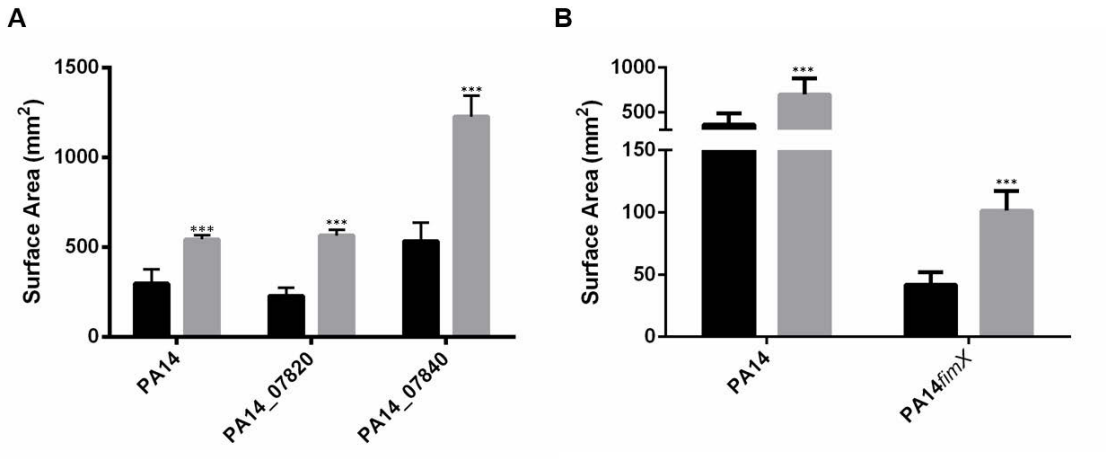


Figure 6-5. The two component sensor/regulator pair PA14_07820/PA14_07840 and FimX are not involved in the twitching motility response of *P. aeruginosa* to GlcNAc.

Subsurface twitching motility-mediated biofilm expansion of (A) PA14, *PA14_07820* and *PA14_07840* and (B) PA14 and PA14*fimX* at the agar/plastic interstitial space after 48 hrs at 37℃ in base media (black bars), or base media supplemented with GlcNAc (50 mM) (grey bars). The mean surface area ± SD for three independent experiments performed in triplicate is shown (*** $p < 0.0001$, Paired Student's two-tailed t-test for GlcNAc compared to base media for either the respective parent wildtype or mutant strain).

6.3.3 The effect of BSA, mucin and tryptone on PilA levels in PAK and PAK*chpC*

Twitching motility levels can be affected by changes in expression of the tfp monomer subunit, PilA, and/or alterations in surface assembly of the tfp. To determine if the mechanism of ChpC-mediated stimulation of twitching motility in response to BSA, mucin and tryptone involved changes in expression and/or assembly of the tfp we carried out PilA ELISAs and whole cell Westerns using cells harvested from confluent lawns of PAK and *chpC* grown on base media, BSA, mucin and tryptone. The ELISAs demonstrated that while *chpC* had decreased levels of surface assembled tfp, as reported previously (Whitchurch et al., 2004), there was no significant alteration in surface assembled tfp in either wildtype or a *chpC* mutant grown on BSA or mucin (Figure 6-6A,B). Surface PilA ELISAs of cells harvested from tryptone plates were initially unable to detect any surface PilA. A significant amount of autolysis was observed in the confluent lawns of cells on tryptone, which suggested that there may be excessive amounts of extracellular DNA (eDNA) present. To attempt to remove this

eDNA cells were harvested from both base media control plates and tryptone plates, treated with DNaseI and then washed in PBS, prior to use in the ELISA. This allowed detection of surface tfp levels in these cells, and revealed that both wildtype and *chpC* cells grown in the presence of tryptone had increased levels of surface tfp compared to cells grown on base media (Figure 6-6C). Whole cell Westerns demonstrated that there was no significant alteration in PilA expression levels for both wildtype and *chpC* grown on base media, BSA, mucin or tryptone (Figure 6-6D).

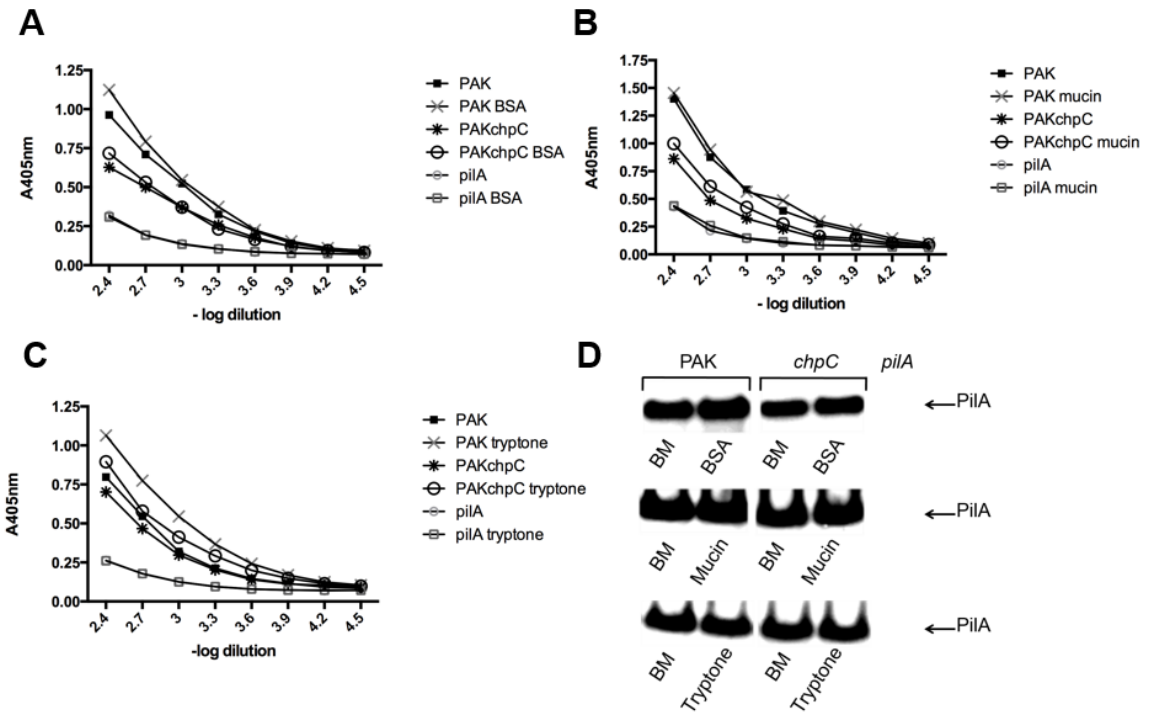


Figure 6-6. The effect of BSA, mucin and tryptone on PilA expression and surface assembly.

Representative results for PilA ELISAs of wildtype PAK, PAK*chpC* and PAK*pilA* cells grown on base media or base media supplemented with (A) BSA; (B) mucin; or (C) tryptone. The mean is presented for one experiment performed in triplicate with these graphs being representative of results obtained from three independent experiments. Equal cell numbers were loaded. (D) PilA Western immunoblot of whole cell lysates from wildtype PAK and PAK*chpC* cells grown on base media or base media supplemented with BSA (0.1%), mucin (0.05%), tryptone (3%), and PAK*pilA* cells grown on base media. Equal cell numbers were loaded.

6.3.4 Intercellular cAMP levels are increased in the presence of BSA, mucin and tryptone

Alterations in icAMP levels appear to correlate with changes in twitching motility levels (Nolan et al., 2012, Inclan et al., 2011, Fulcher et al., 2010), suggesting a role for icAMP in regulation of twitching motility. To investigate the involvement of icAMP in the twitching motility response of *P. aeruginosa* to BSA, mucin and tryptone, icAMP levels were assayed for wildtype PAK and *chpC* cells grown in base media and base media supplemented with BSA, mucin, and tryptone (Figure 6-7). This revealed that BSA, mucin and tryptone-grown PAK and *chpC* cells had increased icAMP levels compared to base media-grown cells.

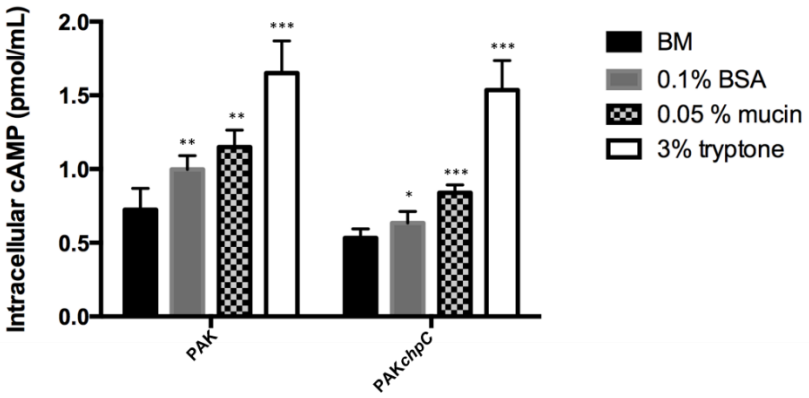


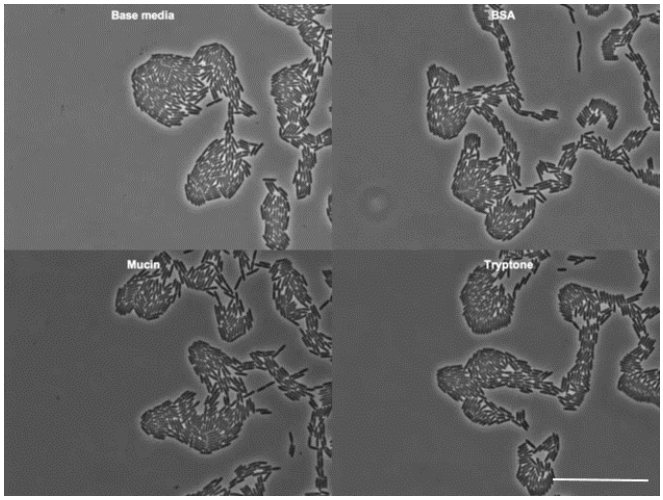
Figure 6-7. BSA, mucin and tryptone increase intercellular cAMP levels.

Intracellular cAMP concentrations of wildtype PAK and PAK*chpC* cells grown in base media (BM) or base media supplemented with BSA (0.1%), mucin (0.05%) or tryptone. Data are presented as mean ± SD for four independent experiments performed in triplicate. (*** $p < 0.0001$; ** $p < 0.005$; * $p < 0.01$, Student's two-tailed t-test compared to base media for PAK or PAK*chpC*).

6.3.5 Quantification of the effect of BSA, mucin and tryptone on twitching motility-mediated expansion of *P. aeruginosa* biofilms at the cellular level

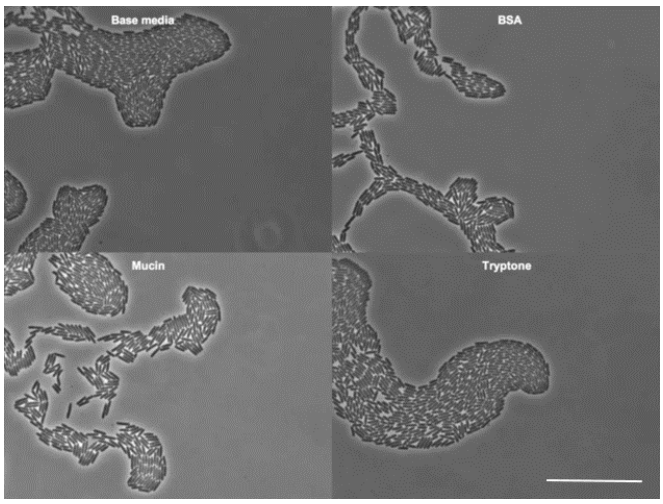
We were interested in understanding how overall biofilm expansion of wildtype PAK was increased by BSA, mucin and tryptone and why overall expansion of PAK*chpC* by these same signals was not stimulated to the same extent (Figure 6-1). This was investigated by analysing time lapse high resolution phase microscopy images with automated computer vision and cell tracking algorithms in Morphometrics. Following capture of biological triplicate sets of time lapse image sequences of the twitching motility-mediated expansion of wildtype PAK

and PAK*chpC* in the presence of base media and base media supplemented with BSA, mucin and tryptone (Movie 6-1 and 6-2), the movement of cells that appeared in the first frame of the time series was tracked using Morphometrics across 50 frames, with each frame being separated by 4 sec intervals (total time = 200 sec). A total of 611-1440 cells were tracked for both wildtype and *chpC* under all conditions. The total distance these cells travelled across the total number of frames that each cell appeared in was used to obtain the average speed of cells in each instance (Figure 6-8). For PAK the average speed of individual cells was significantly increased on BSA, mucin and tryptone compared to PAK on base media. In our subsurface twitching motility assays (Figure 6-1) the overall biofilm expansion of PAK occurred to the greatest extent on tryptone. It was therefore surprising that the average speed of PAK on tryptone was only marginally increased compared to PAK on base media (Figure 6-8). While these results suggest that an increase in average speed may account for the observed increase in overall biofilm expansion of PAK on BSA and mucin, these results cannot explain the significant increase in overall biofilm expansion of PAK on tryptone. These analyses also revealed that PAK*chpC* cells on BSA and mucin had an increase in average speed, but a decrease in average speed on tryptone, compared to PAK*chpC* on base media (Figure 6-8). Thus, as for PAK, while these results suggest that an increase in average speed of PAK*chpC* cells may contribute to the observed increase in overall biofilm expansion on BSA and mucin (Figure 6-1), these observations are unable to explain how individual PAK*chpC* cell movements contribute to an increase in overall biofilm expansion on tryptone. Additionally, these results do not explain why the stimulation of PAK*chpC* overall biofilm expansion on BSA, mucin and tryptone is less than the stimulation of PAK overall biofilm expansion in the presence of the same host-derived signal (Figure 6-1).



Movie 6-1. The effect of serum albumin, mucin and tryptone on *P. aeruginosa* PAK twitching motility.

Time lapse phase microscopy of *P. aeruginosa* PAK twitching motility-mediated biofilm expansion at the interstitial space between a solidified base media or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%) coated microscope slide and a coverslip at 37℃ 4 hrs after inoculation. Scale bar is 20 μm. Original movie 10 mins, played back at 10x (see Appendix 1 for Movie file).



Movie 6-2. The effect of serum albumin, mucin and tryptone on *P. aeruginosa* PAK*chpC* twitching motility.

Time lapse phase microscopy of *P. aeruginosa* PAK*chpC* twitching motility-mediated biofilm expansion at the interstitial space between a solidified base media or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%) coated microscope slide and a coverslip at 37℃ 4 hrs after inoculation. Scale bar is 20 μm. Original movie 10 mins, played back at 10x (see Appendix 1 for Movie file).

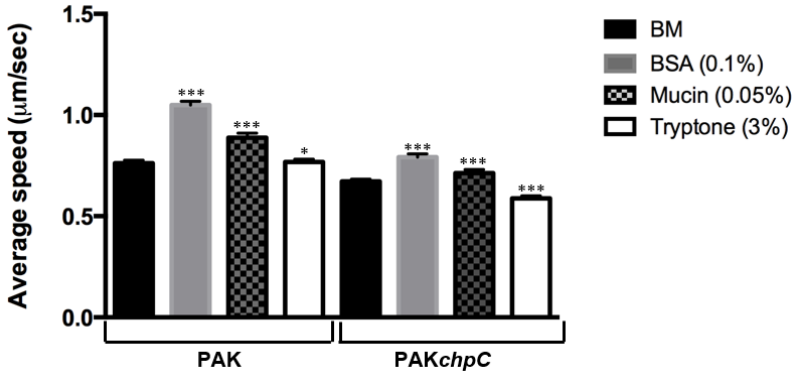


Figure 6-8. The average speed of individual *P. aeruginosa* cells in the presence of base media, BSA, mucin and tryptone.

The average speed of individual PAK and PAK*chpC* cells at the interstitial space between a solidified base media (BM) or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%) coated microscope slide and a coverslip at 37℃ 4 hrs after inoculation. A total of 611-1440 cells were assayed for each strain and each media type. The average speed ± SEM for three independent experiments is shown. (*** $p < 0.0001$; * $p < 0.01$, Student's two-tailed t-test compared to base media for either PAK or PAK*chpC*).

For the purposes of this Chapter we use the term velocity as a measure of the net displacement over time. The net displacement of individual PAK and PAK*chpC* cells on base media, BSA, mucin and tryptone between the first frame of each time series and the last frame that the cell appeared in was calculated (Figure 6-9). This analysis revealed that the velocity of PAK was significantly increased on BSA and mucin, but significantly decreased on tryptone, compared to PAK on base media. As was observed with average speed analyses, an increase in velocity on BSA and mucin may account for some of the observed overall increase in biofilm expansion (Figure 6-1), but again is unable to explain how individual cell movements translate into such a significant increase in overall biofilm expansion on tryptone (Figure 6-1). Surprisingly, while there was no significant difference in velocity of PAK*chpC* on BSA, there was a significant decrease in velocity of PAK*chpC* on mucin and tryptone, compared to PAK*chpC* on base media (Figure 6-9). Thus, while these velocity results suggest that an increase in velocity may account for the observed increase in overall biofilm expansion of PAK on BSA and mucin, they are not able to account for the increase in overall biofilm expansion of PAK on tryptone, nor the increase in overall expansion that is observed for PAK*chpC* on BSA, mucin and tryptone. Additionally, these results are unable to explain why overall biofilm expansion of PAK*chpC* on BSA, mucin and tryptone is not stimulated to the same extent as overall biofilm expansion of PAK in the presence of the same host-derived signal (Figure 6-1).

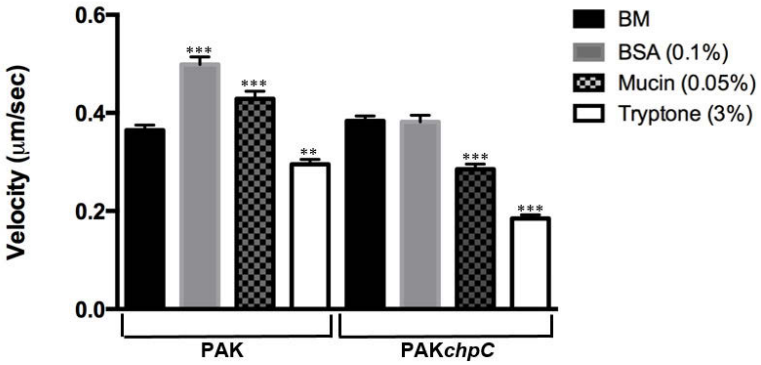


Figure 6-9. The velocity of individual cells in the presence of base media, BSA, mucin and tryptone.

The velocity of individual PAK and PAK*chpC* cells at the interstitial space between solidified base media (BM) or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%) coated on a microscope slide and a coverslip at 37℃ 4 hrs after inoculation for all individually tracked cells. The mean velocity ± SEM for three independent experiments is shown. A total of 611-1440 cells were assayed for each strain and each media type. (*** $p < 0.0001$; ** $p < 0.001$, Student's two-tailed t-test compared to base media for the parent strain; no significant difference was calculated for PAK*chpC* on BSA compared to PAK*chpC* on base media ($p = 0.6604$).

Our results thus far suggest that the observed increase in overall biofilm expansion of PAK and PAK*chpC* may in some cases be due to an increase in average speed and velocity. However, these results have not been able to explain how individual cell movements translate into a significant stimulation of overall biofilm expansion on tryptone for PAK or PAK*chpC*, nor why the overall expansion of PAK*chpC* biofilms is less than the overall expansion of PAK biofilms on BSA, mucin and tryptone.

We considered that differences in the direction of movement of individual cells may contribute to the amount of overall biofilm expansion of PAK and PAK*chpC* on BSA, mucin and tryptone. The ratio of total distance travelled to net displacement for individual cells provides an indication of how frequently a tracked cell undergoes changes in direction. A ratio of 1 demonstrates that a cell did not undergo any changes in direction i.e. the total distance travelled = net displacement. A number greater than 1 shows that a cell did change direction, i.e. net displacement < total distance travelled, and indicates that directional changes were made during the time the cell was tracked. These calculations were performed for PAK and PAK*chpC* cells on base media, BSA, mucin and tryptone (Figure 6-10). This analysis

demonstrated that while there was no significant difference in the ratios for PAK cells on BSA and mucin compared to PAK on base media, there was a significant increase in the amount of directional changes for PAK on tryptone compared to PAK on base media. An increase in the number of directional changes by PAK cells on tryptone may explain why our average speed and velocity results (Figures 6-8 and 6-9) do not account for the observed increase in overall biofilm expansion by PAK on tryptone observed in our subsurface assay (Figure 6-1). Interestingly, these results suggest that PAK*chpC* cells underwent significantly more directional changes on BSA, mucin and tryptone compared to PAK*chpC* cells on base media (Figure 6-10). Remarkably, the ratio for PAK*chpC* cells on tryptone was dramatically higher than the ratio for PAK*chpC* cells on BSA or mucin (Figure 6-10). As an increase in the number of directional changes of individual cells is likely to affect overall biofilm expansion, these results may explain, to some extent, why overall biofilm expansion of PAK*chpC* on BSA, mucin and tryptone is not stimulated to the same extent as PAK.

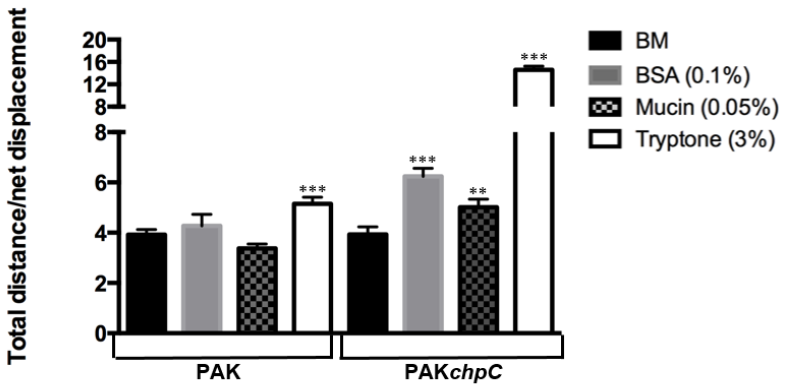


Figure 6-10. The ratio of total distance to net displacement for PAK and PAK*chpC* cells in the presence of base media, BSA, mucin and tryptone.

Ratio of total distance travelled to net displacement of individual PAK and PAK*chpC* cells at the interstitial space between solidified base media (BM) or base media supplemented with BSA (0.1%), mucin (0.05%) and tryptone (3%) coated on a microscope slide and a coverslip at 37℃ 4 hrs after inoculation. The mean ± SEM for three independent experiments is shown. A total of 611-1440 cells were assayed for each strain and each media type. (*** $p < 0.0001$; ** $p < 0.05$, Student's two-tailed t-test compared to base media for the parent strain; no significant difference was calculated for PAK on BSA compared to PAK on base media ($p = 0.4843$) and for PAK on mucin compared to PAK on base media ($p = 0.3195$).

Our results thus far suggest that an increase in average speed and velocity may account for some of the observed increases in overall biofilm expansion of PAK and PAK*chpC* on BSA and mucin. Additionally, our results suggest that an increase in the frequency of PAK*chpC* cell directional changes on BSA, mucin and tryptone may account for the observed decrease in overall biofilm expansion for PAK*chpC* compared to PAK. Due to computer power limitations, these analyses have only been performed on the movement of *P. aeruginosa* cells over 200 seconds. Fluctuations in the speed of *P. aeruginosa* twitching motility over longer time periods have been suggested previously (Semmler et al., 1999), and therefore we reasoned that analysis of a longer time period might better reflect the increase in surface areas we measured in our subsurface twitching motility assays after 48 hrs (Figure 6-1). To test this idea we used low magnification images that we had obtained just prior to capture of our high magnification time series images, to measure the distance that the leading edge of the biofilm had expanded over 600 seconds for both PAK and *chpC* on base media, BSA, mucin and tryptone. We reasoned that, as these low magnification images would allow us to analyse a larger proportion of the overall biofilm, over a longer period of time, the results might be more representative of *P. aeruginosa* biofilm expansion than our high magnification image analyses. The expansion of the leading edge of the biofilm was measured by overlaying low magnification images of the first and last frame of each time series, and false colouring the areas which contained cells in these frames red and green, respectively (Figure 6-11A). Sections which contained cells from both the first and last frame were therefore coloured yellow (Figure 6-11A). The distance that each leading edge raft had expanded over 600 seconds was then measured for PAK and PAK*chpC* on base media, BSA, mucin and tryptone (Figure 6-11B). These results revealed that the expansion distances of the leading edge rafts for both PAK and PAK*chpC* on BSA, mucin and tryptone were all increased compared to the leading edge raft expansion distances for each strain on base media. This suggests that analysis of a larger proportion of the total biofilm, over a longer time period is likely to explain how alterations in individual cell movements translate into an increase in overall biofilm expansion. However, these results still do not explain why overall biofilm expansion of PAK*chpC* is not stimulated to the same extent as the overall biofilm expansion of PAK on BSA, mucin and tryptone.

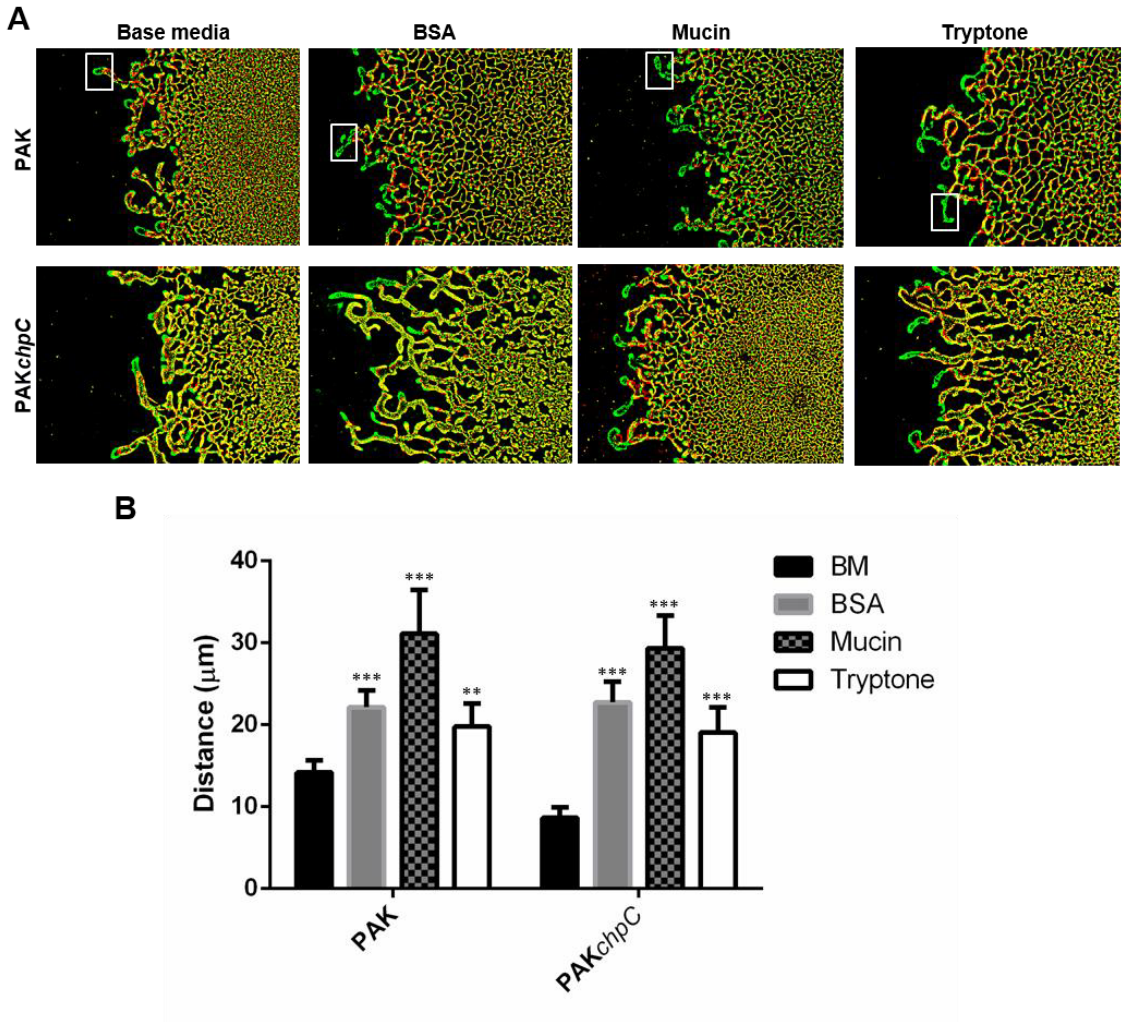


Figure 6-11. Expansion of the leading edge of PAK and PAK*chpC* biofilms on base media, BSA, mucin and tryptone.

Time lapse phase contrast microscopy of *P. aeruginosa* PAK and PAK*chpC* twitching motility-mediated expansion at the interstitial space between a solidified base media (BM) or base media supplemented with BSA (0.1%), mucin (0.05%) or tryptone (3%)-coated microscope slide and glass coverslip of time lapse movies taken over 600 seconds. (A) Represented as an overlay (yellow) of the first (red) and last frame (green). Representative images are shown based upon three independent experiments. White boxes in the top panel outline an example of one leading edge raft which was measured in these analyses. (B) Distances of leading edge raft expansion over a total of 600 seconds. A total of 14-41 leading edge raft distances were measured from three independent experiments. (*** $p < 0.0001$; ** $p < 0.005$ Student's two-tailed t-test compared to base media for either wildtype PAK or PAK*chpC*).

6.4 Discussion

In this Thesis Chapter we investigated the twitching motility response of *P. aeruginosa* to a range of host-derived signals. This allowed identification of a novel signal, GlcNAc, which stimulates twitching motility. The mechanism of twitching motility stimulation by GlcNAc as well as the previously identified stimulants, BSA, mucin and tryptone was also investigated, and ChpC implicated in the twitching motility response to BSA, mucin and tryptone. We also demonstrate that tryptone, but not BSA and mucin, increases the levels of surface assembled tfp, with this effect being mediated via a ChpC-independent mechanism. Additionally, the twitching motility response to BSA, mucin and tryptone was shown to result in an increase in icAMP levels, however this is also not mediated by ChpC.

The Chp system has a putative role in controlling the twitching motility response of *P. aeruginosa* to changes in environmental signals (Whitchurch, 2006), however it is unclear how signal sensing translates into changes in tfp function. In *E. coli* a chemotactic response to chemical gradients is mediated by alterations in the levels of CheY-P, which affects flagella rotation, causing the cell to either swim and continue on the same course, or tumble and change direction (Baker et al., 2006). When *E. coli* is in a uniform environment, cells move in a random walk, quickly alternating between swimming and tumbling to ultimately maintain an overall constant direction (Baker et al., 2006). While this is well studied in *E. coli* these scenarios do not easily translate into alterations in the extension and retraction of the tfp of *P. aeruginosa*. Control of tfp extension and retraction may more closely resemble that of the unidirectional polar flagella in an organism such as *Rhodobacter sphaeroides.* As the flagellum of *R. sphaeroides* is restricted to one direction of rotation (Armitage and Macnab, 1987), the chemotactic response to changes in signal gradients is mediated by stop-start cycles of flagella rotation, as opposed to alterations in rotational direction (Packer et al., 1996). In this organism a number of Che homologs are involved in the response to signal gradients with levels of CheY-P controlling the stopping and starting of flagella rotation (Pilizota et al., 2009, Porter et al., 2011). *R. sphaeroides* is also capable of a chemokinetic response to attractants (Poole et al., 1990), which results in a sustained increase in the rate of flagella rotation and an increased overall rate of swimming (Packer and Armitage, 1994). In the current study our cell tracking analysis, as well as analysis of low magnification images of expansion of the leading edge of the biofilm, suggest that the increase in overall biofilm expansion of PAK and PAK*chpC* may be due, at least in part, to an increase in the average speed and velocity of individual cells. As

BSA, mucin and tryptone were all present at a constant concentration the observed twitching motility response is most likely to be chemokinetic rather than chemotactic, which could result from an increase in the rate of tfp extension and retraction. Although differential rates of *P. aeruginosa* tfp extension and retraction in response to environmental changes have not been reported, this has been observed for *Neisseria gonorrhoeae* (Clausen et al., 2009, Kurre and Maier, 2012).

It has been reported that the expansion of *P. aeruginosa* in the subsurface twitching motility assay produces concentric rings within the expanded zone, which has been suggested to coincide with alterations in the rate of twitching motility and/or periods of clonal growth (Semmler et al., 1999). While our subsurface stab assays were conducted over a 48 hr period, our cell tracking analyses were only performed on cell movements over a period of 200 seconds. These 200 second analyses suggest that an increase in the average speed and velocity of PAK and PAK*chpC*, and an increase in the frequency of directional changes of PAK*chpC*, explains to some extent, the observed differences in overall biofilm expansion for BSA and mucin. These analyses also suggest that an increase in the number of directional changes made by PAK*chpC* on BSA, mucin and tryptone may explain why PAK*chpC* overall biofilm expansion is not stimulated to the same extent as overall biofilm expansion of PAK in the presence of the same host-derived signals. However, while these results suggest that an increase in the frequency of directional changes of individual PAK and PAK*chpC* cells on tryptone may account for the observed average speed and velocity results, we are not able to explain how individual cell movements result in the significant increase in overall biofilm expansion observed in our subsurface stab assays. Initial analyses of cell movements over 600 seconds suggest that tracking more cells over a longer time period is likely to explain how alterations in individual cell movements translate into an increase in overall biofilm expansion. Thus it is likely that the 200 second time periods which we used to track cell movements, particularly for tryptone, were not representative of the movement of cells over a much longer period. To address this further it would be necessary to analyse the movement of PAK and PAK*chpC* cells on base media, BSA, mucin and tryptone over a significantly longer period of time (tens of mins-hrs), and combine the data to provide a better overall picture of the twitching motility response of *P. aeruginosa* to these host-derived signals.

It has also been suggested that cells within the advancing “raft heads”, i.e. the group of cells which initiate biofilm expansion (see white boxed regions in Figure 6-11A), maintain high velocity coherence with neighbouring cells and undergo few directional changes as they

expand into virgin territory. This results in cells travelling in the same direction in the same relative alignment to one another for longer periods within these "raft heads" (Gloag et al., 2013). Our analyses of the ratio of total distance travelled to net displacement for PAK and PAK*chpC* on base media, BSA, mucin and tryptone suggest that an increase in the number of directional changes of PAK*chpC* cells may account for the observed decrease in overall biofilm expansion by PAK*chpC* compared to PAK. We have not been able to determine the velocity coherence values for PAK and PAK*chpC* cells in the presence of these signals, as this would require tracking of all cells within all frames of the time series. However, it is conceivable that in addition to the increased number of directional changes made by PAK*chpC*, a loss of coherence in the "raft heads" as well as an increased rate of cell reversal may account for the decreased stimulation of PAK*chpC* twitching motility on BSA, mucin and tryptone, compared to wildtype. Thus it would be interesting to not only analyse the movement of PAK and PAK*chpC* cells over a longer period of time but to also compare the coherence velocities of the "raft heads" and the number of cell reversals of PAK and PAK*chpC* on BSA, mucin and tryptone. These analyses would require tracking of all cells that appear in all frames of a time series, which could be achieved through optimisation of our computer vision algorithms.

In the current Chapter we observed that in the absence of ChpC, twitching motility was still stimulated to some extent by BSA, mucin and tryptone. As FimX has been reported to be involved in the twitching motility response of *P. aeruginosa* to mucin and tryptone (Huang et al., 2003) it is likely that the observed stimulation of twitching motility by these two signals in the absence of ChpC is due to FimX. As FimX has phosphodiesterase activity against the second messenger c-di-GMP (Kazmierczak et al., 2006) and an apparent role in sensing environmental signals (Huang et al., 2003) it has been suggested that FimX may be involved in both extracellular signal sensing and modulation of intracellular c-di-GMP levels to control *P. aeruginosa* twitching motility (Qi et al., 2011). Thus it is possible that FimX may interface with ChpC/PilI and/or other components of the Chp system to allow alterations in intercellular c-di-GMP levels and tfp function in response to mucin and tryptone. It would be interesting to investigate this further by examining the effect of inactivating both ChpC and FimX on the twitching motility response of *P. aeruginosa* to mucin and tryptone. Additionally, given the putative role of FimX in sensing mucin and tryptone, and the c-di-GMP phosphodiesterase activity of this protein, it would also be informative to determine whether mucin and tryptone affect intercellular c-di-GMP levels via FimX.

As FimX is not implicated in the twitching motility response of *P. aeruginosa* to BSA (Huang et al., 2003), and as some stimulation of twitching motility is still observed in the absence of ChpC, this suggests that additional components are involved in the twitching motility response to this signal. It is also possible that other components, in addition to FimX and ChpC, are involved in the twitching motility response of *P. aeruginosa* to mucin and tryptone. *P. aeruginosa* possesses at least 26 MCPs which sense a range of extracellular signals (Croft et al., 2000, Kato et al., 2008). It is possible that multiple MCPs are linked to ChpA by ChpC and possibly by PilI, to contribute to the extracellular sensing of BSA by ChpC, and extracellular sensing of mucin and tryptone by ChpC and FimX to feed through the Chp system to direct twitching motility. Therefore it would be interesting to investigate the involvement of other MCPs, in addition to PilJ, in responding to BSA, mucin and tryptone.

Additionally, as mucin and BSA are a large glycoprotein and a large globular protein, respectively, and tryptone is a mixture of oligopeptides, it may also be relevant to consider that these extracellular sensors are responding to a smaller component of these signals, which could be produced by one or more of the large number of secreted *P. aeruginosa* proteases (Hoge R et al., 2010). Indeed, previous reports have demonstrated that the twitching motility response of *P. aeruginosa* to phosphatidylcholine (PC) requires degradation of PC by the phospholipase C PlcB to release diacylglycerol (DAG). The long-chain fatty acid (LCFA) moiety of DAG is then involved in directing twitching motility of *P. aeruginosa* up PC gradients (Barker et al., 2004). It would be interesting to investigate if BSA, mucin, and tryptone themselves, or degradation products of these signals are the actual signals sensed by *P. aeruginosa* to stimulate twitching motility. If degradation products are shown to be effective it would also be interesting to identify the secreted proteases, or other secreted factors that are involved in generating these products.

The results in this Chapter also demonstrate that an increase in icAMP levels occurs in cells grown on BSA, mucin and tryptone in both wildtype and a *chpC* mutant. Previously reported data suggest that components of the Chp system control twitching motility in an icAMP-independent manner (Fulcher et al., 2010). While our results demonstrate that ChpC is not involved in increasing icAMP levels in response to these host-derived signals, this does not discount the involvement of other components of the Chp system in modulation of icAMP levels. Our results also suggest that an increase in icAMP levels correlates with an increase in twitching motility which agrees with previously reported work within our laboratory (Nolan et al., 2012) and others (Inclan et al., 2011). As discussed in Chapter 3, the control of *P.*

aeruginosa icAMP levels and twitching motility appears to be very complex and involves multiple signal transduction systems including the Chp system, FimL and the Vfr and icAMP synthesis/degradation pathways. Thus how these extracellular signals bring about an increase in icAMP levels is likely to also be complex. Our results are suggestive of a chemokinetic response to BSA, mucin and tryptone, which may result from an increase in the rate of tfp extension and retraction. One possibility is that this increase in icAMP levels could result in increased activation of Vfr-dependent gene expression of factors which are involved in tfp biogenesis, assembly and/or twitching motility function. Additionally, as a number of ATPases are involved in the extension and retraction of tfp (Whitchurch, 2006), this increase in icAMP levels may inhibit adenylate cyclase generation of cAMP from ATP, providing more ATP for ATPase-mediated tfp extension and retraction.

Interestingly, we demonstrate that GlcNAc stimulates twitching motility of *P. aeruginosa* strain PA14 but not PAK. As phenotypic strain variation is common in this organism this observation is not surprising. Remarkably, a significantly lower (> 20 times) concentration of mucin (0.05%, which is equivalent to 0.9 μM porcine mucin) is required to stimulate twitching motility of PAK, compared to the concentration of GlcNAc (≥ 20 mM) required to stimulate twitching motility of PA14. While it will be important to investigate whether or not mucin also effects PA14 twitching motility, it is logical that a higher concentration GlcNAc would be required to stimulate twitching motility, as GlcNAc is a side-chain of the large glycoprotein mucin. Thus, in addition to investigating the strain-specific twitching motility responses of *P. aeruginosa* to mucin and GlcNAc, it will also be interesting to examine the effect of signal concentration.

GlcNAc-induced stimulation of *P. aeruginosa* twitching motility is likely to be relevant within polymicrobial environments. Gram-positive bacteria are known to shed peptidoglycan, which contains GlcNAc in a polymeric form, in large quantities (Doyle et al., 1988, Mauck et al., 1971). The ability of *P. aeruginosa* to uptake and sense GlcNAc has respective roles in mediating lysis of *Staphylococcus aureus* and *Bacillus licheniformis*, and in stimulating production of the virulence factors elastase and pyocyanin (Korgaonkar et al., 2012, Korgaonkar and Whiteley, 2011). Stimulation of twitching motility by GlcNAc may provide a competitive advantage within a polymicrobial setting by allowing *P. aeruginosa* to navigate closer to the source of GlcNAc, for instance a Gram-positive bacterial cell, providing more effective delivery of virulence factors.

A number of obvious GlcNAc sensing components, including ChpC, FimX and the two component sensor regulator pair PA14_07820/PA14_07840 were shown to not be involved in the twitching motility response of *P. aeruginosa* to this extracellular signal. This suggests that other core components of the Chp system are involved in mediating this response and/or that additional components from outside of this system are involved. One avenue which could be explored would be to use TraDIS (transposon directed insertion sequencing) (also known as Tn-seq) with a pooled PA14 transposon mutant library to identify these components. This technique utilises mass paralleled DNA sequencing to compare the relative frequency of transposon insertions in genes within a pooled mutant population (Barquist et al., 2013). As twitching motility is self-selecting i.e. mutants will either expand away from the site of inoculation or not, this technique could allow identification of genes that are involved in the twitching motility response of *P. aeruginosa* to GlcNAc.

In the current Chapter we have identified a novel host-derived signal, GlcNAc, which stimulates *P. aeruginosa* PA14 twitching motility, and implicated ChpC in the twitching motility response to the previously identified host-derived signals BSA, mucin and tryptone. Our results suggest that the twitching motility response of *P. aeruginosa* to these host-derived signals is chemokinetic, which could possibly result from an increase in the rate of tfp extension and retraction. Overall, our results suggest that a number of complex pathways, including the Chp system and an icAMP-dependent pathway, are involved in controlling the twitching motility response of *P. aeruginosa* to a range of host-derived signals.

Chapter Seven

General Discussion

7.1 Introduction

P. aeruginosa is an opportunistic pathogen that causes acute and chronic infections in a range of immunocompromised patients (Lyczak et al., 2000, Tatterson et al., 2001). The ability of *P. aeruginosa* to exploit damaged epithelia to cause infections in these individuals is a major contributor to morbidity and mortality (Kazmierczak et al., 2001). Formation of biofilms by *P. aeruginosa* is often associated with the chronic nature of infections caused by this pathogen (Costerton, 1999). Within hydrated environments, *P. aeruginosa* forms surface-attached sessile biofilms, and in semi-hydrated environments undergoes rapid twitching motility-mediated active biofilm expansion across surfaces (Stoodley et al., 2002, Semmler et al., 1999). In addition to the involvement of tfp-mediated twitching motility in active biofilm expansion (Semmler et al., 1999), this form of surface translocation is also important in a number of the stages of sessile biofilm formation (Klausen et al., 2003a, Klausen et al., 2003b, Barken et al., 2008, O'Toole and Kolter, 1998). A range of regulatory systems are involved in controlling twitching motility in *P. aeruginosa* (Whitchurch, 2006). While these systems are known to modulate the levels of a number of intracellular signals, and are predicted to sense a number of extracellular signals to regulate twitching motility, there is a great deal that is unknown.

The focus of this Thesis has been on characterising the involvement of small intracellular and extracellular signalling molecules in *P. aeruginosa* twitching motility-mediated biofilm expansion. This included an investigation into the relationship between intracellular levels of the important second messenger, cAMP, and levels of twitching motility-mediated biofilm expansion. The work presented in this Thesis also identifies a novel role for the universal energy currency molecule ATP, which in an extracellular setting was found to be involved in both *P. aeruginosa* sessile biofilm formation and twitching motility-mediated biofilm expansion. Finally, a novel extracellular twitching motility stimulator, GlcNAc was identified, and a detailed molecular and mechanistic analysis of the twitching motility response of *P. aeruginosa* to the host-derived extracellular signalling molecules, mucin, serum albumin and tryptone carried out.

Overall, the results of this Thesis provide further insight into the complex regulation of icAMP levels and twitching motility in *P. aeruginosa* and extend our understanding of the biofilm response of *P. aeruginosa* to a number of important host-derived signals. The key

conclusions and outcomes of this work, as well as future avenues of investigation are discussed below.

7.2 Extragenic suppressor mutations that restore twitching motility to *fimL* mutants of *Pseudomonas aeruginosa* are associated with elevated intracellular cyclic AMP levels

Chapter 3 of this Thesis describes the characterisation of five independent PAO1 *fimL* mutants which had restored twitching motility ability. All of these revertants were found to have increased levels of icAMP above that of the parent *fimL* mutant strain to varying extents. Whole genome sequencing of one revertant identified a SNP in the gene encoding the cAMP phosphodiesterase, CpdA, with further analysis revealing that this SNP resulted in a loss-of-function mutation which was responsible for the observed increase in icAMP levels and restoration of twitching motility. A different mutation in *cpdA* was identified in a second revertant, however it is not clear how this mutation contributes to the observed twitching motility and icAMP phenotypes, and suggests that the primary site of suppressor mutation in this strain occurs elsewhere in the genome. The remaining three revertants had wildtype *cpdA* sequences indicating that the site(s) of suppressor mutation in these strains also occurs elsewhere in the genome. Overall, these results demonstrate that the observed differences in twitching motility and icAMP phenotypes of these *fimL* revertant strains are the result of at least two reversion mechanisms.

A number of complex systems appear to intersect to control the biogenesis, assembly and twitching motility-function of tfp. Modulation of intracellular levels of the second messenger cAMP by a number of these systems also appears to be important in controlling these processes in *P. aeruginosa* however, it is unclear precisely how this occurs. The results presented in this Thesis suggest that an increase in icAMP levels is associated with an increase in twitching motility levels. It has also been shown that low icAMP levels are associated with a decrease in twitching motility (Inclan et al., 2011). Inclan et al. (2011) have also suggested that FimL post-translationally modulates the activity of the adenylate cyclase CyaB to alter icAMP levels and twitching motility. In a further layer of complexity the Chp system has been reported to control tfp assembly in a cAMP-dependent manner, and the twitching motility-mediated function of the tfp in a cAMP-independent manner (Fulcher et al., 2010). Taken together the results of these three studies suggest two possible scenarios. The first of these is

that FimL intersects either directly or indirectly between the Chp system and Vfr/cAMP pathways to control icAMP levels and modulate twitching motility levels. The second possibility is that FimL and the Vfr/cAMP pathways control twitching motility via modulation of icAMP levels, but that these components work separately to the Chp system which controls twitching motility independently of the Vfr/cAMP pathways. While either scenario appears possible, it is also important to consider the strains of *P. aeruginosa* that were used in these various studies. The current study and that of Inclan et al. (2011) used PAO1 and PA103, while strain PAK was used in the study by Fulcher et al. (2010), and thus both models may be correct, but indicative of strain specific regulation of these processes. Overall, these observations suggest that icAMP levels and twitching motility levels correlate. However, the interactions between the Chp system, FimL and Vfr/cAMP pathways which ultimately control twitching motility are extremely complex. Further work is required to fully understand the regulatory networks which control these processes in *P. aeruginosa*. One avenue which could be pursued further is to use whole genome sequencing of the four remaining *fimL* revertants from this Thesis to identify the site(s) of suppressor mutation in these strains. Identification and characterisation of the genes involved in the phenotypic reversion of twitching motility and increase in icAMP levels in these strains is likely to provide further information on the interactions of these regulatory networks to control these processes in *P. aeruginosa*.

To gain further information on the interactions between these complex regulatory systems one could utilise fluorescent protein fusions and/or a bacterial two-hybrid and pull down assay approach. Fluorescent studies have already suggested that FimL and CyaB co-localise to the cell poles (Inclan et al., 2011), however it would be interesting to examine the cellular localisation and/or interactions between FimL and other components in this pathway, such as Vfr and CpdA, using a similar approach. It would also be interesting to examine the interactions with fluorescent fusions of FimL and components of the Chp system. As FimL shares homology with the N-terminal of ChpA, it has been suggested that these two proteins interact within the same signal transduction pathway to control twitching motility and icAMP levels (Whitchurch et al., 2005, Inclan et al., 2011). Additionally, other Chp system components, ChpA, ChpB, ChpC, PilH, PilI, PilG and PilJ have been implicated in separate studies in the control of twitching motility (Darzins, 1993, Darzins, 1994, Darzins, 1995, Whitchurch et al., 2004) and icAMP levels (Fulcher et al., 2010), thus it would be interesting to examine the interactions between these Chp system components and FimL using a fluorescent fusion protein approach. A super resolution microscope, such as a Delta Vision

OMX Blaze (Applied Precision Inc.), could be utilised in such assays to overcome any issues associated with insufficient resolution of individual proteins (Strauss et al., 2012). Fluorescence Resonance Energy Transfer (FRET) could also be utilised to investigate such interactions. Use of a *Pseudomonas* two-hydrid library (Houot et al., 2012) could also provide specific information on the interactions between the proteins in these pathways. It would be interesting to use FimL as bait and screen a *Pseudomonas* two-hybrid library for interacting proteins. Pull down assays using tagged purified proteins could also be used to identify and/or confirm specific protein interactions. These experiments could pinpoint proteins in the Chp system and cAMP/Vfr pathways, as well as novel proteins which interact with FimL and allow more detailed insight into the regulation of twitching motility and icAMP levels in *P. aeruginosa*.

Clearly, the regulation of icAMP levels and twitching motility in *P. aeruginosa* is a complex process. The results of this study have shed some light on this complex area, however a great deal still remains to be done to fully understand how the Chp system, FimL and the cAMP/Vfr pathways intersect to control these important processes in *P. aeruginosa*.

7.3 Extracellular ATP inhibits *Pseudomonas aeruginosa* twitching motility-mediated biofilm expansion and stimulates sessile biofilm formation

Chapters 4 and 5 of this Thesis characterise the effect of the host-derived signal eATP on *P. aeruginosa* biofilm formation. These results demonstrate that eATP inhibits twitching motility-mediated biofilm expansion and stimulates sessile biofilm formation. eATP is a host-derived signal which is released by epithelial cells in response to invading pathogens and/or cell damage (Virgilio, 2007). A number of other host-derived signals, including serum albumin, iron deficiency and lactoferrin, have also been shown to have an inverse effect on *P. aeruginosa* sessile biofilm formation and twitching motility-mediated biofilm expansion. However, unlike eATP these signals have been shown to inhibit sessile biofilm formation and stimulate twitching motility-mediated biofilm expansion and thus it has been proposed that the inverse effect of these signals on biofilm formation and expansion may assist the immune system in removal of *P. aeruginosa* (Hammond et al., 2010, Huang et al., 2003, Singh et al., 2002, Patriquin et al., 2007). Interestingly, the results described in Chapters 4 and 5 of this Thesis suggest that eATP could instead provide a protective advantage for *P. aeruginosa* by inhibiting

twitching motility-mediated biofilm expansion and stimulating formation of a sessile biofilm community, which is likely to be protected from the host immune system. Thus the ability of *P. aeruginosa* to transition from a motile to a sessile mode of life in response to this particular host-derived signal, eATP, is likely to have significant implications for the success of *P. aeruginosa* within an infection setting.

Given the biofilm response of *P. aeruginosa* to a number of host-derived signals, and the potential impact of this response on both the host and bacterium it would be interesting to explore the biofilm response of *P. aeruginosa* to a number of other host-derived signals which have an established role in either sessile biofilm formation or twitching motility-mediated biofilm expansion. Extracellular phosphatidylcholine (PC), which is a major constituent of animal membranes (Geiger et al., 2013) and is present at elevated concentrations in the sputum of CF patients (Meyer et al., 2000), has been shown to stimulate *P. aeruginosa* twitching motility (Barker et al., 2004), however the effect of sessile biofilm formation has not been investigated. Another host-derived signal, oligopeptides, which are present at high concentrations within human urine (Kentsis et al., 2009, Putnam, 1971, Lutz et al., 1972) have been shown, in the form of tryptone, to stimulate *P. aeruginosa* twitching motility (Huang et al., 2003). Given that *P. aeruginosa* biofilm formation is commonly associated with CAUTIs (Stickler, 2008, Donlan, 2001b, Sabbuba et al., 2002) it would be interesting to examine the effect of oligopeptides on *P. aeruginosa* sessile biofilm formation. Additionally, a number of signals have been shown to stimulate dispersal of *P. aeruginosa* from sessile biofilms. These include exogenously added, and endogenously produced NO, the DSF-like molecule *cis*-2-decenoic acid and D-amino acids (Davies and Marques, 2009, Barraud et al., 2006, Kolodkin-Gal et al., 2010), however the effect of these molecules on twitching motility-mediated biofilm expansion has not been examined. Twitching motility has also been implicated in biofilm dispersal (Chiang and Burrows, 2003), thus given that these signals stimulate dispersal of *P. aeruginosa* from sessile biofilms it is possible that this occurs, at least in part, by stimulating twitching motility. As the biofilm response of *P. aeruginosa* to eATP is likely to provide a potential protective effect for the bacterium, it is also possible that these additional host-derived signals could offer a similar adaptive response in mediating a transition between motile and sessile modes of life. By better understanding the nature of the adaptive responses of bacteria to host-derived signals more effective strategies may be developed to combat biofilm-associated bacterial infections.

Stimulation of sessile biofilm formation by eATP has been previously reported for *E. coli*, *A. baumannii*, *S. maltophilia* and *S. aureus* (Xi and Wu, 2010). While some strains of *E. coli*, *A. baumannii*, and *S. maltophilia* possess tfp or tfp-like structures, and varying levels of twitching motility ability (Huang et al., 2006, Xicohtencatl-Cortes et al., 2009, Harding et al., 2013), *S. aureus* does not possess tfp. However, *S. aureus* has been reported to undergo relatively rapid colony spreading across soft agar plates (Kaito and Sekimizu, 2007). Like *P. aeruginosa* each of these bacterial species are important human pathogens (Rabin and Surette, 2012), and thus it would be interesting to examine whether eATP has a similar inverse effect on sessile biofilm formation and twitching motility-mediated biofilm expansion in *E. coli*, *A. baumannii*, *S. maltophilia*, or the colony spreading phenotype of *S. aureus* to determine if a similar adaptive response to eATP occurs in these organisms.

It would also be interesting to understand how eATP specifically controls *P. aeruginosa* twitching motility inhibition and sessile biofilm stimulation. While the inhibition of twitching motility by eATP could decrease active biofilm expansion and thus stimulate cells to begin the process of sessile biofilm development sooner, this may not be the only factor contributing to the stimulation of sessile biofilm formation. Xi et al. (2010) suggest that eATP may induce cell lysis and subsequent eDNA release, with this eDNA contributing to biofilm formation. It would be interesting to examine if a similar effect is observed in *P. aeruginosa* and additionally to determine whether eATP stimulates the production of other EPS constituents such as the Psl and Pel exopolysaccharides, which in turn could stimulate sessile biofilm formation. While there are currently no Pel specific stains, the effect of eATP on Psl production could be examined using a Psl-specific MOA or HHA lectin stain (Ma et al., 2007). Additionally, the effect of eATP on transcription of the Psl (PA2231-PA2245) and Pel (PA3064-PA3058) operons in *P. aeruginosa* could also be investigated with qPCR studies. Our results also demonstrate that eATP needs to be hydrolysed to inhibit twitching motility-mediated biofilm expansion. One approach to determine whether or not eATP is hydrolysed by enzymes within the membrane or within the cytosol of *P. aeruginosa* could be to follow the fate of extracellular [α-^{32}P]ATP. It would also be relevant to identify the enzyme(s) involved in eATP hydrolysis. Possible targets include the ATPases PilB, PilT and PilU, which are involved in tfp retraction (Bertrand et al., 2010, Guzzo et al., 2009). It is conceivable that these enzymes could utilise internalised eATP to enhance the rate of tfp retraction in order to bring about the observed decrease in surface assembled tfp and twitching motility inhibition. It is also possible that the adenylate cyclases CyaA and CyaB (Fulcher et al., 2010, Wolfgang et al., 2003) could utilise

internalised eATP to generate cAMP, which may affect the assembly and function of the tfp. Additionally, as the non-hydrolysable ATP analogue AMP-PNP does not inhibit twitching motility it is plausible that phosphate or adenosine, products of ATP hydrolysis, may be sensed by the cell to control twitching motility. Recent work has demonstrated that another product of ATP hydrolysis, adenosine, affects biofilm formation and a form of flagellum and tfp-mediated motility, swarming motility in *P. aeruginosa* (Sheng et al., 2012). Thus it would also be interesting to also investigate the effect of adenosine on *P. aeruginosa* twitching motility in future work.

The transition from a motile to a sessile mode of life is often associated with a transition between acute and chronic infection states (Gellatly and Hancock, 2013). Given that our results suggest that the biofilm response of *P. aeruginosa* to eATP stimulates a transition to a chronic sessile biofilm mode of life, which is likely to be relevant within an infection setting, it is possible that eATP may also influence the production of other acute and chronic virulence factors. Our results demonstrate that eATP reduces levels of tfp. A decrease in tfp levels has been reported to occur via an RpoN-dependent mechanism in chronic *P. aeruginosa* strains (Mahenthiralingam et al., 1994). It would also be interesting to determine whether eATP also has an effect on the production of other acute virulence factors such as elastase, alkaline protease, pyocyanin, flagellum, LPS and T3SS expression. The RetS/LadS/Gac/Rsm signalling pathway is involved in mediating the transition of *P. aeruginosa* from a motile to a sessile mode of life, and is also involved in the global control of genes associated with either chronic or acute infections (Mikkelsen et al., 2011). As eATP appears to be a signal which triggers a transition between these two modes of life it would be very interesting to determine if we see alterations in expression of these global regulators in the presence of eATP.

Our results also demonstrate that while addition of 10 m eATP completely represses the ability of *P. aeruginosa* to undergo twitching motility, there is still some surface tfp expression. There are a number of possible explanations for these results. It may be that while our Western assay is able to detect surface-associated PilA, these PilA monomers are not assembled into a functional pilus. Additionally, *P. aeruginosa* may require a threshold level of surface-assembled tfp in order to undergo twitching motility. It would therefore be interesting quantify the levels of surface-assembled tfp from cells grown at a range of eATP concentrations (0-5 mM, where cells are still able to undergo twitching motility, and from 5-10 mM where the ability to undergo twitching motility is abolished) and determine whether the level of surface-associated tfp can be correlated to the level of twitching motility.

In addition to exogenous eATP, our results demonstrate that endogenously produced bacterial eATP is important in coordinating both sessile biofilm formation and twitching motility-mediated biofilm expansion. While quorum sensing is important in development of *P. aeruginosa* sessile biofilms (Davies, 1998) the Las and Rhl quorum sensing systems are not involved in controlling *P. aeruginosa* twitching motility (Beatson et al., 2002b). No other small intercellular signalling molecules have been shown to be involved in controlling twitching motility and thus we propose that eATP could be considered to be an important intercellular signalling molecule that controls twitching motility.

Given the propensity of *P. aeruginosa* to infect damaged epithelial cells (Ramphal and Pyle, 1983) the production of high endogenous levels of bacterial eATP that are within the cytotoxic mM range (Di Virgilio, 1998) is likely to play a role in *P. aeruginosa* pathogenesis by inducing local inflammatory damage and fibrosis. It would be interesting to investigate the effect of this endogenously produced bacterial eATP in an epithelial wound model. The ability of *P. aeruginosa* to inhibit the process of epithelial cell wound repair has been examined in *in vitro* MDCK (Madin-Darby Canine Kidney) and A549 cell line models (Kazmierczak et al., 2001, Geiser et al., 2001). It would be interesting to use either of these *in vitro* models to examine the effect of apyrase addition on the ability of *P. aeruginosa* to inhibit wound repair and/or to establish infection at the wound site. As we have detected very high levels of endogenous eATP production by *P. aeruginosa* we would expect that apyrase hydrolysis of this eATP would result in more rapid wound repair and a decreased ability of *P. aeruginosa* to establish an infection at the wound site.

It would also be interesting to further examine the role of endogenous eATP in directing twitching motility-mediated expansion of *P. aeruginosa* biofilms. Our results suggest that addition of high concentrations of eATP removes the self-produced eATP gradient within the active migration zone, and results in cells within the biofilm being unable to determine the direction in which to expand. It would therefore be interesting to use our automated cell tracking algorithms (Chapter 6) to track the movement of individual cells in the presence of a range of eATP concentrations to gain a better understanding of the effect of eATP on directing twitching motility-mediated biofilm expansion. It would also be interesting to quantitate the eATP gradient within *P. aeruginosa* interstitial biofilms formed via twitching motility. This could be achieved by using Nanospray desorption electrospray ionization mass spectrometry (nanoDESI) which allows detection and quantitation of extracellular ionisable molecules that exist within live bacterial colonies grown on agar plates (Watrous et al., 2012).

While a range of host-derived signals have been shown to have an inverse effect on *P. aeruginosa* sessile biofilm formation and active expansion, this work describes for the first time, that a host-derived signal provides a potential protective advantage for *P. aeruginosa* by stimulating sessile biofilm formation and inhibiting active biofilm expansion. Our work also suggests an important role for endogenously produced bacterial eATP not only in directing *P. aeruginosa* multicellular behaviours but also in *P. aeruginosa* pathogenesis by potentially inducing further inflammatory damage and fibrosis. It will be interesting to further characterise the adaptive response of *P. aeruginosa* to eATP and investigate the effect of endogenously produced bacterial eATP in coordinating multicellular behaviours and in pathogenesis. This will provide us with a clearer picture of how *P. aeruginosa* biofilms form and expand, as well as a better understanding of the interactions between *P. aeruginosa* and the host, which may allow for development of more targeted treatments against biofilm-associated infections.

7.4 ChpC controls the twitching motility-mediated expansion of *Pseudomonas aeruginosa* biofilms in response to mucin, albumin and oligopeptides

Chapter 6 of this Thesis describes the identification of a novel extracellular signal, GlcNAc, which stimulates twitching motility-mediated expansion of *P. aeruginosa* biofilms. The presented work also provides an in-depth characterisation of the twitching motility response of *P. aeruginosa* to the host-derived signals mucin, serum albumin and oligopeptides, in the form of tryptone, and goes on to implicate the CheW-homolog, ChpC, in the twitching motility response of *P. aeruginosa* to these signals. The response of *P. aeruginosa* to serum albumin, mucin and tryptone appears to be mediated by an increase in icAMP levels, however this is not controlled by ChpC. Our cell tracking analyses suggest that an increase in the number of directional changes made by PAK*chpC* could explain why the observed stimulation of overall biofilm expansion on serum albumin, mucin and tryptone is less than wildtype. Additionally, an increase in average speed and velocity of individual cells may account for some of the observed increase in overall biofilm expansion of PAK and PAK*chpC*. Importantly, while a number of environmental signals have been shown to affect twitching motility, this Thesis Chapter describes one of only a few examples where environmental signals have been linked to a molecular mechanism which alters twitching motility. This significantly

advances our understanding of the complex regulation of the twitching motility response of *P. aeruginosa* to extracellular cues.

As described in section 7.3, serum albumin is a host-derived signal that has been shown to have an inverse effect on *P. aeruginosa* sessile biofilm formation and active biofilm expansion, which is thought to provide a protective advantage for the host (Hammond et al., 2010, Huang et al., 2003). Both mucin and tryptone have been shown to stimulate twitching motility (Huang et al., 2003), and while the effect of tryptone on sessile biofilm formation has not been examined, mucin has been reported to have both a stimulatory (Landry et al., 2006) and inhibitory (Caldara et al., 2012) effect, depending upon whether mucin was absorbed onto a two-dimensional surface, or retained in a native three-dimensional state, respectively. Given the association of *P. aeruginosa* biofilms with chronic lung and urinary tract infections (Costerton et al., 1999), the biofilm response of *P. aeruginosa* to both mucin and tryptone (oligopeptides) is likely to be relevant within an infection setting. It would therefore be interesting to examine the effect of tryptone on sessile biofilm formation, and additionally to conclusively determine the effect of mucin on this process.

Our results also suggest that other components, in addition to ChpC for serum albumin, mucin and tryptone, and FimX for mucin and tryptone, may be involved in the twitching motility response to these host-derived signals. *P. aeruginosa* possesses 26 MCPs which provides a large repertoire of extracellular sensors, to allow *P. aeruginosa* to respond to alterations in environmental signals and conditions (Kato et al., 2008, Croft et al., 2000). It would be informative to identify the MCPs or other components which associate with FimX and ChpC as this could allow identification of additional MCPs and other proteins which may be signalling through the Chp system. As suggested in section 7.2, to identify interactions between FimL and other proteins, a *Pseudomonas* two-hydrid approach could also be used to identify interactions between specific MCPs, FimX and components of the Chp system. Pull down assays using tagged purified proteins could also be used to confirm specific protein interactions. This would significantly improve our understanding of how the components of environmental sensing signal transduction systems interact to mediate a twitching motility response.

Our cell tracking results suggest that an increase in the average speed and velocity of individual PAK cells, and an increase in the average speed of PAK*chpC* cells, may account for some of the observed increase in overall biofilm expansion on serum albumin and mucin. Additionally, an increase in the frequency of directional changes by PAK*chpC* may explain

why the stimulation of overall biofilm expansion is less than for PAK on serum albumin, mucin and tryptone. While our cell tracking analyses over 200 seconds have not explained how overall biofilm expansion is significantly increased for PAK and PAK*chpC* on tryptone, analyses of low magnification images, taken over 600 seconds, indicate that future analyses of larger numbers of cells, over longer time periods are likely to explain how individual cell movements translate into an increase in overall biofilm expansion on tryptone. These analyses over a longer time period are also likely to allow us to gain a better understanding of the possible fluctuations in twitching motility rates that have been suggested to occur in actively expanding biofilms (Semmler et al., 1999). Additionally, future analysis of velocity coherence of PAK and PAK*chpC* cells within the "raft heads", as well as analysis of the frequency of cellular reversals of these strains in the presence of serum albumin, mucin and tryptone are likely to provide more detail on the contribution of individual cell movements to the observed differences between overall biofilm expansion of PAK and PAK*chpC* in response to these host-derived signals.

Having the ability to track and quantify individual cell movements is a very powerful tool as it allows us to understand the mechanisms behind *P. aeruginosa* twitching motility-mediate biofilm expansion, which is a very complex multicellular behaviour. In this Thesis our computer vision analyses have allowed us to move on from simply demonstrating that the important host-derived signals, serum albumin, mucin and tryptone, stimulate twitching motility, and instead have developed our understanding of how responses by individual cells translate into overall expansion of the entire biofilm community. Further optimisation of our cell tracking algorithms will provide even more detail on this process, and continue to advance our understanding of the biofilm-specific responses of *P. aeruginosa* to environmental cues.

Serum albumin, mucin and tryptone stimulated overall biofilm expansion when present at a constant concentration within the media, suggesting that the twitching motility response of *P. aeruginosa* to these host-derived signals is chemokinetic. In flagella-mediated swimming motility chemokinesis is characterised by an extended period of increased swimming speed which results from an increase in the rate of flagella rotation (Packer and Armitage, 1994). Studies of flagella rotation as well as studies of tfp extension and retraction have used either laser tweezers or fluorescent stains with cells being tethered to a bead or attached to a glass coverslip (Skerker and Berg, 2001, Merz et al., 2000, Packer and Armitage, 1994). These assays do not easily translate into studying the rate of tfp extension and retraction in response to environmental signals within a semi-hydrated environment. It may however be possible to

adapt these assays to grow cells under semi-hydrated conditions in the presence of the extracellular signal, and then transfer these cells into a setup which allows direct examination of tfp extension and retraction. In the absence of this it would appear that the next best approach to determine whether these host-derived signals are likely to be having a chemokinetic effect would be to calculate the twitching motility speed of individual cells over a larger time frame during active biofilm expansion. If the presence of an extracellular signal causes cells to sustain an increased rate of twitching motility this would be indicative of chemokinesis, and an increase in the rate of tfp extension and retraction.

The results presented in Chapter 6 also demonstrate that icAMP levels are increased in cells grown in the presence of serum albumin, mucin and tryptone, but that this effect is not mediated by ChpC. It has previously been reported that addition of exogenous cAMP to a number of Chp system mutants with tfp surface assembly defects is able to restore surface tfp levels back to wildtype (Fulcher et al., 2010). On the contrary, exogenous addition of cAMP was not able to restore the twitching motility phenotypes of these mutants. The authors concluded that the Chp system regulates tfp assembly in a cAMP-dependent manner, and twitching motility in a cAMP-independent manner (Fulcher et al., 2010). Our results demonstrate that ChpC is not involved in the increase in icAMP levels in response to serum albumin, mucin and tryptone. We cannot however rule out the possibility that other components of the Chp system are involved in increasing icAMP levels in response to these signals. Given the complexities of the regulation of icAMP levels and twitching motility as discussed in section 7.2, it is likely that the increase in icAMP levels as a consequence of sensing of these environmental signals occurs through a number of pathways.

In this Chapter we have been able to link the twitching motility response of *P. aeruginosa* to a number of host-derived signals to a specific protein within the Chp chemosensory system. This is significant, as very few linkages have been made to date between environmental cues and the components which sense and respond to these signals to modulate twitching motility. Additionally, our automated computer vision analyses have allowed us to gain insight into how individual cell movements translate into overall biofilm expansion of *P. aeruginosa* in response to a number of host-derived signals.

7.5 Overall conclusions

The work presented in this Thesis has provided insight into the regulation of icAMP levels and twitching motility in *P. aeruginosa* and has characterised the biofilm response of *P. aeruginosa* to a novel host-derived signal, as well as a number of previously identified host-derived signals. Importantly, we have implicated one component of the Chp chemosensory system in the twitching motility response of *P. aeruginosa* to a number of these signals. This is significant because, to date, there have been very few linkages made between environmental cues and the regulatory systems which respond to these signals to modulate *P. aeruginosa* twitching motility.

Prior to this work a number of host-derived signals had been suggested to provide a protective advantage to the host by inhibiting *P. aeruginosa* sessile biofilm formation and stimulating twitching motility-mediated biofilm expansion. The results presented in this Thesis demonstrate, for the first time, that the biofilm response of *P. aeruginosa* to a host-derived signal, eATP, is likely to provide a potential protective advantage for the bacterium. Additionally, our results suggest that high levels of endogenously produced bacterial eATP act to coordinate *P. aeruginosa* biofilm formation and expansion as well as potentially stimulating further local tissue inflammation and fibrosis to promote further *P. aeruginosa* infection. These results suggest that endogenously produced bacterial eATP is an important extracellular molecule which acts to coordinate *P. aeruginosa* complex multicellular behaviours via intercellular signalling as well as potentially contributing to *P. aeruginosa* pathogenesis. This is likely to have significant implications in the development of chronic *P. aeruginosa* biofilm associated infections.

Overall the work presented in this Thesis adds to our understanding of the complex regulation of twitching motility in response to intracellular and extracellular signals. We suggest that the biofilm and twitching motility responses of *P. aeruginosa* to a range of host-derived extracellular signals are likely to contribute significantly to the success of this opportunistic pathogen within an infection setting. While further work is required to fully understand these adaptive responses, the work presented in this Thesis provides a solid basis for further exploration, which may contribute to the development of targeted treatments for biofilm-associated infections caused by *P. aeruginosa* and other pathogens.

Chapter 8

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Appendix 1
Movie Files

Movie 4-1. Exogenous ATP affects twitching motility of *P. aeruginosa* ("Movie 4-1.mov" and "Movie 4-1.avi" in attached DVD). Figure legend on page 82.

Movie 4-2. Extracellular ATP gradients affect twitching motility of *P. aeruginosa* ("Movie 4-2.mov" and "Movie 4-2.avi" in attached DVD). Figure legend on page 86.

Movie 6-1. The effect of serum albumin, mucin and tryptone on *P. aeruginosa* PAK twitching motility ("Movie 6-1.mov" and "Movie 6-1.avi" in attached DVD). Figure legend on page 127.

Movie 6-2. The effect of serum albumin, mucin and tryptone on *P. aeruginosa* PAK*chpC* twitching motility ("Movie 6-2.mov" and "Movie 6-2.avi" in attached DVD). Figure legend on page 127.

[Production Note: Digital copies included in item record.]