Pseudomonas aeruginosa – Host Interactions And The Identification Of Novel Modulators Of Quorum Sensing-Mediated Virulence.

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Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for another degree at the University of Nottingham or any other institute of learning.

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Abstract.

Pseudomonas aeruginosa is a clinically important opportunist pathogen that is responsible for high levels of morbidity and mortality in the nosocomial environment. Due to the intrinsic antibiotic resistance of *P. aeruginosa*, coupled with a genomic flexibility that allows the acquisition of additional resistance mechanisms, treatment options against infections caused by this pathogen are dwindling. Therefore, there is now a drive to develop novel anti-pseudomonal therapeutics. In order to do this, it is important to gain increased understanding of *P. aeruginosa* at the molecular level. To this end, three different aspects of *P. aeruginosa* virulence were studied in this thesis.

Previously, interactions between respiratory pathogens such as *N. meningitidis* and both the host platelet-activating factor (PAFr) and the 37/67 kDa laminin receptor have been demonstrated. *P. aeruginosa* also interacts with the host PAFr, therefore this study investigated whether it also binds the 37/67 kDa laminin receptor via the candidate surface proteins PilQ, the type IV pilus secretin, and OprI, a lipoprotein. Through ELISA and whole-cell pulldown experiments, this work has shown that although *P. aeruginosa* interacts with the 37 kDa laminin receptor precursor, the interaction is mediated only in part by PilQ and OprI and may occur at levels too low to be of physiological significance.

The galactophilic lectin LecA is important in *P. aeruginosa* adhesion to host cells as well as cytotoxicity and biofilm maturation. The expression of the *lecA* gene is subject to complex growth phase-dependent regulation involving RhlR, RpoS and MvaT, and PqsE is also required. However, few direct regulators of the *lecA* promoter have been described. In promoter pulldown experiments, 22 candidate proteins including MifR, FleQ, MvaT, AlgP, RhlR and GbuR were identified bound to the *lecA* promoter region. For 18 of these candidate regulatory proteins, mutation resulted in altered LecA production. The effect of most of these mutations could be partially masked by homeostatic levels of PqsE, confirming its importance in the expression of *lecA*; however, mutation of AlgP and GbuR abolished the

expression of LecA even in the presence of homeostatic levels of PqsE. LecA expression could be rescued in PAO1-W *algP* and *gbuR* mutants by the overexpression of the *pqsE* gene under the control of an IPTG-inducible promoter. Furthermore, FleQ, the major flagellar regulator, bound the *lecA* promoter region and also negatively regulated the production of LecA. Considering that *lecA* expression is indirectly regulated by AlgU and AmpR, the work presented here is consistent with a hypothetical regulatory pathway in which AmpR, SadB, AlgU and AmrZ converge upon FleQ to regulate *lecA* transcription.

PqsE also negatively regulates the activity of the *pqsA* promoter via an unknown mechanism. Promoter pulldown experiments revealed a strikingly complex protein profile at the *pqsA* promoter during peak *pqsA* expression in both the presence and absence of PqsE. A group of 16 candidate regulators of the *pqsA* promoter were chosen for further investigation, such as SadB, ParR, FleQ and FleR. Intriguingly, many proteins that bound the *lecA* promoter also bound the *pqsA* promoter, including AlgP. This suggests that AlgP regulates the activity of the *pqsA* promoter, and therefore *pqsE* expression, and would explain why abrogation of LecA production caused by *algP* mutation can be overcome by the overexpression of *pqsE*. The construction of a *pqsA-lux* bioreporter would enable the impact of the candidate proteins on the activity of the *pqsA* promoter to be determined. The work carried out thus underlines the complexity of the *P. aeruginosa* regulatory networks and contributes through the identification of a number of additional regulatory proteins.

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List of Abbreviations.

3-oxo-C12-HSL	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AHL	*N*-acylhomoserine Lactone
AP	Alkaline Phosphatase
AQ	2-alkyl-4-quinolone
BBB	Blood-Brain Barrier
Bp	Base pair
BSA	Bovine Serum Albumin
C4-HSL	*N*-(butanoyl)-L-homoserine lactone
cAMP	Cyclic Adenosine Monophospate
c-di-GMP	Cyclic Diguanylate
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
ChoP	Phosphorylcholine
cm	Centimetre
CNF	Cytotoxic Necrotising Factor
CRP	cAMP Receptor Protein
Da	Dalton
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced Chemiluminescence
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
GOI	Gene of Interest
h	Hour
HHQ	2-heptyl-4-quinolone
HIF-1	Hypoxia-induced Factor-1
H-NS	Histone-like Nucleoid-Structuring

HRP	Horseradish Peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
LCMS/MS	Liquid Chromatography Mass Spectrometry; Tandem Mass Spectrometry
LPS	Lipopolysaccharide
LTTR	LysR-type transcriptional regulator
μ g	Microgram
mg	Milligram
min	Minute
µl	Microlitre
ml	Millilitre
mRNA	Messenger Ribonucleic Acid
NBT/BCIP	Nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
NCTC	National Collection of Type Cultures
o/n	Overnight
OD	Optical Density
PAF	Platelet-activating Factor
PAFr	Platelet-activating Factor Receptor
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline-Tween-20
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
ppGpp	Guanosine Pentaphosphate
ppm	Parts per Million
PQS	Pseudomonas Quinolone Signal
QS	Quorum Sensing

RBS	Ribosome Binding Site
rcf	Relative Centrifugal Force
RE	Restriction Enzyme
RNA	Ribonucleic Acid
RLU	Relative Light Units
rpm	Revolutions per Minute
RT	Room Temperature
SDS	Sodium-dodecyl Sulphate
SDS-PAGE	Sodium-dodecyl Sulphate Polyacrylamide Gel Electophoresis
Sulfo-SBED	Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate
TISS	Type One Secretion System
TIISS	Type Two Secretion System
TIIISS	Type Three Secretion System
TVSS	Type Five Secretion System
TAE	Tris-Acetic Acid-EDTA Buffer
TLR	Toll-like Receptor
Tn5/7	Transposons
u	Units
UF/uf	Upstream Forward
UR/ur	Upstream Reverse
DF/df	Downstream Forward
DR/dr	Downstream Reverse
w/v	Weight per Volume
v/v	Volume per Volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter One.
General Introduction.

1.1. *Pseudomonas aeruginosa* and Nosocomial Infections.

Pseudomonas aeruginosa is a Gram-negative microorganism notorious for its role in nosocomial infections. Although the bacterium is ubiquitous in the environment owing to its ability to adapt to a range of habitats it rarely infects healthy individuals, instead opportunistically causing infections in those with compromised immunity as a symptom of acquired or genetic conditions such as AIDS, Cystic Fibrosis, and immunodeficiency caused by chemotherapy. Breach of barrier immunity through burns, surgical or trauma wounds and instrumentation (for example ventilation, catheterisation and cannularisation) are also risk factors for nosocomial *P. aeruginosa* infection[1,2]. The high frequency with which *P. aeruginosa* is isolated as a cause of healthcare-associated as opposed to community-associated infections demonstrates the extent to which the bacterium relies on the immunodeficiency of its host[3–6]; nonetheless, a worldwide infection surveillance study carried out between 1997 and 1999 found that, of 70,064 community and nosocomial isolates sampled, approximately 9.4% of these were *P. aeruginosa* strains. In Europe alone, *P. aeruginosa* was identified in 5.6% of blood infections, 22.2% of respiratory infections, 14% of wound infections and 7% of urine infections, highlighting the prevalence of this pathogen[7].

The sizable susceptible host population found in the clinical environment allows *P. aeruginosa* to easily spread and become established, assisted by the high selection pressure exerted by the, until relatively recently, inappropriately excessive use of broad-spectrum antibiotics[8,9]. This allowed the selection of a number of multi-drug resistant clinical strains, not only *P. aeruginosa* but also other opportunists such as *Klebsiella pneumoniae, Staphylococcus aureus* and *Enterobacter cloacae*, amongst others[10].

P. aeruginosa possesses a degree of intrinsic antibiotic resistance due to a combination of the low permeability of its Gram-negative envelope, constitutive expression of multidrug efflux pump genes, and a chromosomal β-lactamase, AmpC. Further resistance can develop in response to environmental stresses exerted by antibiotics via the selection of random mutations that confer resistance, or via the horizontal transfer of β-lactamase genes from other microorganisms. Mutational resistance can occur in several ways, including via the mutation of drug targets, such as DNA topoisomerase, or the derepression of the *ampC* gene via mutations in key regulators. In addition, the production of multidrug efflux pumps can be upregulated, allowing the removal of antibiotics from the cell, whilst downregulation of porin genes leads to the loss of active surface porins, and therefore reduces diffusion of antibiotics into the cell[11]. The OprD porin, for example, is co-regulated with the MexEF-OprN efflux system; mutations in the transcriptional regulator MexT cause downregulation of OprD and upregulation of the MexEF-OprN efflux system, conferring increased resistance to carbapenems (such as imipenem) and chloramphenicol respectively[12]. With the rise of such multi-drug resistant nosocomial pathogens, there are an ever-decreasing number of effective therapeutic options; there is therefore an acute need for new antimicrobials for treatment of *P. aeruginosa* infections.

1.2. *P. aeruginosa* Virulence Factors.

That *P. aeruginosa* is such a successful opportunistic pathogen is due to the fact that it possesses extensive arsenal of virulence factors that have a range of targets in the host. The first step in *P. aeruginosa* pathogenesis is attachment and colonisation of a surface. *P. aeruginosa* possesses a single polar flagellum, which, as well as mediating swimming motility, also enables attachment to host surfaces; the flagellar cap protein FliD, for example, is able to bind respiratory mucins[13]. Similarly, type IV pili, besides being involved in twitching motility, also act as adhesins on epithelial surfaces, where they are believed to bind asialo GM1 residues

that are maximally produced by epithelial cell surfaces undergoing repair[14]. The interactions of pili and flagella with their ligands are particularly of interest given the association of *P. aeruginosa* with the cystic fibrosis lung, since in this environment, mucin production is increased and the lung tissue undergoes constant remodelling due to inflammation[15,16].

Following attachment, *P. aeruginosa* induces the expression of many genes encoding virulence factors that are characteristic of the acute infection stage and promote the establishment of stable colonisation[17]. Some examples of these are shown in Table 1.1.

Table 1.1. Examples of *P. aeruginosa* Virulence Factors. Table continued on the next page.

Virulence Factor	Function	Reference
Motility/Attachment		
Flagella	Swimming & swarming motility; attachment; highly immunogenic	18
Type IV Pili	Twitching motility; attachment	19,20
Glycocalyx	Exopolysaccharide; adhesion, protection against host defences	22
Alginate	Exopolysaccharide; involvement in attachment. Component of biofilms, protection against host immune defences	23–25
Enzymes/Toxins		
LPS	Lipopolysaccharide; highly immunogenic, role as adhesin	21
LecA LecB	Adhesion; involvement in biofilm formation and maturation; cytotoxic effects	26,27
LasA LasB	Enzymes; degrade host tissues individually & synergistically	28–30
ExoS ExoT	Cytotoxins with GTPase-activating & ADP ribosylating activities; cause cell death, interfere with phagocytosis	31,32
ExoU	Cytotoxin with phospholipase activity	
ExoY	Cytotoxin with adenylate cyclase activity; disrupts cellular activities	

Virulence Factor	Function	Reference
Protease IV	Enzyme; degrades host factors such as fibrinogen, plasminogen & immune mediators	33
AprA	Alkaline phosphatase; degrades host factors; role in phosphate acquisition	34
Phospholipase C	Enzyme; induces vascular permeability, causes tissue damage	35
Pyocyanin	Cytotoxic effects include cell damage via inhibition of cellular processes. Involved in iron acquisition	36,37
Siderophores		
Pyoverdin	Involved in iron acquisition	38,39
Pyochelin		
Secretion Systems		
Xcp	Type II secretion system; secretes virulence factors including LasA, LasB, PhoA, ToxA	32
Psc	Type III secretion system; secretes ExoS, ExoT, ExoU and ExoY toxins	
LepB	Type V secretion system; secretes LepA protease	
Antibiotic Resistance		
MexAB-OprM	Efflux pump; resistance to β-lactamases, tetracycline, norfloxacin; role in regulation of QS	40,41
AmpC	Chromosomal β-lactamase	42
Signal Molecules		
Acylhomoserine lactones Alkylquinolones	Cell-cell communication; immune modulation	43

The virulence factors produced by *P. aeruginosa* include numerous exoenzymes such as LasA, LasB and AprA, which are able to cleave host proteins to cause damage to tissue structure, scavenge nutrients and abrogate host immune defences[44]. AprA is able to disrupt immune responses by cleaving excess monomeric flagellin, which is highly immunogenic and would usually stimulate the Toll-like receptor (TLR)-5 dependent immune response[45]; at the same time, by cleaving host

transferrins, which bind iron in the blood, AprA is able to assist in the scavenging of iron from the host via the siderophore pyoverdin[46]. *P. aeruginosa* also produces phospholipases, which can induce vascular permeability[35]. Any kind of tissue damage occurring in this manner can ultimately enable systemic bacterial spread from the local site of infection, and additional damage can be caused by cytotoxic effectors. Exotoxin A causes the death of target host cells[47], whilst the production of many secretion systems allows the delivery of additional effectors that are highly cytotoxic to host cells. In particular, the Xcp type III secretion system delivers the cytotoxins ExoS, ExoT and ExoU directly to a target host cell via a needle assembly. These toxins then disrupt cellular functions by modifying host proteins[32].

P. aeruginosa characteristically causes chronic infections that are associated with the transition from planktonic mode of growth to sessile growth in biofilms, accompanied by a corresponding change in global gene expression. This is representative of adaptation to the environment, and involves the production of fewer secretion systems and cytotoxic mediators, and more proteins involved in biofilm formation and antibiotic resistance[48]. Within biofilms, a bacterial population exists surface-bound and encased in a matrix of exopolysaccharide, extracellular DNA (eDNA) and proteins[49], thus protecting the population from environmental stresses such as the cellular immune response, for example. The composition of biofilms with respect to the proportion of each component within the matrix is dynamic, dependent upon the *P. aeruginosa* strain, age of the biofilm, and the environment in which the biofilm exists[50].

The proteinaceous content of biofilms consists of surface appendages such as type IV pili and flagella, as well as adhesins such as CdrA. These are involved in the initial attachment stages of biofilm formation, mediating both interactions between cells and between cells and surfaces. Pili and flagella are also highly immunogenic[51].

eDNA also plays an important role in biofilm formation, allowing cell-to-cell adhesion, providing a nutrient source during starvation, and increasing resistance to aminoglycosides[52–54]. eDNA is released by

bacterial cells undergoing autolysis, which can occur in response to stressful conditions. However, in biofilms, autolysis is likely to be under the control of additional regulatory mechanisms that coordinate the programmed cell death of distinct bacterial populations[55–57]. DNA from host cells undergoing apoptosis can also enhance biofilm formation, particularly since interactions with biofilm components can lead to the apoptosis of neutrophils[58,59].

P. aeruginosa can synthesise three types of exopolysaccharide, Pel, Psl and alginate; however, alginate production is generally more predominant in biofilms produced by mucoid mutant strains that emerge later on in infection, particularly within the cystic fibrosis lung, and contributes to the formation of much more adhesive, thicker, biofilms with a more defined structure[50,60]. The switch to mucoidy from the Pel/Psl-producing phenotype most commonly occurs via mutations in the *mucA* gene, which encodes a negative regulator of the regulatory sigma factor AlgU. This leads to the overexpression of *algU*, and in turn, the overexpression of the alginate biosynthetic genes[61].

On the other hand, biofilms of non-mucoid strains are formed predominantly with Pel and Psl polysaccharides, as opposed to alginate. Although functionally redundant, the relative level of production of Pel compared with Psl is dependent upon the strain[50]. Alginate, Psl and Pel are involved in bacterial adhesion, and maintenance of the biofilm architecture; however, these exopolysaccharides also protect the bacterial population from antibiotics and the immune response[51]. *P. aeruginosa* growing within a biofilm is markedly more resistant to clearance compared with planktonic cells, mainly due to the 'buffer effect' conferred by the outer layers of a multi-layered biofilm; bacteria and matrix components in these layers in effect 'soak up' antibacterial effectors thus protecting bacteria inside the biofilm[62]. For this reason, along with the utilisation of multiple antibiotic resistance mechanisms, *P. aeruginosa* biofilms are notoriously difficult to eradicate.

LecA and LecB are also important virulence factors involved in biofilm formation. Besides acting as cytotoxins and cellular adhesins

targeting host cells, LecA and LecB are lectins that are able to specifically bind galactose and fucose respectively[26]; these lectins are therefore able to mediate intercellular interactions. LecA and LecB may also bind galactose and mannose residues within Psl polysaccharide, contributing to the stability of the biofilm structure[63,64]. LecA has been demonstrated to be important in biofilm maturation, whereas LecB is more important during biofilm formation[27]. The regulation of LecA and its role in *P. aeruginosa* biofilms will be discussed further in Chapter 4.

1.3. Interactions Between *P. aeruginosa* and the Host.

To be successful pathogens, bacteria must adapt to environments within the host and evade immune defences. *P. aeruginosa* is renowned for successfully adapting to almost any niche, not least due to the genetic flexibility of its large arsenal of virulence factors. This is particularly highlighted by the different mechanisms of pathogenesis employed at different sites of infection[22]. Most commonly, *P. aeruginosa* colonises the lungs, burn wounds, the cornea and the urinary tract, diverse environments in which the responses to initial infection vary. Some of the host-pathogen interactions that occur during *P. aeruginosa* infection are summarised in Figure 1.1.

The cornea, for example, is an immunologically privileged site[65]. In order to protect the delicate tissues, immune responses here are tightly controlled, through pathways that encourage tolerance to novel antigens and immunosuppression of T cells, since excessive or inappropriate inflammation could have a severe impact on eyesight. In the eye, the first line of defence against invading pathogens consists of secreted IgA antibodies and lysozyme. Pathogen-associated molecular patterns (PAMPs) are then recognised by TLRs, which are expressed on the surface of those cells most likely to encounter invading microorganisms or viruses, such as corneal epithelial cells.

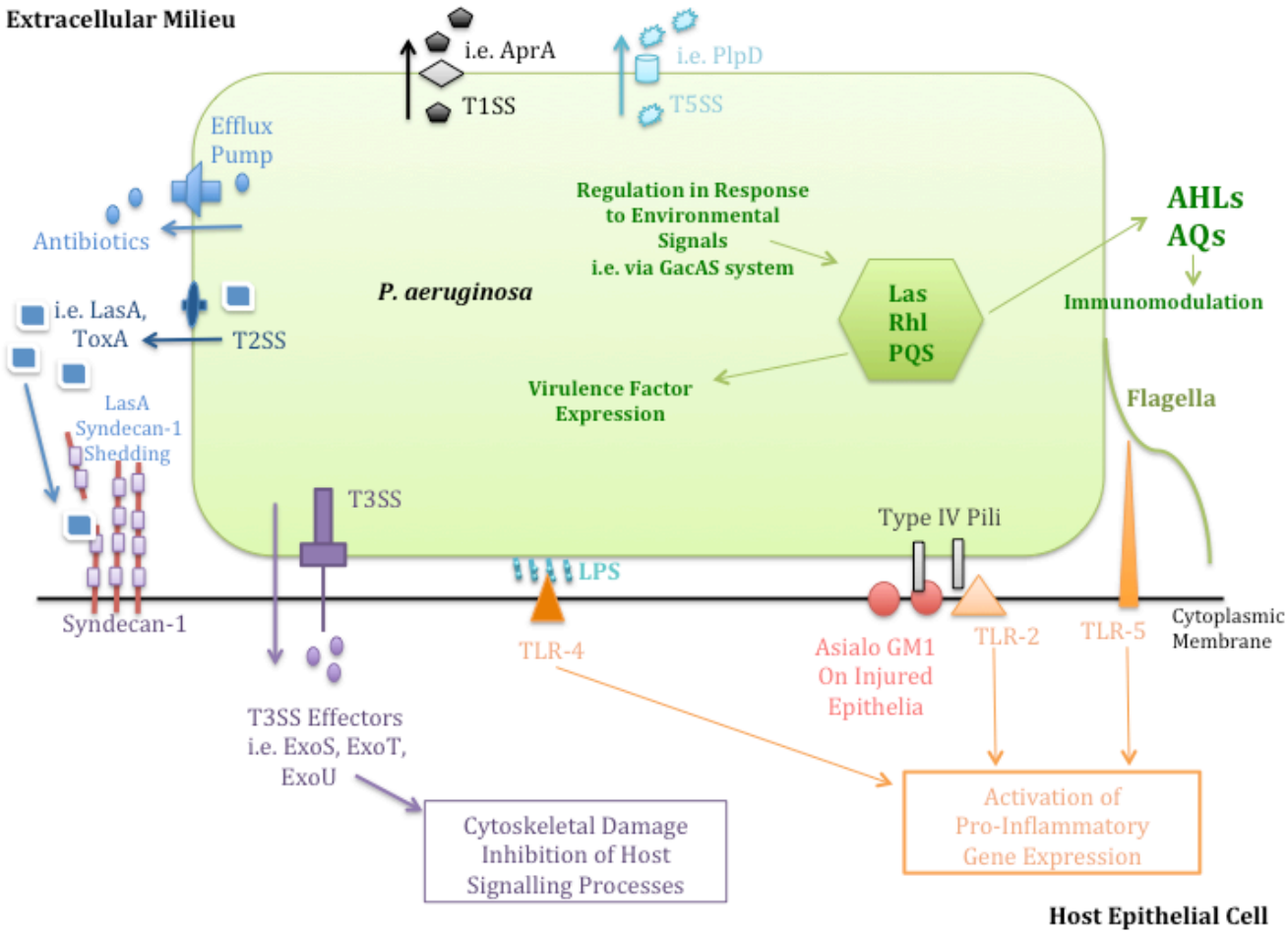


Figure 1.1. A General Overview of *Pseudomonas*-Host Interactions. *P. aeruginosa* is able to bind host epithelial cells via asialo GM1 and CFTR (not shown). Type IV pili–mediated binding of asialo GM1 results in activation of TLR-2 and subsequent activation of the immune response. Flagella and LPS similarly stimulate TLR signalling. In acute infections, *P. aeruginosa* expresses type III secretion systems (T3SS), which directly deliver cytotoxic effectors to host cells. Further secretion systems secrete effectors into the extracellular milieu where they are able to degrade extracellular membrane components; LasA cleaves elastin but also causes syndecan-1 shedding from host cells, allowing immune evasion. Expression of virulence factors is highly controlled according to environmental signals – not all virulence determinants are expressed simultaneously. Adapted from[44].

On contact with PAMPs, TLRs initiate signalling cascades that lead to the induction of cytokine and chemokine release, promoting the influx of B- and T-lymphocytes to the local area in a specific and controlled manner. A careful balance between protecting the integrity of the tissues whilst effectively clearing the infection is thus maintained[66].

However, *P. aeruginosa* infections occur preferentially following damage to corneal tissue[67], initiating a cycle in which the inflammatory response against the tissue damage is exacerbated by the presence of infection, which itself causes more damage. Clearance of *P. aeruginosa* infections in corneal keratitis is dependent on a rapid, early response by T lymphocytes and influx of macrophages which ingest the bacteria; however, in contact lens wearers in particular, the immune response can be sustained over a longer period of time, characterised by the release of inflammatory cytokines[68].

In burns patients, on the other hand, the outer barrier against pathogens is disrupted, and the underlying tissues exposed. Investigation of the immune response in burn injuries has revealed that within burns there is a reduced distribution of antibodies and complement cascade components, which promote opsonisation and clearance of pathogens; neutrophil chemotaxis to the area is also reduced, and *P. aeruginosa* is able to take advantage of the compromised local immune responses to establish infection[69–71]. Exoenzymes and toxins secreted via type III secretion systems then facilitate destruction of tissues and systemic spread of infection[22].

P. aeruginosa lung infections are also seen in individuals with either systemic immune dysfunction, such as AIDS patients, or those who have been ventilated, where a direct route to the lung tissue is provided for opportunistic pathogens such as *P. aeruginosa*. In the immunocompetent host these infections are generally acute, and are characterised by the production of a large number of immunogenic and cytotoxic virulence factors by *P. aeruginosa*, again including type III secretion systems and their effectors, which can compromise the integrity of the epithelial barrier[72]. The pathogen has been shown to adhere to the

cystic fibrosis transmembrane regulator (CFTR) on epithelial cell surfaces via lipopolysaccharide (LPS). However, this attachment initiates the host response; *P. aeruginosa* are internalised into the epithelial cell and an intracellular signalling cascade mediates the apoptosis of the infected cell, particularly when the epithelial cell layer has already been disrupted[73–75]. The CFTR protein can also promote the internalisation of *P. aeruginosa* into epithelial cells at sites of infection other than the lung[76,77].

At the same time, *P. aeruginosa* pili are able to bind epithelial cells via asialo GM1, which is complexed with TLR-2, whilst flagellin is recognised by both TLR-2 and TLR-5; LPS and Exoenzyme S can also be recognized by TLRs[44]. In response to TLR signalling and tissue damage, downstream signal transduction events trigger the release of cytokines and chemokines, and influx of cellular mediators of immunity such as neutrophils, T cells and macrophages. An increase in airway mucus secretion traps bacteria and apoptotic host cells on the epithelial cell surface, allowing their removal via the action of the mucociliary escalator[72].

In any acute *P. aeruginosa* infection accompanied by an effective immune response, the infection should theoretically remain localised and be cleared by the immune system, often in combination with antibiotic therapy. However, highly cytotoxic bacterial phenotypes associated with the production of exotoxins and exoenzymes can facilitate invasion and sepsis, and also provoke highly inflammatory immune responses that contribute to tissue pathology; *P. aeruginosa* is also able to induce local immunosuppression of phagocytosis. In combination, these features of pathogenesis lead to increased morbidity and mortality[78–80].

In the cystic fibrosis lung, however, the conditions are such that a chronic *P. aeruginosa* infection is established, despite the presence of an apparently functional host immune system and the reduced virulence of the *P. aeruginosa* strain[80]. Studies have established that the reason for this is most likely multifactorial[72]. In cystic fibrosis, a defective CFTR protein causes abnormal osmolarity, which leads to the production of thick mucus in which bacteria and debris get trapped. Antimicrobial peptides are

inactivated by the conditions, and the defective CFTR protein is not able to mediate the internalisation of bound bacteria. Simultaneously, both bacterial exoproducts and the immune response cause inflammation and tissue damage, beginning a cycle of tissue damage and remodelling that potentiates further inflammation, mucin secretion and bacterial colonisation[81,82]. *P. aeruginosa* trapped on the epithelial surface is then able to form its characteristic biofilms (which may also be suspended in mucus in the form of microcolonies rather than surface bound[83]) and begin a persistent infection in which it adapts to the extracellular milieu; variants cultured from cystic fibrosis sufferers are found to be reduced in virulence due to mutations accumulated over time that favour the development of a less immunogenic and less cytotoxic phenotype, further reducing the ability of the immune response to recognise the infection[84]. Whilst the immune response in the cystic fibrosis lung appears to be intact, it is in fact characterised by the excessive infiltration of neutrophils, even in the absence of invading pathogens. These neutrophils produce large amounts of inflammatory mediators and induce further immune responses, all of which cause the inflammation and tissue damage required for *P. aeruginosa* colonisation. Recruited macrophages are unable to successfully kill pathogens following phagocytosis or present antigens to T-lymphocytes; the T-lymphocyte response itself has been shown to be inappropriate in cystic fibrosis models, causing defects in adaptive immunity. Studies have postulated that the defects in the immune responses of cystic fibrosis sufferers are as a result of disruptions in key signalling processes due to the CFTR mutation[85].

Indeed, regardless of infection site, *P. aeruginosa* must be able to subvert host processes in order to evade the immune response. As well as through passive mechanisms, such as downregulating flagella and altering the LPS structure to avoid recognition[21,86,87], *Pseudomonas* is also actively able to modify the response to infection. Syndecans are heparan sulphate proteoglycans expressed on the surface of epithelial cell surfaces and have a role in maintaining the homeostasis of host tissues. When shed from the cell surface, syndecans are able to bind components of the extracellular

matrix, antimicrobial peptides, chemokines and cytokines in order to modulate the inflammatory response. However, studies have shown that *P. aeruginosa* LasA is able to upregulate the shedding of syndecan-1 in order to promote its own survival[88–90]. Similarly, *P. aeruginosa* LasB and protease IV are able to cleave surfactant proteins, which usually bind bacteria and cause agglutination, enhancing phagocytic killing and clearance[91,92]; LasB is also able to inhibit the chemotaxis of monocytes to the site of infection[93], whilst protease IV has additional activity against complement components and IgG antibodies[33]. Pyocyanin, the *P. aeruginosa* phenazine, is able to abrogate macrophage activity and cause apoptosis, which then leads to the release of intracellular inflammatory mediators[398]. *P. aeruginosa* is also able to invade natural killer T cells and induce their apoptosis[95]; in this instance, *P. aeruginosa* takes advantage of the host's protective apoptotic pathways in order to evade immune clearance. Interestingly, a *P. aeruginosa* PA14 strain has also been found to repress the expression of a gene encoding a host antimicrobial peptide in a *Drosophilia* model of infection[96], whilst Galle *et al.*[97] showed that, although the process of type III secretion results in caspase activation and subsequent activation of the IL-1β cytokine, the type III-secreted exoenzyme S is subsequently able to repress this activation.

P. aeruginosa infections are associated with diverse chemokine and cytokine responses, and many studies have been carried out to elucidate their role in the clearance of infection; however, these are complex and highly dependent on the type and site of infection[44]. Studies have confirmed that *P. aeruginosa* virulence factor production can vary amongst isolates from different infection sites. In *in vitro* experiments, Woods *et al.*[98] observed that the highest levels of protease were produced by isolates from the blood compared with isolates from the urinary tract, pneumonia and wounds, suggesting the importance of proteases such as elastase in the degradation of tissues during the spread of infection; however, when isolates from chronic lung infections were compared with acute pneumonia isolates, chronic infection isolates produced lower levels

of all virulence factors investigated. The highest level of elastase was produced by an acute pneumonia isolate, whilst the highest level of phospholipase C was produced by a wound isolate. On the other hand, Hamood *et al*.[99] found that although the levels of some virulence factors varied between isolates from different infection sites, similar levels of elastase and phospholipase C were produced by isolates from the urinary tract, respiratory tract and wounds, while the highest levels of exotoxin A were produced by wound isolates. Mittal *et al.*[100] also revealed that urinary tract isolates produce varying levels of the different virulence factors, despite the fact that they were all isolated in the same way; however, those strains that produced high levels of haemolysin were associated with a higher risk of spread of infection to the renal tissue, implying an important role for haemolysins in tissue damage and subsequent systemic infection. Taken together, these results suggest that *P. aeruginosa* adaptation to the extracellular milieu via the production of different types of virulence factors may be important, and highlight the role of the individual virulence factors *in vivo*. Ultimately, the outcome of infection, whether acute or chronic, is determined by the balance between both *P. aeruginosa* virulence factors and host immunity mediators as summarised in Figure 1.2. This is illustrated by the high prevalence of *P. aeruginosa* infections amongst individuals with underlying defects in both barrier immunity and innate and adaptive immune responses.

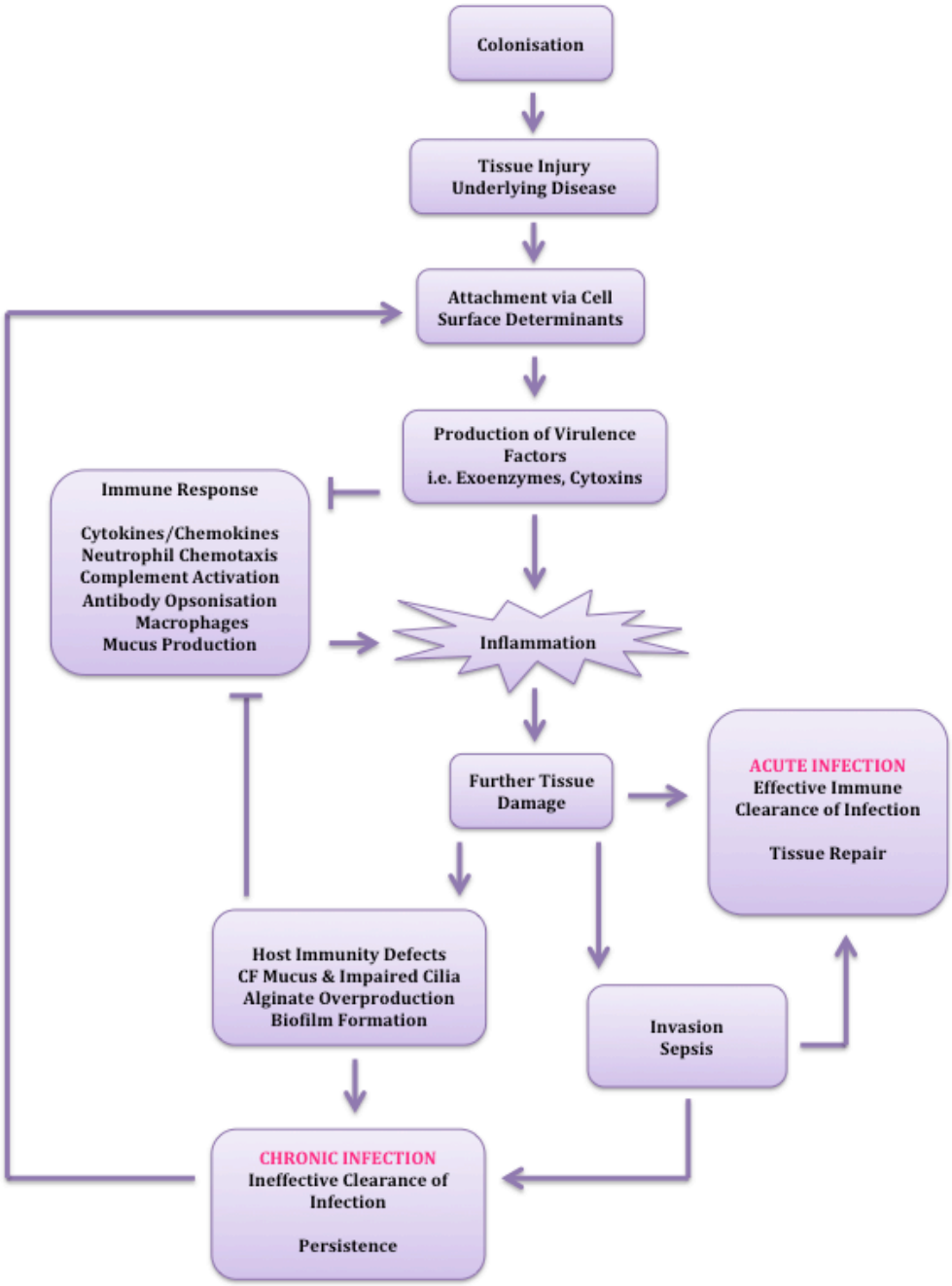


Figure 1.2. Pathogenesis of *P. aeruginosa* Infections. Following colonisation of host epithelial surfaces, *P. aeruginosa* requires the presence of injured tissue (e.g. surgical wounds) or underlying disease (i.e. cystic fibrosis (CF)) in order to attach to host cells and establish infection. In immunocompetent hosts, the innate immune response is triggered; mediators vary dependent upon the site of infection. *P. aeruginosa* virulence factors are able to dampen the immune response, for example via cleavage of antibodies or inhibition of neutrophil activity. The combination of host immune response and bacterial exoproducts leads to inflammation and tissue damage. Local infections can be cleared by the immune system in immunocompetent hosts, but tissue damage may lead to invasion and sepsis, requiring antibiotic treatment. Ineffective clearance of infection occurs in immunocompromised hosts, such as AIDS patients, or in CF sufferers. In the absence of effective immune responses, chronic infection can be established. Diagram adapted from [395].

1.4. The Regulation of *P. aeruginosa* Virulence.

The very large number of virulence factors produced by *P. aeruginosa* is clearly a key factor in its ability to cause infections so efficiently, but the genetic flexibility of the bacterium is also vastly important. With a genome 6.3 million base pairs in size, consisting of 5,570 predicted open reading frames, it is vital that the genome is tightly regulated to ensure that expression of specific genes occurs only at appropriate times; indeed, many *P. aeruginosa* genes are expressed in a growth phase-dependent manner. That approximately 8.4% of the *P. aeruginosa* genome consists of genes with predicted regulatory functions highlights the importance of transcriptional and post-transcriptional regulation in this microorganism[101]. Furthermore, the *P. aeruginosa* genome encodes numerous two-component systems (approximately 64 sensor kinases and 72 response regulators), which allow the environmental sensing and subsequent response necessary for adaptation to the extracellular milieu[102]. Classical two-component systems generally consist of a cytoplasmic membrane-associated sensor kinase protein and a response regulator, which transmits the effect of the stimulus to the target genes[103]. The PhoP-PhoQ system, for instance, consists of the PhoQ sensor kinase and the PhoP response regulator and is primarily involved in regulating the response to low magnesium conditions; however, it has also been shown to regulate the expression of *P. aeruginosa* genes encoding virulence determinants such as those required for twitching motility and biofilm formation[104].

One of the best characterised two component systems is the GacA-GacS system. In this system, GacA is the response regulator which, upon activation by its cognate sensor kinase GacS, induces the transcription of two small regulatory RNAs, RsmY and RsmZ[105]. These are able to bind and sequester RsmA, an RNA-binding protein that post-transcriptionally regulates target mRNA by binding in the region of the ribosome binding site to prevent translation. When RsmY and RsmZ titrate RsmA, RsmA target mRNA is derepressed and translation occurs[106,107]. The GacA-GacS

two component system is closely associated with *P. aeruginosa* virulence, having been found necessary for pathogenicity in mice models[108]. It is required for the expression of a range of virulence genes, including those for the production of pyocyanin and hydrogen cyanide, and also regulates the *rhlR* and *lasR* genes themselves, making it an important determinant of cell density-dependent gene regulation[109]. GacA mutants have also been found to be reduced in biofilm formation[110]. As shown in Figure 1.3, the GacA-GacS system is itself additionally regulated by two distinct hybrid sensor kinases, RetS and LadS, which consist of both histidine kinase and response regulator domains[111,112].

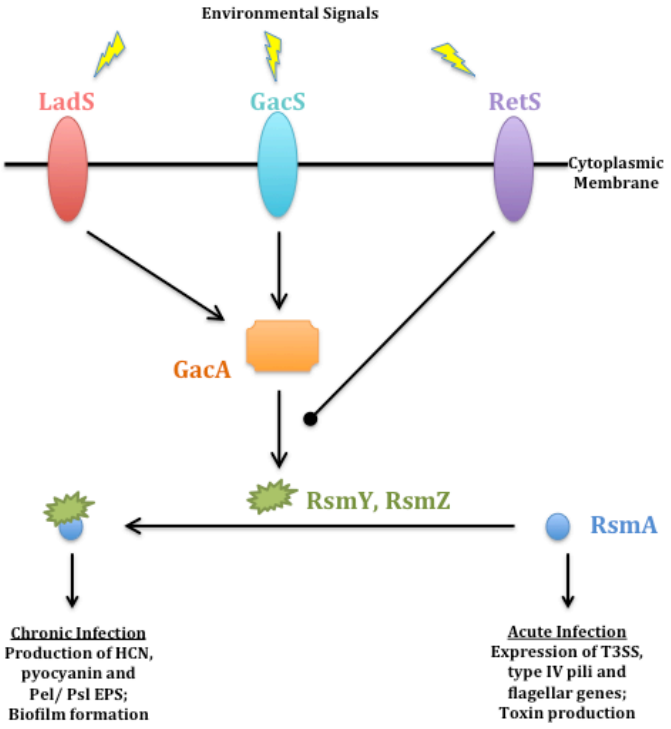


Figure 1.3. The GacA-GacS System. The GacS sensor kinase, in combination with the LadS sensor kinase, activates the GacA response regulator in response to unknown signals. GacA then activates the expression of the regulatory RNAs, RsmY and RsmZ, which inhibit the activity of the small RNA binding protein, RsmA. This leads to the expression of genes involved in chronic infection, such as those encoding biofilm components. The RetS sensor kinase acts in the opposite manner, repressing GacA activation and therefore derepressing RsmA. RsmA then represses the expression of chronic infection mediators, whilst acute infection mediators such as type III secretion systems (T3SS) and their effectors are produced. Thus, GacAS, RetS and LadS direct the outcome of infection. Adapted from [102]

Together with the Gac-Rsm pathway, RetS and LadS are key regulators of *P. aeruginosa* pathogenesis. RetS negatively regulates the expression of genes expressed during chronic infection, such *pel* and *psl*, and promotes the expression of genes encoding acute effectors. A *retS* mutant has a hyperadhesive phenotype, and biofilm formation is markedly increased due to increased expression of *psl*. However, mediators of acute infection such as type III secretion systems are downregulated in the mutant, and the strain is attenuated in a mouse model of acute pneumonia[111]. LadS, on the other hand, functions in an opposing manner to RetS and favours the establishment of chronic infections. A *ladS* mutant is defective in biofilm formation and demonstrates increased cytotoxity due the upregulation of type II secretion systems and cytotoxic mediators of acute infection such as ExoU. LadS mutants also downregulate the transcription of *rsmZ*, whilst RetS mutants upregulate it[112]. Figure 1.3 illustrates the importance of the Gac-Rsm-RetS-LadS system in maintaining the balance between acute and chronic infection; although both RetS and LadS signals converge onto the Gac-Rsm pathway, the predominance of one or the other directs the expression of the virulence factor genes associated with each infectious state. However, the stimuli to which GacS, RetS and LadS respond in order to mediate pathogenesis are not yet known.

The importance of the Gac-Rsm-RetS-LadS system is evident in the *P. aeruginosa* PA14 strain. This strain has a mutation within the *ladS* gene, therefore PA14 has a highly virulent phenotype but does not form biofilms, since chronic infection mediators are repressed by RetS via RsmA[113]. Biofilm formation is under the control of many additional regulators; c-di-GMP-responsive transcriptional regulators such as PelD, which regulates Pel biosynthesis[114], and FleQ, which is able to both positively and negatively regulate both flagellar gene expression and *pel* expression dependent on c-di-GMP levels[115,116]. Furthermore, RpoS regulates the *psl* operon[117], whilst MvaT negatively regulates the expression of the *lecA* and fimbrial *cup* genes, determinants involved in biofilm formation[118,119]. That mutation of the *ladS* gene inhibits biofilm

formation to the extent that it does, despite the presence of other regulators, indicates the importance of the Gac-Rsm-RetS-LadS system at the top of the regulatory hierarchy. Quorum sensing is also important in biofilm formation, since mutants within the *las* or *rhl* genes have been observed to form altered biofilms *in vitro*; however, this is highly dependent upon the environmental conditions used[120–122].

Via the action of RsmY and RsmZ, the Gac-Rsm pathway integrates with the quorum sensing system to add another level of regulation to the expression of virulence factor genes in *P. aeruginosa*[106,109]; two component systems are therefore part of a wider regulatory network in *P. aeruginosa* involving transcriptional factors, regulatory RNAs and sigma factors, all of which form an intricate system for the effective regulation of the large genome.

1.5. The Role of Quorum Sensing in the Regulation of Virulence.

Much of the gene expression in *P. aeruginosa* is subject to regulation by the quorum sensing (QS) systems, which have been extensively reviewed elsewhere[43,123–126]. In quorum sensing, individual bacteria produce diffusible chemical signal molecules, called autoinducers, which accumulate in the environment. As the population increases, the bacteria sense the concentration of autoinducers as they accumulate to a critical threshold in the environment, in turn allowing sensing of the population density and induction of changes in gene expression. Quorum sensing allows appropriate regulation of genes necessary in infection, for example those encoding virulence factors, whilst integrating other stimuli from the environment, such as stress, temperature and nutrient availability. Expression of specific genes is thus modulated as dictated by the extracellular milieu.

In *P. aeruginosa*, three overlapping quorum sensing systems have so far been described, as summarised in Figure 1.4. The first two, the *las*

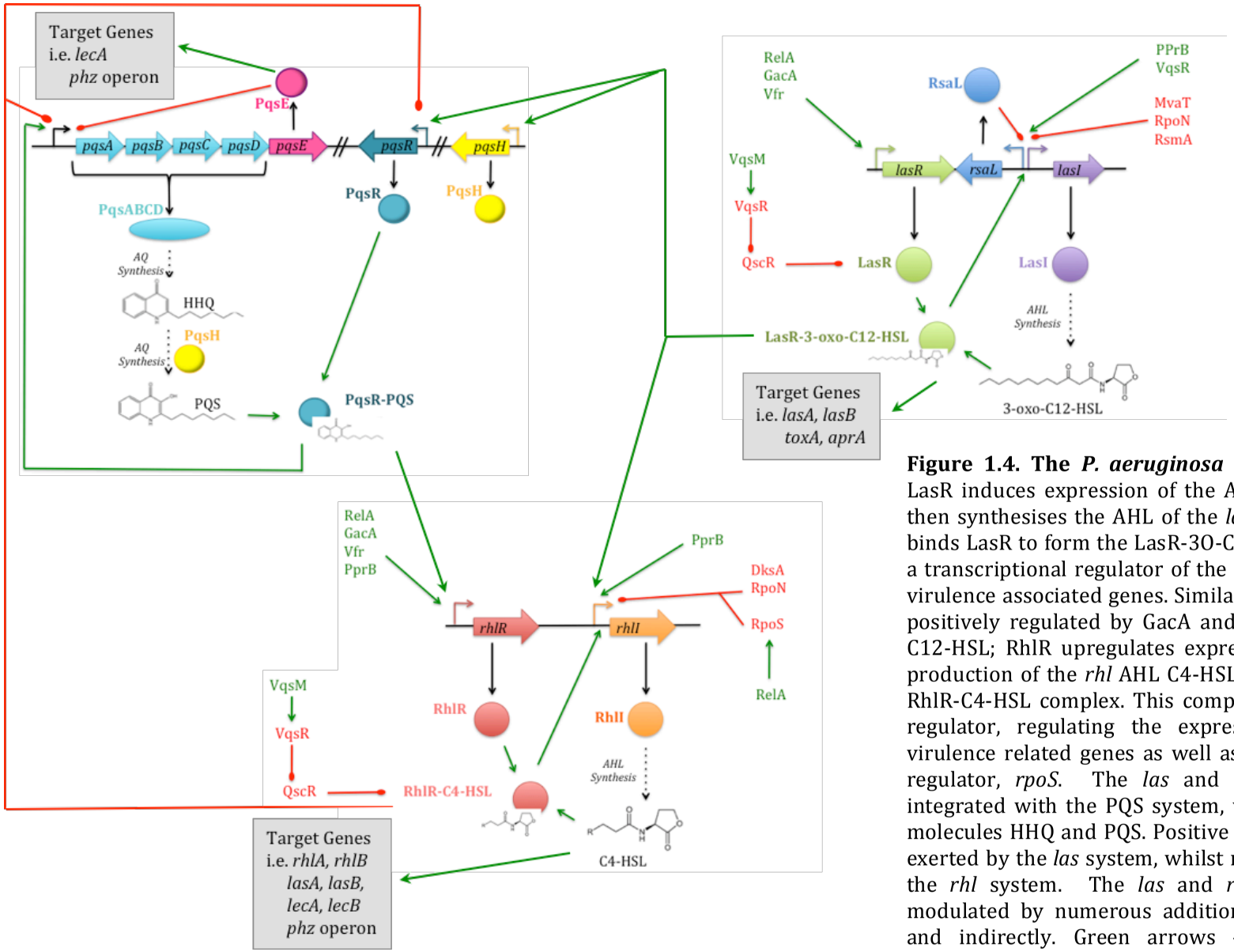


Figure 1.4. The *P. aeruginosa* Quorum Sensing Systems. LasR induces expression of the AHL synthase gene *lasI*. LasI then synthesises the AHL of the *las* system, 3O-C12-HSL. This binds LasR to form the LasR-3O-C12-HSL complex that acts as a transcriptional regulator of the *rhl* system, and a number of virulence associated genes. Similarly to the *las* system, RhlR is positively regulated by GacA and Vfr as well as LasR-3-oxo-C12-HSL; RhlR upregulates expression of *rhlI* and increased production of the *rhl* AHL C4-HSL leads to formulation of the RhlR-C4-HSL complex. This complex acts as a transcriptional regulator, regulating the expression of a further set of virulence related genes as well as the cell density-dependent regulator, *rpoS*. The *las* and *rhl* systems are also fully integrated with the PQS system, which utilises the AQ signal molecules HHQ and PQS. Positive control of the PQS system is exerted by the *las* system, whilst negative control comes from the *rhl* system. The *las* and *rhl* systems themselves are modulated by numerous additional regulators both directly and indirectly. Green arrows – positive regulation; red arrows – negative regulation. Diagram adapted from [6,180,396]

and *rhl* systems, utilise *N*-acylhomoserine lactones (AHLs) as the autoinducers and so are AHL-dependent; the third system utilises 2-alkyl-4-quinolones (AQs) as the autoinducers and so is AQ-dependent. AHL-dependent quorum sensing under the control of the *las* and *rhl* systems has classically been thought of as hierarchical; the *las* system exerts control over the *rhl* system[127,128]. LasR, the intracellular transcriptional regulator of the *las* system, is produced in response to environmental stimuli and upstream regulatory signals before then activating transcription of the *lasI* gene, coding for the LasI autoinducer synthase. LasI synthesises the cognate AHL *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which forms a complex with LasR; this allows it to further activate target promoters including those of *lasI*, creating a positive feedback loop, and *rhlR*, which encodes the intracellular transcriptional regulator of the *rhl* system. RhlR activates expression of *rhlI*, coding for the autoinducer synthase RhlI, responsible for the synthesis of *N*-(butanoyl)-L-homoserine lactone (C4-HSL); C4-HSL then complexes with RhlR, allowing it to bind target promoters[129–132]. Historically, the *rhl* system was associated with a distinct regulon, encompassing a certain subset of virulence factors including rhamnolipids, pyocyanin, LecA and hydrogen cyanide, which are not under the control of the *las* system. However, certain virulence factors regulated by the *las* system can also be controlled by the *rhl* system, such as LasA and LasB[132,133].

This classical view of the quorum sensing cascade dictates that, since LasR sits at the top of the cascade, it should be absolutely required for the expression of the *rhlRI* genes and therefore the *rhl*-associated virulence factors. However, LasR mutants have been isolated that are able to produce RhlR-regulated products, such as pyocyanin, LecA and C4-HSL in a LasR-independent manner[134]. A recent study challenged the classical view of the *las*–*rhl* hierarchy by characterising the transcriptome of a *lasR* mutant strain[135]. Intriguingly, a range of quorum sensing-controlled virulence factors were found to be produced in stationary phase despite the lack of functional LasR, and the study concluded that RhlR is able to

induce the expression of a set of genes previously thought to be exclusively LasR-regulated. This included the *lasI* gene, indicating a pathway through which *P. aeruginosa* is able to overcome the effects of mutations in the *lasR* gene.

The *las* and *rhl* systems are fully integrated with the AQ-dependent quorum sensing system, adding another level of complexity to the *P. aeruginosa* regulatory network. AQ-dependent quorum sensing utilises two main AQ signal molecules; 2-heptyl-4-quinolone (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone Signal, PQS)[136,137]. The products of the *phnAB* genes synthesise anthranilate[138–140], which is then converted to HHQ by the products of the *pqsABCD* genes. This operon encodes an anthranilate coenzyme A ligase (PqsA), two β-keto acyl acyl carrier protein synthases (PqsB and PqsC) and a FabH1-homologous transacetylase (PqsD), which act in concert to incorporate the fatty acid octanoic acid and an intermediate molecule, 2-aminobenzoylacetate, into HHQ. The resulting HHQ molecules are then converted to PQS by the mono-oxygenase PqsH[136,141,142]. The *pqsABCDE* genes are under the positive transcriptional control of PqsR (also known as MvfR), which is itself positively regulated by LasR; PqsR also positively regulates the transcription of *pqsH* and the *rhl* system. Conversely, RhlR represses the transcription of both *pqsR* and *pqsABCDE*, highlighting the complexity of the interactions between systems[143–146]. PqsR is bound by HHQ and PQS, which behave as coactivators, thus forming an autoregulatory loop[147]; however, the PQS molecule also has many additional functions. Besides acting as a signal molecule and regulator of gene expression, PQS also has iron chelating properties[143] and is involved in the formation of membrane vesicles[148], as well as mediating both autolysis and the reduction of metabolism in response to stress under certain conditions[56].

The *pqsE* gene completes the *pqs* operon, although the function of the 34 kDa PqsE protein has not yet been effectively resolved. Since a *pqsE* mutant is still able to produce PQS[149], it seems unlikely that PqsE is involved in the synthesis of the AQ signal molecules; studies have, however, shown that PqsE appears to function as a quinolone response

protein in the respect that it is required for the response of cells to PQS. A *pqsE* mutant strain has reduced virulence in mouse models comparable with that of a *pqsR* mutant[150], and it has been shown that PqsE is able to regulate the production of pyocyanin, rhamnolipids and elastase in a RhlR-dependent manner[151], as well as having involvement in AQ-independent *lecA* expression and swarming motility[152]. Furthermore, PqsE is also required in combination with PQS for biofilm formation[152]. Farrow *et al.* [151] hypothesised that PqsE might exist as part of a regulatory hierarchy; following upregulation of *pqsE* expression by PQS and PqsR, PqsE is able to regulate a subset of virulence-associated genes that assist *P. aeruginosa* in pathogenesis. To investigate this, Rampioni *et al.*[152] carried out transcriptomic analysis of a *P. aeruginosa* strain with an IPTG-inducible conditional mutation in the *pqsE* gene, and found that when *pqsE* was overexpressed, the activity of the *pqsA* promoter was abolished. This suggests a role for PqsE in the negative regulation of the *pqsA* promoter, and, since PqsE exists in an operon under the control of the *pqsA* promoter, implies that PqsE creates a negative feedback system. The role of PqsE in the negative regulation of the *pqsA* promoter will be discussed further in Chapter 5.

Whilst PqsE is clearly an important determinant of *P. aeruginosa* virulence, the mechanism through which it does this is still elusive. Attempts to study the crystal structure of the protein have characterised the active site and revealed that PqsE has low levels of hydrolase activity, and is able to use phosphodiesters, thioesters and nucleic acids as substrate; nonetheless, the physiological role of PqsE *in vivo* still remains to be elucidated[153,154].

1.6. Modulation of the QS Response in *P. aeruginosa*.

The *las* and *rhl* systems are themselves directly and indirectly regulated by a number of additional independent proteins besides two component systems, meaning that quorum sensing is just one part of a

much wider regulatory network consisting of many regulators; some of these regulators are illustrated in Figure 1.4.

Vfr is a cyclic adenosine monophosphate (cAMP) receptor protein (CRP) that is required for the expression of many virulence factor genes[150,151], whilst cAMP is an important intracellular second messenger that serves to add an additional level of control to gene regulation, since CRP proteins are only fully active when complexed with cAMP[155]. As a result, elevated intracellular cAMP levels often correlate with changes in gene expression. Fuchs *et al.*[156] characterised Vfr as a global regulator that is able to regulate the expression of a large number of genes in a cAMP-dependent manner, and this has been confirmed in a number of studies[157–160]. However, Vfr is also able to regulate the *lasR* gene in a cAMP-independent manner, binding the promoter directly and therefore sitting at the top of the quorum sensing cascade[156,161]. Direct interaction has also been demonstrated between Vfr and the *rhlR* promoter[162].

Vfr has also been shown to have a role in the negative regulation of the stationary phase sigma factor, RpoS[157]. Interestingly, RpoS itself is integrated into the quorum sensing system, and is able to regulate the production of a number of virulence factors, such as LecA and rhamnolipids[163,164]. In a study by Latifi *et al.*[127], the expression of *rpoS* was shown to be dependent upon RhlR and C4-HSL, identifying *rpoS* as a quorum sensing-controlled gene. However, Whiteley *et al.*[165] showed that actually, RpoS appears to negatively regulate *rhlI*, controlling its expression such that it is not transcribed prior to stationary phase. This controversy was explored by Schuster *et al.*[166], who showed that RpoS does indeed regulate the majority of its target genes only in stationary phase. Through transcriptomic microarray comparison of a PAO1 *rpoS* mutant with its parent strain, the study identified 3 classes of genes; those controlled only by quorum sensing, those controlled only by RpoS, and those controlled by both. Subsequently, a model was developed that hypothesised that it could be possible for both the scenarios of Latifi *et al.*[127] and Whiteley *et al.*[165] to occur simultaneously, with RpoS able to indirectly regulate the expression of genes via the regulation of the *rhlR*

and *lasR* genes, and vice versa; some genes might be indirectly regulated by the quorum sensing system via the regulation of *rpoS* expression. This model represents the overlap between the quorum sensing and RpoS regulons, and again highlights the complexity of gene expression in *P. aeruginosa*[166].

There are a range of additional mechanisms through which *P. aeruginosa* controls the quorum sensing system. QscR was identified by Chugani *et al.*[167] as a LuxR homolog with similarity to RhlR and LasR, but lacking a cognate autoinducer synthase. Further investigation revealed that a *qscR* mutant produced AHLs earlier in growth than the wild type, and found QscR to be involved in the repression of several quorum sensing-controlled genes, including those involved in pyocyanin biosynthesis. Ledgham *et al.*[168] then demonstrated that QscR is able to repress the activities of LasR and RhlR by forming heterodimers with these proteins which can then dissociate in the presence of high levels of 3-oxo-C12-HSL and C4-HSL. This suggests that QscR plays a role in repressing the quorum sensing system until the levels of autoinducer molecules have reached a critical threshold, preventing its premature induction. Following induction of quorum sensing, LasR is able to upregulate the expression of a gene encoding another regulator, VqsR, which represses *qscR*[169,170]. The expression of *vqsR* is negatively regulated by yet another regulator, VqsM; in a *vqsM* mutant, production of both virulence factors and AHL signalling molecules was reduced, but could be restored to some extent by overexpression of *vqsM*[171]. Demonstrating the extent of the integration of the QS system with other regulatory elements in *P. aeruginosa,* the *qscR* gene was also found to be regulated by the GacA-GacS system [168].

Another important regulator of QS is RsaL, which represses the *lasI* promoter directly. The expression of *rsaL* is activated by LasR; however, the *rsaL* and *lasI* genes are adjacent to each other, transcribed in opposite directions, and separated by an intergenic region containing a bidirectional promoter to which LasR binds, activating expression of both genes. As the levels of 3-oxo-C12-HSL increase, so too do the levels of

RsaL, which binds the *lasI* promoter and represses it along with its own promoter. This creates a negative feedback loop and has a homeostatic effect, ensuring levels of 3-oxo-C12-HSL are maintained at a constant level[172,173]. Rampioni *et al.*[174] investigated the role of RsaL further, showing that it is able to function as a global regulator in its own right to alter the expression of around 130 genes independently of *lasI*.

Along with cAMP, guanosine tetraphosphate (ppGpp) also functions as a second messenger and has some involvement in gene regulation. ppGpp is synthesised by a ribosome-associated protein, RelA; in conditions of nutritional stress – more specifically, amino acid starvation – RelA activity is upregulated and the intracellular levels of ppGpp increase, leading to the arrest of protein synthesis[175]. Van Delden *et al.*[176] found that, when *relA* is overexpressed, the expression of *rpoS, lasR* and *rhlR* can be activated prematurely, even in conditions of low cell density, suggesting that RelA is involved in the regulation of quorum sensing independent of cell density in environments where nutrients are scarce. This strategy allows the production of virulence factors such as exoenzymes, thus facilitating the acquisition of the nutrients required to allow the *P. aeruginosa* population to increase to its threshold level.

In addition, the post-transcriptional regulator, RsmA, generally has a negative effect on genes such as *lasI* and *rhlI*, but has also been found to act as a positive regulator of swarming and rhamnolipid production[177,178]. RsmA is regulated by the regulatory RNAs RsmY and RsmZ, which are antagonised via the GacA-GacS system, as discussed in Section 1.4.

Finally, the MexAB-OprM efflux pump has been postulated to regulate the *las* quorum sensing system to some extent. In a *mexB* mutant unable to express the genes encoding the MexAB-OprM efflux pump, the activity of the *las* system is upregulated, and it was found that in this strain LasR was able to respond more readily to 3-oxo-AHL molecules other than 3-oxo-C12-HSL. Aside from demonstrating how AHL cross-talk between bacterial species such as *P. aeruginosa* and *Vibrio* species, for example, can occur, the study concluded that MexAB-OprM is able to regulate the response of the *lasR* system to AHLs by regulating the

intracellular levels of 3-oxo-C12-HSL proportionally with other signalling molecules via its efflux activity[41]. This might allow *P. aeruginosa* more control over its response to signals from other bacteria in a multi-species environment, such as within biofilms.

There are a number of additional mechanisms of regulation of the QS system that occur transcriptionally, post-transcriptionally, and post-translationally; these have been reviewed elsewhere[179,180]. This integration of regulatory signals at various levels is important in ensuring that genes are expressed only when required, and expression of any one gene is often regulated by more than one mechanism in order to achieve this. Indeed, switching on the quorum sensing system upon entry to stationary phase is a huge undertaking that requires tight control, not least because quorum sensing controls the expression of approximately 10% of the *P. aeruginosa* genome[181]; expression of such a large number of genes is hugely energetically demanding and comes at a great nutritional cost. This explains the presence of separate levels of regulation in *P. aeruginosa*; firstly, overall regulation of the quorum sensing system and its initial activation, and secondly, regulation of the quorum sensing-controlled genes themselves[27]. For many, but not all, of the genes in this class, the presence of quorum sensing signals alone is not sufficient to induce their expression.

1.7. Modulation of Host Defence Mechanisms by Quorum Sensing.

In 2008, Le Berre *et al.*[182] found that the most clinically pathogenic *P. aeruginosa* strains isolated from patients with ventilator-associated pneumonia had increased quorum sensing activity, and also tended to produce elevated levels of elastase and pyocyanin, quorum sensing-regulated virulence factors that are important in the initial establishment of infection. This suggests that quorum sensing is an important aspect of *P. aeruginosa* pathogenesis, with a key role as a virulence determinant. A recent study inoculated lung epithelial cells with wild type *P. aeruginosa* membrane vesicles and compared their effects with membrane vesicles

from a *lasR* mutant strain; the wild type vesicles induced an inflammatory response that was not seen with the Δ*lasR* vesicles[183], again highlighting a role for quorum sensing and quorum sensing-controlled products in host-pathogen interactions. Far from modulating only *P. aeruginosa* gene expression, the quorum sensing system is also involved in altering the host response to infection. The *las* autoinducer 3-oxo-C12-HSL is able to function in an immunomodulatory manner, able to both stimulate and repress the inflammatory response via changes in the cytokine profiles. This occurs in a dose-dependent manner, and is most likely highly dependent upon the type of host cell involved[184–186]. Lawrence *et al.*[187] revealed an additional function for 3-oxo-C12-HSL as a vasorelaxant, inhibiting the contraction of arterial smooth muscle; however, no such roles have been identified for C4-HSL[188].

Similar characteristics have been attributed to PQS and HHQ, the signal molecules of the alkyl quinolone system. PQS, along with 3-oxo-C12-HSL, is able to exert an immunosuppressive effect on dendritic cells[189], which can impact on antigen presentation and the induction of appropriate immune responses. Furthermore, both PQS and HHQ repress the production of antimicrobial peptides and phagocytic killing through interference with the activation of the host transcription factor, HIF-1, which is essential for clearance of *P. aeruginosa* corneal infections due to its role in immunomodulation[190,191]. HIF-1 consists of two subunits, of which HIF-1α is predominantly responsible for transcriptional regulator activity during hypoxia. Hypoxia refers to the low oxygen conditions in infected and inflamed tissues, caused in part by increased metabolic activities of both host and bacterial cells[192]. In hypoxic environments, PQS is able to trigger the degradation of HIF-1α, in turn downregulating the expression of HIF-1 target genes, such as those encoding antimicrobial peptides[193]. Interestingly, it has also been shown that *P. aeruginosa* is able to detect the activation of HIF-1α through the recognition of HIF-1-related metabolites and upregulate *lecA* expression in response[194]. A recent study has also compared the transcriptome of a monocyte-macrophage cell line in response to the culture supernatants of a Δ*pqsA* strain in the presence

and absence of AQs, finding that a large number of host genes that were up or downregulated in response to AQs were known to be involved in mediating the immune response to infection[195].

These are examples of the ways in which *P. aeruginosa* is able to take advantage of host processes for its own benefit, and reveals how it has become such a successful opportunistic pathogen; further examples will be discussed in Chapter 3.

1.8. Emerging Therapeutics Against *P. aeruginosa* infections.

As mentioned previously in Section 1.1, the intrinsic antibiotic resistance of *P. aeruginosa* and the emergence of multi-drug resistant strains is rendering the majority of available antibiotics ineffective in the clinical environment[11]. Particularly in the treatment of *P. aeruginosa* infections in the cystic fibrosis lung, novel approaches have to be found to combat increasing resistance profiles. Cystic fibrosis infections are now predominantly treated with combination therapy, using a mixture of antibiotics that would otherwise have no effect when used alone[196]. The development of new antibiotics is expensive and takes many years; indeed, many newer antibiotics are derivatives of existing drugs, target *Pseudomonas* via the same mechanism, and are therefore vulnerable to selecting for resistant strains in the same way as their predecessors[197].

One of the few antibiotics still in use against *P. aeruginosa* infections is colistin, a polymyxin that is synthesised naturally by a *Bacillus* species. Previously, colistin use was withdrawn due to its high cytoxicity. However, it is now known as a drug of last resort, highlighting the desperate need for new anti-pseudomonal therapeutics[197].

Recently, a promising new antibiotic has been developed that is highly specific for *Pseudomonas aeruginosa*. This drug, POL7080, is a peptide mimetic and is based on the protein structure of the host antimicrobial peptide, protegrin-1. However, studies have determined that whilst protegrin-1 forms pores in the bacterial outer membrane, POL7080 functions by binding LptD, an outer membrane protein involved

in LPS transport. Rather than damaging existing outer membranes, this peptidomimetic appears to interfere with outer membrane biogenesis. In initial experiments, POL7080 was active against many *P. aeruginosa* isolates that were already resistant to other classes of antibiotics, and the drug has recently shown low toxicity in phase II clinical trials[198–200].

The screening of natural compounds for antimicrobial properties is a method that is also being used to identify inhibitors of the quorum sensing systems. Many of the new approaches to identifying new therapeutics against *P. aeruginosa* infections utilise the 'anti-pathogenic drug principle'[201], which proposes that the abrogation of virulence or specific steps in pathogenesis will prevent the establishment of infection without killing the bacterium directly. This reduces the amount of selection pressure on the pathogen and therefore reduces the emergence of resistance to the drug.

As discussed in Section 1.5, the quorum sensing system is a key determinant in the expression of virulence factor genes and therefore the pathogenesis of infection. Various studies have shown that interference with the quorum sensing systems can lead to attenuation of *P. aeruginosa* virulence in animal models[17,202,203], therefore there is immense interest in the development of treatments that inhibit either the synthesis of signal molecules, the activity of signal molecules themselves, or the activity of the quorum sensing transcriptional regulators, LasR and RhlR[201].

Hentzer *et al.*[204] carried out a study that identified a natural furanone-derived compound as an inhibitor of quorum sensing. *In vitro*, an analogue of this compound reduced the expression of a number of quorum sensing-controlled virulence factors, without affecting the growth of *P. aeruginosa*. *In vivo*, the compound was able to inhibit quorum sensing in the respiratory tract following administration intravenously, without killing the bacteria. Attenuation of virulence by disrupting the production of virulence factors could render *P. aeruginosa* more vulnerable to clearance by the immune system; in addition, interference with the ability of *P. aeruginosa* to form biofilms would allow the infection to subsequently be treated with lower doses of antibiotics than the

bacterium might otherwise be sensitive to when growing within biofilms[205].

In cystic fibrosis, the growth of *P. aeruginosa* in biofilms is highly problematic. Many studies have identified natural compounds that are able to disperse biofilms or prevent their formation, making them an attractive prospect for treatment of cystic fibrosis lung infections. For example, Richards *et al.*[206] carried out structure-activity relationship studies to identify compounds similar to oroidin, which can disperse biofilms in marine environments, and the compounds identified were shown to disperse *P. aeruginosa* biofilms at low doses. In addition, Johansson *et al.*[207] developed glycopeptide dendrimers specific to *P. aeruginosa* LecB, a fucose-specific lectin involved in biofilm development. Again, these glycopeptides were able to disperse biofilms and prevent their formation in a LecB-specific manner. Similar results were obtained by Chemani *et al*.[26], who observed that administration of LecA and LecB inhibitors in a mouse model of *P. aeruginosa* infection reduced inflammation and tissue injury, whilst Hauber *et al*.[208] have shown that inhalation of fucose and galactose sugars might be a new approach to the treatment of respiratory biofilms.

In addition, a novel drug based on the structure of alginate has a bacteriostatic effect on *P. aeruginosa*, disperses and prevents the formation of biofilms, and also potentiates the activity of antibiotics to which *P. aeruginosa* might otherwise be resistant. This drug, OligoG, has also successfully passed phase II clinical trials[209].

Other natural products are also being investigated; garlic extract contains ajoene, which has quorum sensing-inhibitory activities against *P. aeruginosa*, as do propolis, a compound produced by bees, and fungal compounds produced by *Penicillium* species, for example[205,210]. Progress in this field clearly relies on an in-depth understanding of the molecular events occurring during infection, both in prokaryotic and eukaryotic cells, and any new treatments are highly likely to be administered in combination with established antimicrobial drugs.

1.9 Overall Aims.

The work presented in this thesis focuses on three distinct projects. In Chapter 3, the ability of *P. aeruginosa* to interact with the human 37 kDa laminin receptor precursor and the host carbohydrate-binding lectin galectin-3 will be explored through the use of ELISA and whole cell pulldown techniques, with a view to determining whether these determinants play a role in *P. aeruginosa* colonisation of damaged and repairing tissues. Chapter 4 will investigate the regulatory network controlling the expression of the *lecA* gene, a key virulence factor in *P. aeruginosa* pathogenesis. Using a promoter pulldown approach, the study will attempt to identify novel regulatory proteins binding to the *lecA* promoter. Finally, the work discussed in Chapter 5 will also make use of the promoter pulldown technique in order to address the PqsE-dependent negative regulation of the *pqsA* promoter, of which little is currently understood. Ultimately, this thesis aims to further understanding of *P. aeruginosa* pathogenesis and the complex regulatory networks that allow it to be such an effective opportunist pathogen.

Chapter Two.

Materials and Methods.

2.1. Bacterial Strains, Plasmids and Culture Techniques.

Bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2. All *E. coli* and *P. aeruginosa* strains were grown at 37°C either in Luria-Bertani (LB) broth with vigorous shaking at 200 rpm, or on LB agar plates. LB medium was prepared as described by Sambrook *et al*.[211]. The wild type *Haemophilus influenzae* strain was grown under similar conditions, either on agar plates or in broth cultures made using Brain-Heart Infusion medium supplemented with haemin (10 μg/ml) and NAD (10 μg/ml). Stock solutions of antibiotics were prepared according to manufacturer's instructions, filter sterilised, and added to media as appropriate. Unless otherwise stated, all antibiotic concentrations were used as follows. For *E. coli* - carbenicillin (Cb) 50 μg/ml; chloramphenicol (Cm) 20 μg/ml; ampicillin (Ap) 50 μg/ml; gentamicin (Gm) 25 μg/ml; tetracycline (Tc) 10 μg/ml; nalidixic acid 15 μg/ml. For *P. aeruginosa* - Cb 100 μg/ml; Cm 400 μg/ml; Gm 25 μg/ml; Tc 125 μg/ml; streptomycin (Sm) 1 mg/ml. Growth of cultures was measured using a Novaspec II Visible Spectrometer (Pharmacia LKB Ltd)) to obtain optical density (OD) readings at a wavelength of 600nm. All chemicals were purchased from Sigma Aldrich unless otherwise stated.

Table 2.1. Bacterial Strains Used In This Study.

Strain	Description	Source or Reference
DH5α	*Escherichia coli* strain used as host of pBluescript vector in genetic manipulation and electroporation	212
S17-1 λpir	Donor *E.coli* strain used for conjugation of suicide vector pME3087 into PAO1	213
PAO1-L	Wild type *P. aeruginosa* PAO1 strain, Lausanne laboratory subline	214
PAO1-N	Wild type *P. aeruginosa* PAO1 strain, Nottingham laboratory subline	

Strain	Description	Source or Reference
PAO1-W	Wild type *P. aeruginosa* PAO1 strain, Washington laboratory subline	214
NCTC 12934	Wild type *P. aeruginosa* reference strain	NCTC
PAO1-L Δ*oprI*	PAO1-L containing an in-frame deletion within the *oprI* gene	This Study
PAO1-L Δ*pilQ*	PAO1-L containing an in-frame deletion within the *pilQ* gene	
PAO1-L Δ*oprI*Δ*pilQ*	PAO1-L containing in-frame deletions within the *oprI* and *pilQ* genes	This Study
PA14	Wild type *P. aeruginosa* strain	215
Rd-KW20	Wild type *Haemophilus influenzae* reference strain	216
PAO1-N Δ*mvaT*	PAO1-N with a deletion in the *mvaT* gene	118
PAO1-N *pqsEind*	PAO1-N with an IPTG-inducible conditional mutation in the *pqsE* gene; Sm^R	152
PAO1-L ΔPA3697	PAO1-L with a deletion in the PA3697 gene	This Study
PAO1-L Δ*vfr*	PAO1-L with a deletion in the *vfr* gene	
PAO1-L ΔPA2117	PAO1-L with a deletion in the PA2117 gene	
PAO1-L Δ*fleQ*	PAO1-L with a deletion in the *fleQ* gene	
PAO1-L *pqsEind*	PAO1-L with an IPTG-inducible conditional mutation in the *pqsE* gene; Sm^R	
PAO1-W *pqsEind*	PAO1-W with an IPTG-inducible conditional mutation in the *pqsE* gene; Sm^R	
Δ*vfr pqsEind*	PAO1-L Δ*vfr* with an IPTG-inducible conditional mutation in the *pqsE* gene; Sm^R	
Δ*fleQ pqsEind*	PAO1-L Δ*fleQ* with an IPTG-inducible conditional mutation in the *pqsE* gene; Sm^R	
PAO1-W Δ*glpR*	PW7082 *glpR*-B11::IS*phoA*/hah; Tc^R	217
PAO1-W Δ*gbuR*	PW3586 *gbuR*-F09::IS*phoA*/hah; Tc^R	
PAO1-W Δ*dtcD*	PW9686 PA5166-D02::IS*lacZ*/hah; Tc^R	
PAO1-W Δ*pilR*	PW8661 *pilR*-F12::IS*phoA*/hah; Tc^R	
PAO1-W Δ*algD*	PW10273 *algB*-G04::IS*lacZ*/hah; Tc^R	
PAO1-W Δ*mifR*	PW10331 PA5511-A04::IS*phoA*/hah; Tc^R	

Strain	Description	Source or Reference
PAO1-W Δ*algP*	PW9843 *algP*-H02::IS*lacZ*/hah; Tc^R	217
PAO1-W ΔPA257*2*	PW5317 PA2572-D10::IS*lacZ*/hah; Tc^R	
PAO1-W ΔPA4132	PW8001 PA4132-B12::IS*phoA*/hah; Tc^R	
PAO1-W ΔPA0588	PW2078 PA0588-G01::IS*lacZ*/hah; Tc^R	
PAO1-W ΔPA0178	PW1308 PA0178-D05::IS*phoA*/hah; Tc^R	
PAO1-W ΔPA3729	PW7314 PA3729-D02::IS*phoA*/hah; Tc^R	
PAO1-W Δ*bfmR*	PW7935 PA4101-F02::IS*phoA*/hah; Tc^R	
PAO1-W ΔPA0236	PW1419 PA0236-E09::IS*lacZ*/hah; Tc^R	
PAO1-W ΔPA0141	PW1236 PA0141-E12::IS*lacZ*/hah; Tc^R	
PAO1-W Δ*cbrB*	PW8965 *cbrB*-G03::IS*phoA*/hah; Tc^R	
PAO1-W ΔPA5208	PW9769 PA5208-H12::IS*lacZ*/hah; Tc^R	
PAO1-W Δ*amgR*	PW9752 *amgR*-C09::IS*lacZ*/hah; Tc^R	
PAO1-W ΔPA4352	PW8349 PA4532- B11::*lacZ*/hah; $Tc^{R,}$	
PAO1-W Δ*erdR*	PW7120 PA3604-E03::*lacZ*/hah; Tc^R	
PAO1-W Δ*sadB*	PW10007 *sadB*-H09::*phoA*/hah; $Tc^{R,}$	
PAO1-W Δ*fleR*	PW2984 *fleR*-C08::*lacZ*/hah; $Tc^{R,}$	
PAO1-W Δ*phoB*	PW10033 *phoB*-F02::*phoA*/hah; $Tc^{R,}$	
PAO1-W ΔPA1799	PW4167 PA1799-G12::*phoA*/hah; $Tc^{R,}$	
PAO1-W Δ*glpR pqsEind*	*glpR*-B11::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	This Study
PAO1-W Δ*dctD pqsEind*	PA5166-D02::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W Δ*pilR pqsEind*	*pilR*-F12::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	

Strain	Description	Source or Reference
PAO1-W Δ*algB pqsEind*	*algB*-G04::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	This Study
PAO1-W Δ*mifR pqsEind*	PA5511-A04::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W Δ*algP pqsEind*	*algP*-H02::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA2572 *pqsEind*	PA2572-D10::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA4132 *pqsEind*	PA4132-B12::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA0588 *pqsEind*	PA0588-G01::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA0178 *pqsEind*	PA0178-D05::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA3729 *pqsEind*	PA3729-D02::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W Δ*bfmR pqsEind*	PA4101-F02::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA0236 *pqsEind*	PA0236-E09::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W Δ*cbrB pqsEind*	*cbrB*-G03::IS*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA5208 *pqsEind*	PA5208-H12::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W Δ*amgR pqsEind*	*amgR*-C09::IS*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R	
PAO1-W ΔPA4352 *pqsEind*	PA4532-B11::*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R,	
PAO1-W Δ*erdR pqsEind*	PA3604-E03::*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R,	

Strain	Description	Source or Reference
PAO1-W Δ*sadB pqsEind*	*sadB*-H09::*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R,	This Study
PAO1-W Δ*fleR pqsEind*	*fleR*-C08::*lacZ*/hah, Δ*pqsEind;* Sm^R, Tc^R,	
PAO1-W Δ*phoB pqsEind*	*phoB*-F02::*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R,	
PAO1-W ΔPA1799 *pqsEind*	PA1799-G12::*phoA*/hah, Δ*pqsEind;* Sm^R, Tc^R,	

Table 2.2. Plasmids Used In This Study.

Plasmid	Description	Source or Reference
pBluescript II KS+ (pBls)	pBluescript vector incorporating the multiple cloning site, *bla* resistance gene and *lacZ* gene for blue-white screening; Cb^R	218
pDM4	Suicide vector incorporating a multiple cloning site, *sacB* and *cat* genes; Cm^R	219
pME3087	Suicide vector incorporating a multiple cloning site and a *tet* resistance gene; Tc^R	220
pRB201	Upstream region of *pilQ* cloned into MCS of pBls; Cb^R	This Study
pRB202	Downstream region of *pilQ* cloned into MCS of pBls; Cb^R	This Study
pRB203	1kb Δ*pilQ* construct cloned into MCS of pBls; Cb^R	This Study
pRB204D	pDM4::Δ*pilQ.* *pilQ* gene incorporating in-frame deletion of central region cloned into pDM4; Cm^R	This Study
pRB204M	pME3087::Δ*pilQ* *pilQ* gene incorporating in-frame deletion of central region cloned into pME3087;Tc^R	This Study
pRB301	Upstream region of *oprI* cloned into MCS of pBls; Cb^R	This Study
pRB302	Downstream region of *oprI* cloned into MCS of pBls; Cb^R	This Study

Plasmid	Description	Source or Reference
pRB303	1kb Δ*oprI* construct engineered into MCS of pBls; Cb^R	This Study
pRB304D	pDM4::Δ*oprI.* *oprI* gene incorporating in-frame deletion of central region cloned into pDM4; Cm^R	This Study
pRB304M	pME3087::Δ*oprI.* *oprI* gene incorporating in-frame deletion of central portion engineered into pME3087; Tc^R	This Study
pDM4::*pqsEind*	pDM4 derivative for the generation of strains with an IPTG-inducible *pqsE* deletion; Cm^R	152
pDM4::Δ*vfr*	pDM4 derivative for the generation of the Δ*vfr* strain	G.Rampioni, unpublished
pRB401	pBls derivative for the generation of the Δ*vfr* strain	This Study
pRB402	pME3087 derivative for the generation of the Δ*vfr* strain	
pRB501	pBls derivative for the generation of the ΔPA3697 strain	
pRB502	pME3087 derivative for the generation of the ΔPA3697 strain	
pRB601	pBls derivative for the generation of the Δ*fleQ* strain	
pRB602	pME3087 derivative for the generation of the Δ*fleQ* strain	
pRB701	pBls derivative for the generation of the ΔPA2117 strain	
pRB702	pME3087 derivative for the generation of the ΔPA2117 strain	
pRB801	pBls derivative for the generation of the Δ*gbuR* strain	
pRB802	pME3087 derivative for the generation of the Δ*gbuR* strain	
pBK-miniTn7ΩGm	Delivery vector carrying a miniTn7 element containing a MCS and gentamicin resistance marker; Ap^R, Gm^R	221

Plasmid	Description	Source or Reference
pUX-BF13	Helper plasmid for containing transposition genes *tnsABCDE* required for chromosomal integration of miniTn7 element	222
miniCTX::*pqsA-lux*	Reporter plasmid containing the *pqsA* promoter region fused to the *lux* reporter; Tc^R	143
pRB901	miniCTX::*pqsA-lux*-derived reporter plasmid in which the *tet* gene is spliced and inactivated by the insertion of a functional *bla* gene; Cb^R	This Study
pRB902	pBls-derived plasmid containing the P*pqsA-lux* BamHI-XbaI fragment derived from miniCTX::*pqsA-lux*; Cb^R	
pRB903	pBls-derived plasmid containing the P*pqsA-lux* PstI fragment derived from miniCTX::*pqsA-lux*; Cb^R	
pRB1000	pBK-miniTn7ΩGm-derived plasmid containing the *pqsA-lux* PstI fragment derived from miniCTX::*pqsA-lux*; Gm^R	

2.2. DNA Manipulation.

2.2.1 Isolation of DNA.

Genomic DNA was prepared from overnight bacterial cultures using a Wizard Genomic DNA purification kit (Promega). Bacterial plasmids were similarly isolated from overnight cultures using either the QiaPrepspin Miniprep kit for high-copy plasmids, or the Midiprep kit for low-copy plasmids, while DNA fragments obtained during cloning procedures were purified by either Qiaquick gel extraction or PCR purification kits (all Qiagen). All procedures were carried out according to manufacturer's instructions. Final resuspensions and elutions of DNA were in H_2O. DNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

2.2.2. Digestion and Ligation of DNA.

Cloning of DNA into vectors was carried out by digestion and subsequent ligation of fragments. All DNA restriction digests were carried out according to manufacturer's instructions, using restriction enzymes (New England Biolabs) paired with the correct buffer for optimum enzyme activity. DNA was then purified, the concentration determined, and used in ligation reactions. Electroligase (New England Biolabs) was used in reaction mixtures according to manufacturer's instructions.

2.2.3. DNA Gel Electrophoresis.

DNA gel electrophoresis was carried out according to Sambrook *et al.*[211]. Unless otherwise stated, 1% w/v agarose was dissolved in 1x TAE buffer (40mM Tris, 20mM acetic acid and 1mM EDTA). 10 µg/ml ethidium bromide was added to visualise DNA. DNA loading dye (6x stock solution; 40% w/v sucrose and 0.4% w/v orange G in 1x TAE buffer) was added to samples as required. To determine DNA fragment sizes, a 1 kb plus DNA ladder (Invitrogen) was used.

2.2.4. Polymerase Chain Reaction (PCR).

To amplify DNA, PCRs were set up according to a protocol adapted from Saiki *et al.*[223], using either the Expand High Fidelity PCR system (Roche) for mutagenesis, or the GoTaq flexi PCR kit (Promega) for amplicon generation and routine PCR screening. Both kits were used according to manufacturer's instructions. In a total volume of 50 µl, reaction mixtures included 2.5 u/µl Taq DNA polymerase, 1mM of each primer required, 200 mM dNTPs and 1% v/v DMSO along with 1x PCR buffer provided with the polymerase. $MgCl_2$ was added at concentrations varying from 1 mM to 3 mM dependent on the template. Thermal cycling was carried out using a Progene PCR Thermal Cycler (Techne). DNA was denatured at 96°C for 5 minutes, followed by 30 cycles of denaturation at

96°C for 30 seconds, annealing at 50-70°C for 45 seconds (dependent on primer properties), and elongation at 72°C for 1 minute 30 seconds. The final elongation step was carried out at 72°C for 10 minutes, followed by cooling to 4°C. Primer sequences used in this study are given in Table 2.3.

Table 2.3. Primer Sequences Used in This Study. Specific sites for restriction enzyme (RE) digest are highlighted in green. The prefix 'B-' indicates that the primers are 5' biotinylated.

Primer Name	Primer Sequence 5 '- 3'	RE
OprI.uf	TATTCTAGAAGGTGTCCGAGTGGTTGAAG	XbaI
OprI.ur	TATGGATCCAACGTTGTTCATCGTGTTCCC	BamHI
OprI.df	TATGGATCCAGCCGCAAGTAATAGGTCTCACGA	BamHI
OprI.dr	TATCTCGAGGAACAAGAAGTTCGGCATCG	XhoI
PilQ.uf	TATTCTAGACGATCTGCAGTCCTACATGG	XbaI
PilQ.ur	TATGGATCCGCCACTGTTCATCGTCCGAC	BamHI
PilQ.df	TATGGATCCATCGGTCGCTGATATCGAAC	BamHI
PilQ.dr	TATCTCGAGTTCGAGAATTTCCTGGACGA	XhoI
B-LecPro_f	TATTTCAGCCGACTTCTGCGTTCCTT	
B-LecPro_r	TATGATTGATCTCCGATATATGAATT	
PA3697_uf	TATGGATCCGAACGAGTGGAAGGAAGAACA	BamHI
PA3697_ur	TCAGAGGATGTTCGCCTTGCGCTGGCTCATCGGCAA	
PA3697_df	ATGAGCCAGCGCAAGGCGAACATCCTCTGACCGTTC	
PA3697_dr	TATAAGCTTCATAGGCTGCGCAGAGGAA	HindIII
Vfr_uf	TATTCTAGAAGAGTTTGCGCCCCTTTTAT	XbaI
Vfr_dr	TATGTCGACATCGCCGAGATCAAGAAGG	SalI
PA2117_uf	TATGGATCCGAAGTGGAACGGACTCATGC	BamHI
PA2117_ur	CTACGACGAGCTCTGCCGTTTGAACGCCATGTTCCC	
PA2117_df	ATGGCGTTCAAACGGCAGAGCTCGTCGTAGAGTCGG	
PA2117_dr	TATAAGCTTCGGATTTCGTCGCCTTCG	HindIII
FleQ_uf	TATGGATCCGTCAAGATCGACCAGGCCAT	BamHI
FleQ_ur	TCAATCATCCGACAGGGTTTCGCGCCACATTTTGAT	
FleQ_df	ATGTGGCGCGAAACCCTGTCGGATGATTGACAGGTC	
FleQ_dr	TATAAGCTTCGATCACCTCACGCCAGA	HindIII
GbuR_uf	TATGGATCCTTGAAGGTGTTGATCGCGA	BamHI
GbuR_ur	TCAGGCCAGGCTCATCAAGGCGTTGGACACGGC	
GbuR_df	GTGTCCAACGCCTTGATGAGCCTGGCCTGAGCC	
GbuR_dr	TATTCTAGACGAACACGGCGCGCTGATCAT	XbaI
PA3583_f	CGACGAGCTGAAGAGCAAG	
PA3583_r	AGGTCCAGGTGAACCTTGTG	
PA5166_f	ATCTCGAGCAGATCAGCGAC	

Primer Name	Primer Sequence 5 ’- 3’	RE
PA5166_r	AACAGTTCGCTTTCCACCAC	
PA4547_f	GGACCCACATCAGTTGAACC	
PA4547_r	CCCTGCTTGTCTTCGATAGC	
PA5483_f	CCGAAAACCCAAGAACAGAC	
PA5483_r	GACGCGTTCGTATTCCTTGT	
PA5511_f	GAGTTGGCGAAGGATCTCTG	
PA5511_r	AGGGTGCCTACGACTTCATC	
PA5253_f	CTTGGTTGCGGCAGGTTT	
PA5253_r	TCGCTTGTAGCACATCGAAC	
PA2572_f	GAGGAGCTAAAGCGCAGCTA	
PA2572_r	GGTCGATGAGATTTCCCAGA	
PA4101_f	GGAGTTGTCCCGGATGTGT	
PA4101_r	CGGTCTTGATGTACTCCGGT	
PA4132_f	GAGGCATGACAACACGGTC	
PA4132_r	CGTTTCTTCTCGTCGTCCAT	
PA0588_f	AGCTTCTCGTAGGACAGCCA	
PA0588_r	CTGTACCCCTCGATCCTCAA	
PA0178_f	TCAGCGGAATCACGTAGTTG	
PA0178_r	GGGTCAGCGTGGAAAAGAT	
PA3729_f	TCCTCGAAGCTCTTCTCCAG	
PA3729_r	GGCGATCAAGAAGAAGAACG	
PA0236_f	TGTTCATGGGACGGGTTC	
PA0236_r	CTATTCGAACGCTGATGTCG	
PA0141_f	GCTTCTTGTCGTCGGACTTG	
PA0141_r	CATAGAGTGGGGGACTGCAT	
PA5200_f	ATTTCTGTGTACGGCCAGTG	
PA5200_r	AGCAGATGGATCGCCTTCTA	
PA1422_f	GATTCCACGGTGAGCGAC	
PA1422_r	GAGGAAATGCTCAAGATCGC	
PA5208_f	AAATAGGCACCGCAGAACTC	
PA5208_r	TGGGTGGCCTATACTTTTCG	
PA4726_f	ATCAACCTGCTCTCCAATGC	
PA4726_r	GAGCCGATGATGCCTATCTC	
PA1099_f	CAATTGCAGTTGCGCTCTC	
PA1099_r	CCGACAGGCGATAGTAGAGG	
PA5360_f	AAGACCATGGTTGGCAAGAC	
PA5360_r	GTCTTGGTTCACGACGGATT	
PA4352_f	GCAACCTGCTGATCTTCCC	
PA4352_r	GTGGTACGCAATGCGAAGAT	
PA3604_f	GACTGACGTCGAGGAGGAAG	
PA3604_r	GAACGCGGTGTACGAGGTAT	
PA5436_f	GAACGAATGCTCGGCAAG	
PA5346_r	GCTTCCAGCACCTTTTTCAC	
PA1799_f	AGCTTTTCCACCAGCGAATA	
PA1799_r	GCATATAATGCCAGCCGATT	

Primer Name	Primer Sequence 5 '- 3'	RE
Hah-138	CGGGTGCAGTAATATCGCCCT	
Lac-148	GGGTAACGCCAGGGTTTTCC	
B-PqsA_prof	AATTCTGCAAATGGCAGGCGAGG	
B-PqsA_pror	TATAAGCTTTGGACATGACAGAACGTTC	
Bla_f	CGGCCGGTGGCACTTTTCGGGGAAAT	EagI
Bla_R	CGGCCGGATTTTGGTCATGAGATTAT	EagI
BlaSeq_f	ATTGCTAACGCAGTCAGGCACCGTG	
BlaSeq_r	AATCCGTTAGCGAGGTGCCGCCGGC	
PpqsActx_f	CAGGGAAGCTTGCAAATGGCAGGCGA	HindIII
PpqsActx_r	ACCTCCTGCAGGTTGGCCAATGTGGA	PstI
T7 forward	TAATACGACTCACTATAGGG	
M13 reverse	GGAAACAGCTATGACCATG	

2.2.5. DNA Sequencing.

All DNA sequencing was carried out by the DNA Sequencing Laboratory, Queen's Medical Centre, University of Nottingham using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit and 3130xl Genetic Analyser. Sequencing results were analysed using the Chromas Lite software package (Technelysium) and the NCBI BLAST tool[224].

2.3. Cloning and Mutagenesis.

2.3.1. Construction of Plasmids.

All primers used in construction of plasmids are listed in Table 2.3.

2.3.1.1. Construction of pRB201, pRB202, pRB203, pRB204D and pRB204M.

To make pRB201 and pRB202, PCRs using the primer pairs PilQ.uf/PilQ.ur and PilQ.df/PilQ.dr were carried out to give the PCR products *pilQ*.U (516 bp upstream fragment) and *pilQ*.D (515 bp downstream fragment) respectively. pilQ.U was double-digested with XbaI

and BamHI, whilst pilQ.D was digested with BamHI and XhoI. Each fragment was then ligated into similarly digested pBluescript giving pRB201 (pilQ.U) and pRB202 (pilQ.D).

pRB201 was subsequently digested using XbaI and BamHI, and the resulting ~500 bp insert containing pilQ.U was ligated with similarly digested pRB202 to make pRB203, a pBls derivative containing a 1,031 bp Δ*pilQ* construct.

Digestion of pRB203 with XbaI and XhoI yielded a fragment approximately 1 kb in size. This was ligated into similarly digested pDM4, resulting in the suicide vector pRB204.D, containing the Δ*pilQ* construct.

The Δ*pilQ* suicide vector pRB204.M was constructed by digestion of pRB203 with XbaI and KpnI, followed by ligation of the ~1 kb fragment into similarly digested pME3087.

2.3.1.2. Construction of pRB301, pRB302, pRB303, pRB304D and pRB304M.

The 514 bp *oprI*.U (upstream) and 529 bp *oprI*.D (downstream) PCR products were obtained using the primer pairs OprI.uf/OprI.ur and OprI.df/OprI.dr respectively. These products were double digested with XbaI and BamHI (*oprI*.U) or BamHI and XhoI (*oprI*.D) before ligation into similarly digested pBluescript to give pRB301 and pRB302 respectively.

The ~500 bp XbaI-BamHI fragment resulting from the digest of pRB301 was then ligated into similarly digested pRB302, resulting in pRB303, a pBls derivative containing a 1,043 kb Δ*oprI* construct.

pRB303 was subsequently digested with XbaI and XhoI, yielding an ~1kb fragment containing the Δ*oprI* construct. This was ligated into similarly digested pDM4, resulting in the suicide vector pRB304.D.

pRB304.M was constructed by digestion of pRB303 using XbaI and KpnI, to release a ~1 kb fragment which was then ligated into similarly digested pME3087, creating a suicide vector containing the Δ*oprI* construct.

2.3.1.3. Construction of pRB401 and pRB402.

To construct pRB401, a 1,479 bp Δ*vfr* construct was cut from pDM4::Δ*vfr* (G. Rampioni, unpublished) using the restriction enzymes XbaI and SalI. The resulting fragment was ligated into similarly digested pBluescript, giving pRB401.

pRB401 was then digested with KpnI and XbaI to release the Δ*vfr* fragment for subsequent ligation into pME3087, also digested with KpnI and XbaI; this resulted in a suicide vector containing the Δ*vfr* construct.

2.3.1.4. Construction of pRB501 and pRB502.

The ΔPA3697 construct was made by amplifying the 447 bp upstream and the 450 bp downstream fragments of PA3697 using the primer pairs PA3697_uf/PA3697_ur and PA3697_df/PA3697_dr respectively. These fragments were then combined and used as template in a second PCR, using the primer pair PA3697_uf/PA3697_dr, resulting in the 897 bp ΔPA3697 construct.

The ΔPA3697 fragment was digested using HindIII and BamHI before being ligated into similarly digested pBluescript to make the plasmid pRB501.

The ~1 kb ΔPA3697 fragment was then released from pRB501 by double digestion with BamHI and HindIII. The resulting fragment was ligated into similarly digested pME3087 to make the suicide vectors pRB502, containing the ΔPA3697 construct.

2.3.1.5. Construction of pRB601 and pRB602.

The Δ*fleQ* construct was made using the primer pairs FleQ_uf/FleQ_ur and FleQ_df/FleQ_dr to obtain a 488 bp upstream and a 491 bp downstream fragment respectively. These were subsequently used as template in a second PCR with the primer pair FleQ_uf/FleQ_ dr to give a 979 bp Δ*fleQ* product.

The Δ*fleQ* fragment, was digested using HindIII and BamHI before being ligated into similarly digested pBluescript to make the plasmid pRB601.

The Δ*fleQ* fragment was released from pRB601 by digestion with XbaI and KpnI. The resulting ~1kb fragment was ligated into similarly digested pME3087 to make the pRB602 suicide vector containing the Δ*fleQ* construct.

2.3.1.6. Construction of pRB701 and pRB702.

The ΔPA2117 construct was obtained first using the primer pairs PA2117_uf/PA2117_ur and PA2117_df/PA2117_dr to give the 572 bp upstream and the 457 bp downstream fragments. These were then used as template in a subsequent PCR with the primer pair PA2117_uf/PA_2117_dr to create the 1,029 bp ΔPA2117 PCR product .

The ΔPA2117 fragment was subsequently digested with HindIII and BamHI before ligation with similarly digested pBluescript to make pRB701.

pRB701 was then digested using XbaI and KpnI to yield the ~1 kb fragment containing the ΔPA2117 construct; this was ligated into similarly digested pME3087 to make the suicide vector pRB702, carrying ΔPA2117.

2.3.1.7. Construction of pRB801 and pRB802.

The Δ*gbuR* construct was obtained by amplifying the 517 bp upstream and 496 bp downstream fragments using primer pairs GbuR_uf/GbuR_ur and GbuR_df/GbuR_dr respectively. These fragments were combined and used as template in a second PCR using primer pairs GbuR_uf/GbuR_dr, giving the 1, 013 bp Δ*gbuR* product.

The Δ*gbuR* product was digested using BamHI and XbaI and then ligated into similarly digested pBluescript to make pRB801.

pRB801 was then digested using XbaI and KpnI , and the resulting ~1 kb fragment was ligated into similarly digested pME3087 to make the suicide vector pRB802, containing the Δ*gbuR* construct.

2.3.1.8. Construction of pRB901, pRB902, pRB903 and pRB1000.

To create pRB901, the *bla* gene of pBluescript was amplified by PCR using the primers Bla_F and Bla_R to produce a 1,097 bp PCR product. This fragment was digested with EagI. At the same time, the miniCTX::*pqsA-lux* bioreporter[143] was linearised to a single ~13 kb fragment by restriction digest also using EagI. The two fragments were ligated to give pRB901, a miniCTX::*pqsA-lux* derivative containing the *bla* resistance marker.

To make pRB902, miniCTX::*pqsA-lux* was digested with XbaI and EagI. This created three fragments approximately 1 kb, 3 kb and 8 kb in size. The 8 kb fragment containing the *pqsA-lux* construct was ligated into pBluescript digested with XbaI, creating pRB902, a pBls derivative carrying the *pqsA-lux* XbaI fragment.

pRB903 was made by PstI digestion of pRB902, yielding a ~7.5 kb fragment that was subsequently ligated into similarly digested pBls, creating pRB903, a pBls derivative containing a shortened *pqsA-lux* construct.

pRB1000 was then made by PstI digestion of pRB903, followed by ligation of the ~7.5 kb *pqsA-lux* fragment into similarly digested pBK–miniTn7ΩGm[221]. This created pRB1000, a bioreporter containing *pqsA-lux* within the miniTn7 insertion element.

2.3.2. Preparation of Electrocompetent Cells.

Electrocompetent *E. coli* DH5α, *E. coli* S17-1 λ*pir* and *P. aeruginosa* were prepared according to the protocols developed by Sambrook *et al.*[211]. To prepare *E. coli* electrocompetent cells, an overnight culture was diluted 1 in 1000 with fresh LB medium and grown to an OD_{600nm} of 0.6-0.8. Cells

were harvested by centrifugation (3,702 rcf, 10 minutes at 4°C) and kept on ice throughout 2 washes in ice cold sterile H_2O and 2 washes in ice cold sterile 10% w/v glycerol. Cells were resuspended in ice cold 10% w/v glycerol and aliquoted in 50 µl volumes before being snap frozen in liquid nitrogen and stored at −80°C. Electrocompetent *P. aerguinosa* were similarly prepared directly from overnight cultures.

2.3.3. Transformations.

To transform electrocompetent strains, 50 µl of electrocompetent cells were combined with either 2 µl plasmid DNA or ligation mixture and electroporation carried out according to Sambrook *et al.*[211].

Where *E. coli* was transformed with pBluescript-derived vectors, clones carrying the plasmid were selected by carbenicillin (50 µg/ml) and blue-white screening, which was facilitated by addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to LB agar plates at a concentration of 50 µg/ml. Successful insertion of the DNA fragment into the multiple cloning site results in disruption of the *lacZ* gene within pBls, resulting in white colonies; no insertion results in blue colonies.

Where *E. coli* was transformed with pDM4 or pME3087, positive clones were selected with chloramphenicol (20 µg/ml) or tetracycline (10 µg/ml) respectively.

P. aeruginosa transformants were selected with either 125 µg/ml tetracycline to select miniCTX::*pqsA-lux*, 100 µg/ml carbenicillin to select pRB901, or 25 µg/ml gentamicin to select pRB1000.

2.3.4. Gene Replacement in *P. aeruginosa*.

2.3.4.1. Conjugative Mating Experiments.

In-frame deletion mutants were created in the PAO1-L background by allelic exchange using the suicide vectors derived from pDM4 and pME3087. Allelic exchange was achieved in biparental mating

experiments between the *E. coli* S17-1 λ*pir* donor strain (grown at 37°C overnight) and the *P. aeruginosa* recipient strain (grown at 42°C overnight) as described elsewhere[178]. Briefly, 5 ml of donor and recipient overnight cultures were mixed. Cell pellet were washed twice in LB broth before a final resuspension in 100 µl LB broth. 25 µl volumes were spotted onto LB plates and incubated at 37°C for 4-8 hours, before the bacterial cells were washed from the plate and serially diluted and plated on LB plates containing the appropriate antibiotic.

2.3.4.2. Sucrose Counter-Selection of In-Frame Deletion Mutants and *pqsEind* Conditional Mutants.

Following conjugations in which pDM4-derived vectors were used, transconjugants were selected by chloramphenicol (400 µg/ml) and nalidixic acid (15 µg/ml) . Following PCR screening for the presence of the appropriate insert, sucrose counter-selection was carried out using a method adapted from Westfall *et al.*[225], using 5-10% w/v sucrose to obtain the required isogenic mutants in which allelic exchange had occurred by recombination. Mutants were identified by replica plating to identify loss of chloramphenicol resistance, followed by PCR screening.

For creation of *pqsEind* strains with an IPTG-inducible conditional mutation in the *pqsE* gene, following sucrose selection successful recombination events were selected by loss of chloramphenicol resistance, accompanied by the acquisition of streptomycin (1 mg/ml) resistance. Induction of *pqsE* expression was achieved by growth of the *pqsEind* strains in the presence of 1 mM IPTG.

2.3.4.3. Enrichments.

Following conjugations in which pME3087-derived vectors were used, transconjugants were selected by tetracycline (125 µg/ml) and chloramphenicol (10 µg/ml) and isogenic mutants were obtained by enrichment for tetracycline-sensitive cells. Briefly, strains grown in LB

were inoculated with a bacteriostatic concentration of tetracycline (20 μg/ml) and incubated at 37°C with shaking for one hour. Carbenicillin was then added at a bactericidal concentration of 2 mg/ml and incubated at 37°C with shaking for a further 6 hours. Following 3 enrichment cycles, cultures were grown on LB agar, and colonies obtained then replica-plated on LB agar with or without tetracycline (125 μg/ml) to screen for loss of tetracycline resistance. Tetracycline sensitive colonies were then screened by PCR for the presence of the mutated gene.

2.3.5. Verification of Transposon Mutant Genotypes.

Transposon mutants obtained from the University of Washington were confirmed upon receipt for correct insertion of the Tn5 element by PCR and subsequently grown on tetracycline (5 μg/ml) according to advice given by the Manoil group, University of Washington Genome Centre.

2.4. Promoter Pulldown Experiments.

Promoter pulldown experiments were adapted from Koskenkorva *et al.*[226], with some modifications. Briefly, biotinylated promoter fragments were obtained by PCR amplification of the desired DNA sequence using 5' biotinylated primers (Sigma Aldrich) with the Expand High Fidelity PCR system (Roche) under the thermal cycling conditions described in Section 2.2.4. Specificity and purity of the PCR product was confirmed by agarose gel electrophoresis before promoter DNA was pooled and purified by phenol chloroform extraction and ethanol precipitation, as described by Sambrook *et al.*[211]. DNA was then quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Each DNA sample contained approximately 100 μg promoter DNA.

Cell extracts were prepared from *P. aeruginosa* cultures grown to the required OD_{600nm} in 400 ml LB broth. Following centrifugation to pellet (10,285 rcf, 10 minutes at 4°C), cells were resuspended in 2 ml lysis

buffer (100 mM NaCl, 10 mM Tris HCl pH8, 1 mM EDTA, 0.05% v/v Triton X-100) and sonicated on ice. Cell lysates were centrifuged as previously to remove cell debris, and supernatants 2 ml in volume containing proteins were retained. 20 µl PMSF (100 mM stock prepared in isopropanol) and 10µl fish sperm DNA (10 mg/ml stock prepared in H_2O) were added and the cell lysates stored at -80°C.

Using a magnetic rack, 100 µl Streptavidin-coupled magnetic Dynabeads M-280 (Invitrogen) were washed three times with 2x wash buffer (2 M NaCl, 10 mM Tris pH7.4, 1 mM EDTA) before incubation with 100 µg promoter DNA for 20 minutes with light shaking at room temperature. Following 3 more wash steps using 1x wash buffer, the beads were incubated with the cell lysates for 4 hours with light shaking at room temperature. After 5 washes with lysis buffer, the beads were resuspended in 20 µl elution buffer (1.2M NaCl, 10 mM Tris HCl pH8, 1 mM EDTA, 0.05% v/v Triton X-100) and incubated with shaking at room temperature for 10 minutes. The magnetic beads were removed and the resulting protein samples stored at -20°C prior to SDS-PAGE analysis.

2.5. SDS-PAGE.

SDS-PAGE was carried out according to manufacturer's instructions using the NuPAGE system (Invitrogen). Precast 4-12% gradient Bis-Tris gels were used in combination with MES-SDS running buffer in an XCell SureLock MiniCell, and gels were stained overnight in colloidal Coomassie blue (0.12% w/v Coomassie blue G250, 10% w/v ammonium sulphate, 10% v/v phosphoric acid, 20% v/v methanol in H_2O) before destaining in H_2O. For size determination, a ColourPlus prestained broad range protein ladder (New England BioLabs) was used.

2.6. LCMS/MS Protein Identification.

Protein bands of interest were cut from the Coomassie-stained Bis-Tris gels and sent for LCMS/MS analysis at the University of Leicester

Proteomics Facility. Following in-gel trypsin digestion, LCMS/MS was performed and data extracted. Charge state deconvolution and deisotoping was not performed. LCMS/MS data was analysed using Mascot (Matrix Science) set up to search UniProt for all entries classed under *Pseudomonas*. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion mass tolerance of 10.0 ppm. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification, and oxidation of methionine and acetyl of the N-terminus were specified as variable modifications. To validate the LCMS/MS based peptide and protein identifications, Scaffold (Proteome Software Inc.) was used with the UniProt database[227]. Peptide identifications were assigned using the PeptideProphet algorithm[228] and accepted if they were established at 95% probability or more, whilst protein identifications were assigned using the ProteinProphet algorithm[229] and were accepted if they were established at 95% probability or more containing at least 3 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. To compare the abundance of proteins between samples, data was normalised; the sums of the unweighted spectrum counts for each sample were scaled, and the scaling factor used to adjust the unweighted spectrum counts to a normalised quantitative value. Gene function information was obtained from the PseudoCap annotation project (www.pseudomonas.com)[230].

2.7. Mutant Characterisation.

2.7.1. Twitching Motility.

For twitching motility assays, the protocol was adapted from Patriquin *et al.*[231]. LB broth was solidified with 1% w/v agar and plates containing 25 ml of medium were stab-inoculated with 1 µl of overnight culture. Plates were incubated for 24 hours at 37°C, before the agar was

removed and twitching motility zones visualised using 1% w/v crystal violet staining.

2.7.2. ELISA for Bacterial Binding to the 37/67kDa Laminin Receptor and Galectin-3.

For these experiments, the protocol was adapted from Orihuela *et al* [232] and the assays to investigate each strain binding to either galectin-3 or the laminin receptor were carried out in triplicate. Briefly, a 96-well microtitre plate (Amino Immobiliser, Nunc) was coated at 4˚C overnight with 200 µl of either recombinant galectin-3 (Sigma Aldrich) or recombinant 37 kDa laminin receptor precursor (prepared as previously[232]), both at a concentration of 5 µg/ml in phosphate buffered saline (PBS) or 50mM carbonate buffer (142 mM $NaHCO_3$, 8 mM Na_2CO_3, pH 9). 200 µl 1% w/v BSA in PBS without laminin receptor or galectin-3 was added to control wells. Following washing (3 times) with PBST (PBS with 0.5% v/v Tween–20), the plate was blocked with 1% w/v BSA in PBST for 2 hours at room temperature.

P. aeruginosa mutant derivatives, or *H. influenzae* cells were harvested from LB plates and washed in PBST before a final resuspension in either carbonate buffer or PBS. The optical density of the samples was standardised to OD_{600nm} between 0.02 and 1.0 in a total volume of 1 ml. For digoxygenin labelling of cells, 10 µg digoxygenin (Roche) was added to the samples and incubated for 2-16 hours at room temperature. Bacterial cells were then washed three times in PBST to remove unbound digoxygenin before a final resuspension in PBS with 1% w/v BSA.

The coated and blocked microtitre plate was washed briefly with PBST before wells were inoculated with 200 µl of digoxygenin-labelled bacterial cells. The plate was then incubated between 2 to 5 hours at room temperature, and stored at 4°C overnight. After further washing in PBST (5 cycles), 200 µl polyclonal anti-digoxygenin fab fragment-conjugated horseradish peroxidase- or alkaline phosphatase-conjugated antibody was

added for 90 min (1:5,000 in 1% w/v BSA in PBST, Roche). 200 µl of either ABTS substrate for HRP detection (Bio-Rad; colourless to green colour change) or NBT/BCIP for AP detection (Roche; colourless to yellow colour change) was added and the colour change developed. Optical density readings were taken when colour change was observed, at a wavelength of 405nm with a ELx800 Absorbance Microplate Reader with Gen5 software (Biotek).

2.7.3. Laminin Receptor-Cell Pulldown Assay.

Bacterial strains were harvested from LB agar plates and washed in PBST 3 times. The final suspension of bacterial cells was adjusted to an OD_{600nm} of 0.3 before the bacterial cells were pelleted by centrifugation for 1 minute at 3823.5 rcf and the pellet resuspended to a total volume of 1 ml using 1% w/v BSA in PBS containing 20µg of recombinant laminin receptor. After 3 hours incubation at room temperature, the cell suspensions were washed 5 times in PBST to remove unbound laminin receptor, with the final resuspension of the pellets in 100 µl 1x SDS sample loading buffer (prepared from a 4x stock; 40% v/v glycerol, 240 mM Tris HCl pH 6.8, 8% w/v SDS, 0.04% w/v bromophenol blue and 5% v/v β-mercaptoethanol). The samples were sonicated on ice, centrifuged briefly at 3823.5 rcf, and then boiled at 100°C for 5 minutes. Purified laminin receptor was used as a positive control during SD-PAGE analysis. Bacteria only (no laminin receptor added) were used as a negative control.

Samples were analysed by SDS-PAGE as described in Section 2.5, with some changes; 20µl of each sample was run on NuPAGE 4-20% gradient precast Tris-Glycine (Novex, Invitrogen) with Tris-glycine buffer. One gel was then stained using Coomassie Blue stain (0.1% w/v Coomassie Blue G250, 50% v/v methanol, 10% v/v glacial acetic acid) and then destained using destain solution (40% v/v methanol, 10% v/v glacial acetic acid). The second gel was transferred to a nitrocellulose membrane (using a semi-dry transfer chamber (Bio-Rad) and semi-dry transfer

buffer (48 mM Tris, 39 mM glycine, 20% v/v methanol). The membrane was then blocked in 1% w/v BSA in PBST for 1 hour, before being probed with rabbit anti-laminin receptor antibody (1:2,000 in 1% w/v BSA in PBST) at room temperature for 1 hour. The membrane was then washed 4 times with PBST before application of the secondary antibody, AP-conjugated anti-rabbit antibody (1:1,200 in PBST containing 5% w/v milk powder) for 1 hour at room temperature. After a final round of 4 washes with PBST, the blot was developed with NBT/BCIP substrate (NBT/BCIP tablets (Roche) dissolved in H_2O) until bands were observed.

2.7.4. Western Blotting for the Presence of LecA.

Overnight LB broth cultures of *P. aeruginosa* were washed 3 times in ice cold PBS and the OD_{600nm} adjusted to 1.0 in a total volume of 1 ml. Following centrifugation at 6797.4 rcf, 4°C for 2 minutes, the cell pellets were resuspended in 100 µl 1x SDS sample loading buffer. The samples were sonicated on ice, boiled for ten minutes and then stored at -80°C. LecA standard (Sigma Aldrich) was used as a positive control.

Samples were analysed by SDS-PAGE on precast 12% Bis-Tris gels using the NuPAGE system (Invitrogen) as described in Section 2.5; subsequently, gels were either stained with colloidal Coomassie blue or electophoretically transferred to nitrocellulose membrane in NuPAGE Transfer buffer for 1 hour at 30V. The membrane was stained with Ponceau S (0.5% w/v Ponceau S, 10% v/v acetic acid in H_2O) to visualise protein before blocking at 4°C overnight in PBST containing 5% milk powder. After washing with PBST, the membrane was incubated for one hour at room temperature with rabbit anti-LecA primary antibody (1:1,000 in PBST with 5% w/v milk powder), prepared by Keith Bishop at the University of Nottingham. The membrane was washed again 3 times in PBST before incubation for one hour with goat anti-rabbit HRP-conjugated secondary antibody (Amersham; 1:10,000 in PBST with 5% w/v milk powder). Following 8 wash steps in PBST, bands were visualised on the membrane according to manufacturer's instructions

using enhanced chemiluminescence (ECL) HRP substrate (Pierce), and Hyperfilm ECL (Amersham).

2.7.5. *pqsA-lux* Reporter Assay.

Activity of the *pqsA* promoter was studied using *pqsA-lux* promoter fusions. PAO1-N *pqsA-lux* strains were grown either in 96 well flat bottom microtitre plates (black/clear isoplates, Perkin Elmer Life Sciences) at 37°C, or in culture flasks with shaking at 37°C. Serial readings were taken over time for growth at OD_{600nm} and for bioluminescence (in relative light units, RLU). Readings were taken in both instances using the Infinite 200 microplate reader (Tecan). Where strains were grown in 96 well microtitre plates, overnight cultures were standardised to an OD_{600nm} of 1.0, and diluted 1 in 100 in growth medium. 200µl of culture was added to each well, and serial growth and bioluminescence readings taken at half hourly intervals. Where strains were grown in culture flasks, overnight cultures were standardised to an OD_{600nm} of 0.05 and 200µl samples transferred to a 96 well microtitre plate at 0 hours, 2 hours, and hourly thereafter for growth and bioluminescence readings. Data analysis was carried out using Excel software (Microsoft).

Chapter Three

The Interaction Between *Pseudomonas aeruginosa* and the 37/67 kDa Human Laminin Receptor.

3.1 Overview.

As described in Chapter 1, *P. aeruginosa* must be highly adaptable to the many niches within the human body, whether it is the lung, the urinary tract or the skin. Whichever niche the bacterium colonises during the course of infection, it must overcome a barrage of innate and adaptive immune responses, both cellular and humoral, and to do this it has evolved myriad virulence factors. However, this is not unique to *P. aeruginosa*, since numerous other species are able to behave in an opportunistic manner and produce many virulence factors allowing them to do so. Since conservation of virulence factors between species is evident (for example, in type III secretion systems[233]), understanding the mechanisms of pathogenesis in other infections can give some insight into the pathogenesis of *P. aeruginosa*, and in particular allow the identification of homologous virulence factors.

3.1.1. The Role of Opportunistic Pathogens in Bacterial Meningitis.

Neisseria meningitidis, *Streptococcus pneumoniae* and *Haemophilus influenzae* are microorganisms that are frequently found as commensal bacteria in the human nasopharynx. As with *P. aeruginosa*, these microorganisms do not cause disease in the majority of cases; indeed, up to 10% of the healthy population can carry *N. meningitidis* asymptomatically[234]. Despite this, *N. meningitidis*, *S. pneumoniae* and *H. influenzae* are frequently identified as the most common causes of bacterial meningitis[235], highlighting their ability to behave as opportunists and cause disease. In contrast with *P. aeruginosa*, these pathogens do not cause disease solely in immunocompromised hosts. Research has shown

that, in the majority of cases, bacterial meningitis occurs in otherwise healthy individuals with no underlying medical conditions[235].

N. meningitidis, S. pneumoniae and *H. influenzae* most commonly cause meningitis by invading the intravascular space from the nasopharyngeal mucosa. Following survival and replication in the blood, causing bacteraemia, the microorganisms are able to cross the endothelial blood brain barrier (BBB) to enter the cerebrospinal fluid of the subarachnoid space in the brain. This constitutes entry to the central nervous system, where the bacteria replicate and cause a catastrophic inflammatory response, resulting in the symptoms associated with meningitis[236]. A very limited number of microorganisms are able to cross the BBB by adhesion to and invasion of endothelial cells within the BBB, suggesting that a specific mechanism involving tissue tropism is involved[237].

3.1.2. Bacterial Invasion of the Blood Brain Barrier.

The details surrounding bacterial invasion of BBB endothelial cells are not clearly understood. Each of the pathogens associated with meningitis generally adheres to and invades cells through a distinct mechanism[236]; however, there are some shared features. *N. meningitidis*, *S. pneumoniae* and *H. influenzae* all express phosphorylcholine (ChoP) on their cell surfaces. ChoP can be found either as a component of lipid membranes, as in *S. pneumoniae*, or associated with surface structures; for example, in *N. meningitidis*, ChoP is found on type IV pili, whilst in *H. influenzae* it is part of outer membrane lipooligosaccharides[238–240].

Bacterial ChoP provides another means through which bacteria can abrogate host immunity for their own advantage, since it acts as a bacterial mimic of phosphatidylcholine, a component of host cell plasma membranes. This allows invading bacteria to escape recognition by the immune system[241,242]. However, ChoP is also a mimic of the host chemokine, platelet-activating factor (PAF). PAF is a phospholipid mediator of many host processes that occur both in healthy tissues and

during disease; PAF is thought to be involved in the regulation of immune and inflammatory responses, since it is a potent activator of polymorphonuclear leukocytes, monocytes and macrophages[243].

PAF signalling occurs via the PAF receptor (PAFr) on host cell membranes. PAFr is one of a group of receptors that modulate their own presence on the cell surface in response to stimulation by the cognate ligand, causing subsequent desensitisation of the host cell to the molecule, thus preventing multiple signalling events; this is particularly useful in the case of PAF, which is one of the most potent phospholipid agonists known and is able to mediate hosts processes at very low concentrations[243,244]. This downregulation is thought to occur via receptor endocytosis; upon stimulation of PAFr by PAF, phosphorylation cascades are triggered. β-arrestins are recruited to the cell membrane where they associate with the C-terminus of PAFr, mediating its endocytosis, with its ligand, into clathrin-coated vesicles. Receptors are then either targeted for degradation or dephosphorylated and recycled to the apical cell membrane[245].

However, *S. pneumoniae* has been shown to bind via ChoP to PAFr on host endothelial cells before inducing its own β-arrestin-mediated endocytic uptake. The bacterium is then killed in lysosomes, 'recycled' to the apical membrane, or targeted to the basal membrane by transcytosis and released into the extracellular space, the latter being the process most likely to allow crossing of the BBB into the subarachnoid space[246–248]. Interestingly, invasion of the BBB does not occur in mice deficient in PAFr, which also show improved survival following sepsis, suggesting that the interaction between ChoP and PAFr is an important determinant of pathogenesis[246]. *N. meningitidis* and *H. influenzae* have also been shown to interact with host PAFr during meningeal infection[249,250]. On the other hand, whilst PAFr contributes to invasive *S. pneumoniae* infections in the lungs, it has been shown to have a negligible effect on the outcome of *H. influenzae* pneumonia[251], demonstrating the ways in which pathogenesis can differ between sites of infection.

It has been determined that *P. aeruginosa* also expresses ChoP epitopes on the cell surface. In isolates from acute infections, ChoP levels are high at 22°C but negligible at 37°C. The temperature-dependent variation in ChoP expression may contribute to the ability of *P. aeruginosa* to cause invasive infections, protecting it from immune recognition. Interestingly, some chronic infection isolates have been identified that are able to produce ChoP even at 37°C, suggesting that ChoP may be important in the persistence of infection rather than the establishment of infection. When both acute and chronic ChoP-positive isolates were treated with free PAFr, their ability to adhere to and invade lung epithelial cells was diminished[252]. Van Zoelen *et al.*[253] then showed that *P. aeruginosa* exhibits enhanced invasion and dissemination in PAFr-deficient mice, suggesting that the PAFr-mediated immune response is highly important in control of *P. aeruginosa* infections. That acute isolates are able to produce lower amounts of surface-associated ChoP at 37°C may indeed be a means of evading immune clearance early in infection.

3.1.3. The Role of the 37/67 kDa Human Laminin Receptor in Meningitis.

The use of similar mechanisms of pathogenesis between several different species of phylogenetically unrelated bacteria in meningitis led to a search for further shared virulence factors. Thus, Orihuela *et al.*[232] found that *N. meningitidis, S. pneumoniae* and *H. influenzae* are able to bind the 37 kDa laminin receptor precursor present on the surface of brain endothelial cells within the blood brain barrier, mediating initial adhesion with the host cell.

Each strain binds the laminin receptor via a different surface exposed virulence determinant. *S. pneumoniae* does so using an adhesin, choline binding protein A (CbpA)[254], whilst *N. meningitidis* utilises PilQ, an outer membrane secretin involved in type IV pili biogenesis[255] and PorA, a phase variable outer membrane porin that is also targeted by the host

antibody response[256257]. *H. influenzae* binds via OmpP2, an outer membrane porin and adhesin[258,259].

Identification of these interactions may have implications for the development of broad range therapeutics against meningitis, since binding of the bacteria was inhibited in competitor studies by recombinant soluble laminin receptor or anti-laminin receptor antibody, and deletion of the *cbpA, pilQ, porA* or *ompP2* genes resulted in decreased binding of the strains to recombinant laminin receptor in ELISA. Findings were further reinforced by *in vivo* studies utilising *S. pneumoniae cbpA* mutants in which substitutions had been introduced for two amino acid residues found to be required for laminin receptor binding; mice infected with the *S. pneumoniae cbpA* mutants did not develop meningitis, despite a sustained high bacterial titre in the blood. In contrast, those mice infected with wild type *S. pneumoniae* developed meningitis at much lower levels of bacteraemia[232].

Mahdavi *et al.* (personal communication) hypothesised that prior to *N. meningitidis* invasion, binding of ChoP to PAFr (in combination with the interaction of type IV pili with the host CD46 receptor) mediates initial contact between the bacterium and the BBB[260,261]. Subsequently, retraction of the pili and binding of Opa/Opc adhesins to their respective ligands brings the host cell and pathogen into closer proximity[262]. Host response is induced by bacterial lipooligosaccharide and mediated by PAFr signalling[239,263]. Subsequently, the upregulation of cell surface 37/67 kDa laminin receptor during inflammation allows tighter adhesion to meningococcal PilQ or PorA, thus facilitating invasion. Research towards the understanding of the impact of these interactions in the context of the wider immune response has been enhanced with the finding that the host secretes galectin-3, a carbohydrate-binding lectin specific for β-galactosides within LOS to neutralise it and attract proinflammatory cells to the site of infection[264]; however, *N. meningitidis* and other pathogens are thought to utilise galectin-3 in infection, using it as a 'bridging' factor between the laminin receptor and either bacterial lipid A or membrane-associated LOS[265–267]. Galectin-3 is involved in a

range of biological processes within mammalian cells and tissues, having been found both intracellularly and extracellularly. When located inside the cell, galectin-3 can regulate the events involved in cell proliferation, survival and apoptosis, whilst secretion into the extracellular matrix allows it to play roles in cell adhesion and signalling. Galectin-3 also modulates the immune response to infection and has been shown to be involved in angiogenesis and tumour metastasis[399]. Given its importance in cell and tissue homeostasis, the ability of bacterial pathogens to perhaps utilise galectin-3 in infection is intriguing.

It must be noted the evidence surrounding the aforementioned ability of *N. meningitidis* to bind the host CD46 receptor using type IV pili is conflicting[268,269]. In addition, *N. meningitidis* PilQ is likely to be masked by the cellular polysaccharide capsule and therefore will only be available for interaction with the 37/67 kDa laminin receptor when the bacterium is acapsulate following phase variation of the capsular genes[270]. Despite this, a non-piliated non-capsulate *N. meningitidis pilE* mutant strain is unable to bind endothelial cell surfaces despite intact expression of the *pilQ* gene, suggesting that PilQ alone may not be sufficient for *N. meningitidis* interaction with the 37/67 kDa laminin receptor[271].

3.1.4. The Role of the 37/67 kDa Laminin Receptor in Other Infections.

Intriguingly, the 37/67 kDa laminin receptor is not targeted only by bacteria. The Sindbis virus[272], Venezuelan Equine Encephalitis virus[273], Tick-borne Encephalitis virus[274], Adeno-associated viruses[275] and the Dengue virus[276] have all been shown to target the host laminin receptor in infection, as well as the prion protein PrP[277]. In addition, *Escherichia coli* is able to upregulate the production of the 37/67 kDa laminin receptor and invade host cells in a CNF-dependent manner; CNF, the *E. coli* cytotoxic necrotising factor, is a cell-associated toxin expressed by pathogenic *E. coli* strains that are able to cause meningitis[278].

The broad range of pathogens that utilise the laminin receptor during infection and the fact that the laminin receptor is highly conserved amongst eukaryotes[279] offers the tantalising opportunity to move towards a comprehensive treatment based around abrogation of a common feature of pathogenesis, regardless of pathogen type. Exciting though this is, much more research is required to elucidate the role of the laminin receptor in infections, including detailed study of intracellular signalling events and the impact on the wider system of interfering with the laminin receptor function, which will be discussed in Section 3.1.6.

3.1.5. *P. aeruginosa* Interaction with the 37/67 kDa Laminin Receptor.

The pathogens previously found to interact with the laminin receptor are predominantly neurotropic; however, similarly to *P. aeruginosa, S. pneumoniae, N. meningitidis* and *H. influenzae* also colonise the respiratory tract, and similar interactions with host PAFr have been demonstrated for all[249,251,252,280]. Since the laminin receptor is ubiquitous throughout human tissues, it is important to determine its role in other infections. To this end, Mahdavi *et al.* (personal communication) have assessed the role of the receptor in *P. aeruginosa* infections and have hypothesised that *P. aeruginosa* interacts with the laminin receptor and also with galectin-3, since the mature 67 kDa laminin receptor is able to form heterodimers with galectin-3[281]. Gupta *et al.*[266] also revealed that *P. aeruginosa* is able to bind galectin-3 via LPS during corneal infection.

Pilot experiments shown in Figure 3.1 comparing bacterial binding to the recombinant 37 kDa laminin receptor precursor and galectin-3 indicated that the *P. aeruginosa* PAO1-N and PAO1-L strains, along with the NCTC 12934 strain, were able to bind both the laminin receptor and galectin-3 at levels comparable with the *N. meningitidis* M58 strain; in contrast, *P. aeruginosa* PA14 showed only very low level binding to galectin-3, and no binding to the 37 kDa laminin receptor precursor (J. Mahdavi, personal communication).

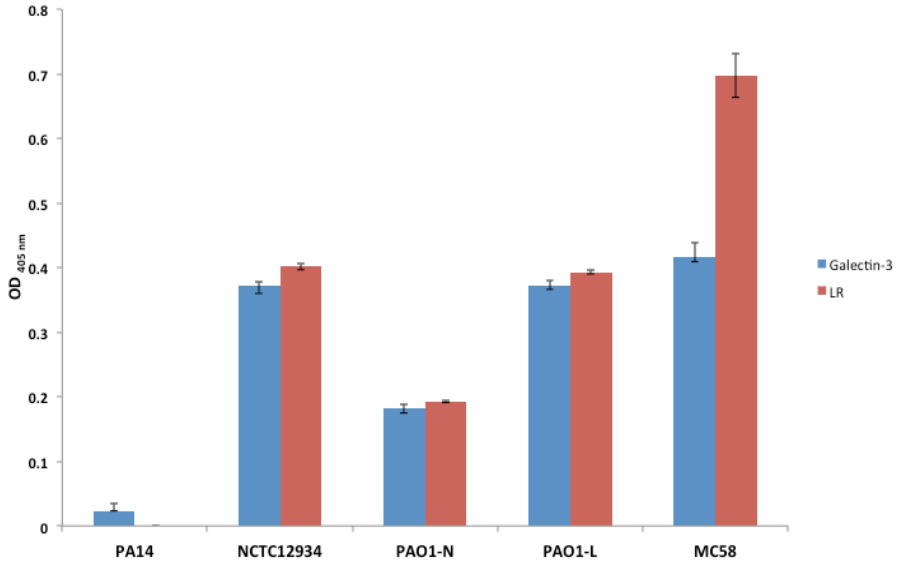


Figure 3.1. *P. aeruginosa* Interactions with the 37 kDa Laminin Receptor Precursor and Galectin-3. Pilot ELISA experiments were carried out by Mahdavi *et al.* (personal communication) to investigate the ability of the *P. aeruginosa* wild type strains PA14, NCTC 12934, PAO1-N and PAO1-L to bind recombinant 37 kDa laminin receptor precursor (LR) and galectin-3 compared with MC58, a *N. meningitidis* strain previously shown to have high affinity for both host determinants[232].

Further experiments suggested the existence of two candidate proteins that could potentially mediate these interactions. These proteins were identified in retagging experiments adapted from Orihuela *et al.*[232], in which the 37 kDa laminin receptor precursor coupled to a sulfo-SBED crosslinker was combined with *P. aeruginosa* NCTC 12934 cells. When *P. aeruginosa* binds the laminin receptor, the crosslinker allows biotin tagging of the ligands, which can then be purified using streptavidin-coated magnetic beads and analysed by LCMS/MS following SDS-PAGE. Proteins matching most strongly with the peptide sequences were investigated for function, and those most likely to be surface exposed *in vivo* were chosen as candidate laminin receptor-binding proteins (Mahdavi *et al.*, personal communication).

The first *P. aeruginosa* candidate protein identified was PilQ. PilQ is encoded by the 2,145 bp *pilQ* gene, the final gene in the *pilMNOPQ* operon. These genes are involved in the biosynthesis of type IV pili, appendages found on the surface of *P. aeruginosa* that are responsible for twitching

motility and adhesion. PilQ itself is a component of a multimeric transmembrane secretin which creates a large pore in the outer membrane through which the pilus extrudes as it is assembled[282,283]. The 77 kDa PilQ monomer is made up of distinct domains. The large C-terminal domain tends to be conserved amongst secretins, and is embedded in the outer membrane; this domain mediates the formation of the ring-shaped homomultimeric complex, creating the outer membrane pore. The C-terminal domain is connected via a linker to the N-terminal domain, which sits in the periplasmic space and is conserved only between secretins performing the same secretory functions. Indeed, the closest homologue of *P. aeruginosa* PilQ is the PilQ protein of *N. meningitidis;* it is proposed that the C-terminal regions would be responsible for mediating interactions with the 37/67 kDa laminin receptor[284].

The second candidate is the outer membrane lipoprotein OprI, encoded by the 252 bp *oprI* gene. This 8 kDa lipoprotein can associate with peptidoglycan, anchoring the outer membrane to the peptidoglycan layer below. OprI has predominantly been studied as an immunogenic vaccine component, since its lipid tail binds to the host TLR molecules and thus induces an immune response[285]. In fusion proteins containing both the *oprI* gene product and a specific antigen, the OprI protein confers an adjuvant effect on the fused antigen, increasing its immunological potency and eliciting a protective immune response[286]. OprI has also been proposed as a *P. aeruginosa* receptor for the host antimicrobial peptide, hRNase 7. This is found in epithelial tissue, and, together with RNase activity, is able to induce localised membrane damage in bacterial cells[287,288]. Furthermore, Wessel *et al.*[289] have identified the OprI-peptidoglycan tethering interaction as a structural feature that regulates the formation of bacterial outer membrane vesicles in response to PQS. Similar to PilQ, OprI is conserved amongst fluorescent pseudomonads, suggesting it plays roles in key beneficial bacterial processes that offset its high immunogenicity[290].

3.1.6. The 37/67 kDa Laminin Receptor.

The 37/67 kDa laminin receptor is encoded by a gene of 888 bp predicted to produce a protein of 32 kDa; however, the gene product is actually 37 kDa in size[291]. It is believed that the initial precursor 37 kDa protein then undergoes modification to become the mature 67 kDa receptor, although the 37 kDa laminin receptor has been identified in plasma membrane fractions alongside the mature isoform[277]. That the mature protein is a modified form of the precursor is supported by the fact that a number of antibodies against the 37 kDa protein also recognise the 67 kDa protein, suggesting that the maturation process includes either homodimerisation or heterodimerisation with galectin-3, and other post-translational modifications, such as acylation. However, the exact mechanism is still unclear[281,292]. Fatehullah *et al.*[293] compared the receptor activities of both the precursor and mature forms, finding that both are able to bind laminin but via different mechanisms, reflecting a conformational change that occurs during maturation.

The 37/67 kDa laminin receptor has myriad roles within human tissues, both in health and disease, which must be considered when contemplating the laminin receptor as a therapeutic target in infections. For example, the 37 kDa precursor has been characterised as having very high similarity to the p40 ribosomal protein, and thus has been postulated to be part of eukaryotic translational machinery[294]. Indeed, phylogenetic analysis by Ardini *et al.*[279] suggested that the 37 kDa precursor was originally a ribosomal protein that acquired the ability to bind laminin over time. Venticinque *et al.*[295] recently demonstrated that the 37/67 kDa laminin receptor colocalises with α-tubulin microtubules, interacting with both tubulin and ribosomes to anchor the translational machinery to the cytoskeleton.

The 37/67 kDa laminin receptor has been assigned further roles. Besides being involved in angiogenesis, the laminin receptor was also thought to be involved in anchoring cells to the extracellular matrix and maintaining tissue architecture; proliferating cells *in vitro* express high

levels of laminin receptor on the cell surface, which is reduced when cells become contact-inhibited, implying a role in tissue repair. The laminin receptor is also able to enhance the susceptibility of laminin to enzymatic degradation during tissue remodelling[296]. However, it has also been implicated in cancer, and there is a positive correlation between increased cell surface expression of the 37/67 kDa laminin receptor and the metastatic potential of cancer cells[281]. This suggests that the 37/67 kDa laminin receptor has more involvement in cellular motility than stabilisation with the extracellular matrix. Venticinque *et al.*[295] showed that when cells were cultured on laminin-coated plates, the rearrangement of cytoskeletal actin filaments induced the formation of lamellipodia, membrane structures that mediate cell motility. Transmembrane laminin receptors also colocalised with the cell membrane termini of the actin filaments, suggesting that the 37/67 kDa laminin receptor directly interacts with both intracellular actin and extracellular laminin to play a role in cellular motility. The 37/67 kDa laminin receptor may also interact with elastin[297].

Laminin itself is a high molecular mass glycoprotein that is found on the cell membrane as a heterotrimer assembled into an asymmetric cross conformation with three short arms and one long arm. These arms are able to bind cells and extracellular matrix components, including other laminin molecules. Laminin, along with other components of the extracellular matrix such as collagen and elastin, acts as a scaffold for tissue development and is distributed throughout tissues dependent upon the stage of development and tissue function. *In vivo*, laminins perform essential functions in influencing cell survival, differentiation, migration and tissue maintenance[281]. Furthermore, production of laminin is upregulated in remodelling airways in response to inflammation[298–300], which might in turn prompt a concomitant increase in 37/67 kDa laminin receptor production.

The mature 37/67 kDa laminin receptor has been postulated to contain a number of different domains which all have different ligand binding specificities, as summarised in Figure 3.2. Three laminin binding

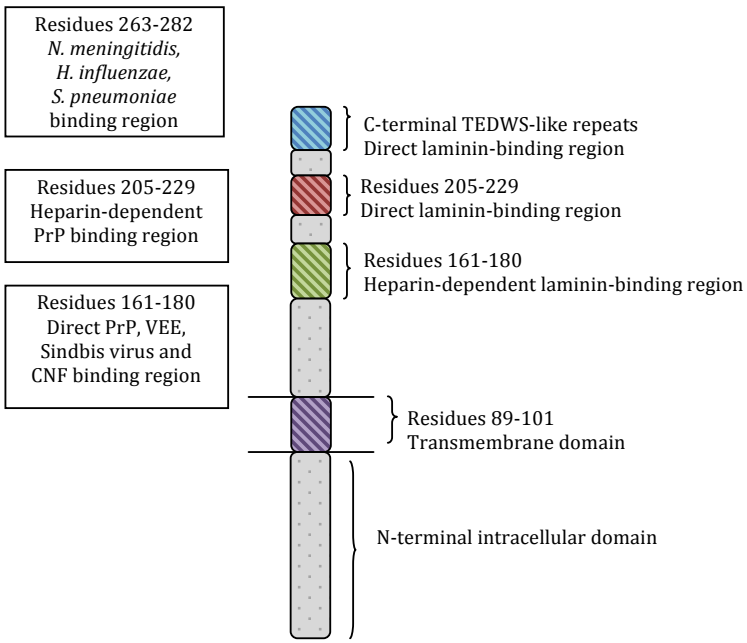


Figure 3.2. The Structure of the 37/67 kDa Laminin Receptor. Following maturation processes and targeting to the plasma membrane, three regions of the laminin receptor are involved in binding laminin, both directly and indirectly; these regions are also believed to play a role in binding a number of pathogens. The intracellular domain is associated with signalling components which regulate cellular activities such as filopodia formation. For simplicity, the receptor is shown as a monomer. Numbers denote the amino acid residues. PrP-Prion protein, VEE-Venezuelan Equine Encephalitis virus, CNF-*E. coli* Cytotoxic Necrotising Factor. Diagram adapted from [397].

sites have been characterised within the 37 kDa laminin receptor precursor, one of which is dependent on heparan sulphate, a component of the host extracellular matrix[301]. The intracellular domain is associated with signalling components that might modulate gene expression and cell motility via signal transduction pathways[302]; Moodley *et al.*[303] revealed that reduction of 37/67 kDa laminin receptor gene expression via siRNA transfection of cancer cells triggered apoptosis, suggesting that the laminin receptor has some involvement in maintaining cell viability. Certainly, previous studies have shown that cell detachment from the extracellular matrix can lead to programmed cell death[304].

Given the conservation and importance of the 37/67 kDa laminin receptor in the host, coupled with the presence of laminin in tissues associated with *P. aeruginosa* infections, the potential interaction of *P. aeruginosa* with this protein and any effects arising from this interaction could have interesting roles in the pathogenesis of this important opportunist.

3.1.7. Aims of this Chapter.

The aim of the work presented in this Chapter was to further investigate the interactions of the host 37/67 kDa laminin receptor and galectin-3 with the *P. aeruginosa* proteins PilQ and OprI. To elucidate the mechanisms through which these interactions occur, as well as the purposes they serve during infection, in-frame deletion mutations in the *pilQ* and *oprI* genes were created in the PAO1-L background and characterised for recombinant 37 kDa laminin receptor precursor and galectin-3 binding properties in both ELISA experiments adapted from Orihuela *et al*.[232] and whole cell-laminin receptor pulldowns. The findings indicate a low level of *P. aeruginosa* interaction with both the laminin receptor and galectin-3 that is likely to be non-specific.

3.2. Results.

3.2.1. Construction of *P. aeruginosa* Δ*oprI*, Δ*pilQ*, and Δ*oprI*Δ*pilQ* Mutants.

To investigate the role of the PilQ and OprI proteins in specific *P. aeruginosa* interactions with the 37 kDa laminin receptor precursor, single in-frame deletion mutants were generated in the *P. aeruginosa* PAO1-L background. According to the pilot experiments by Mahdavi *et al*. (personal communication; see Figure 3.1, Section 3.1.5), the PAO1-L strain displayed the highest levels of binding to the 37/67 kDa laminin receptor and galectin-3 when compared with the PA14 and PAO1-N strains. The *P. aeruginosa* strain NCTC 12934 also showed a level of binding similar to that of PAO1-L; however, the PAO1-L strain was chosen because it is a widely used laboratory strain and reasonably well characterised. The NCTC 12934 strain, despite being a reference *P. aeruginosa* strain, has not been so well characterised and so there is no genome information available.

In-frame mutagenesis of the *pilQ* and *oprI* genes was carried out by deletion of the central region of the genes by PCR, as shown in Figure 3.3. To create an in-frame deletion, 2 sets of primer pairs were designed. The first pair, the upstream forward (UF) and upstream reverse (UR) primers, amplify the upstream region of the gene to be mutated. This creates a PCR product incorporating the flanking sequence upstream of the gene, the start codon, and part of the beginning of the gene, usually around five codons. The second primer pair consists of the downstream forward (DF) and downstream reverse (DR) primers, amplifying a region such that the resulting PCR product includes the final few codons of the target gene, the stop codon, and the flanking sequence downstream of the gene. The primers also allow cloning of restriction sites into the ends of each fragment. The restriction sites in the UR and DF primers must be the same, in order to allow ligation of the two fragments in order to create the

deletion construct; the restriction sites in the UF and DR primers, however, allow ligation of the constructs into cloning vectors in the correct orientation and are usually distinct from each other.

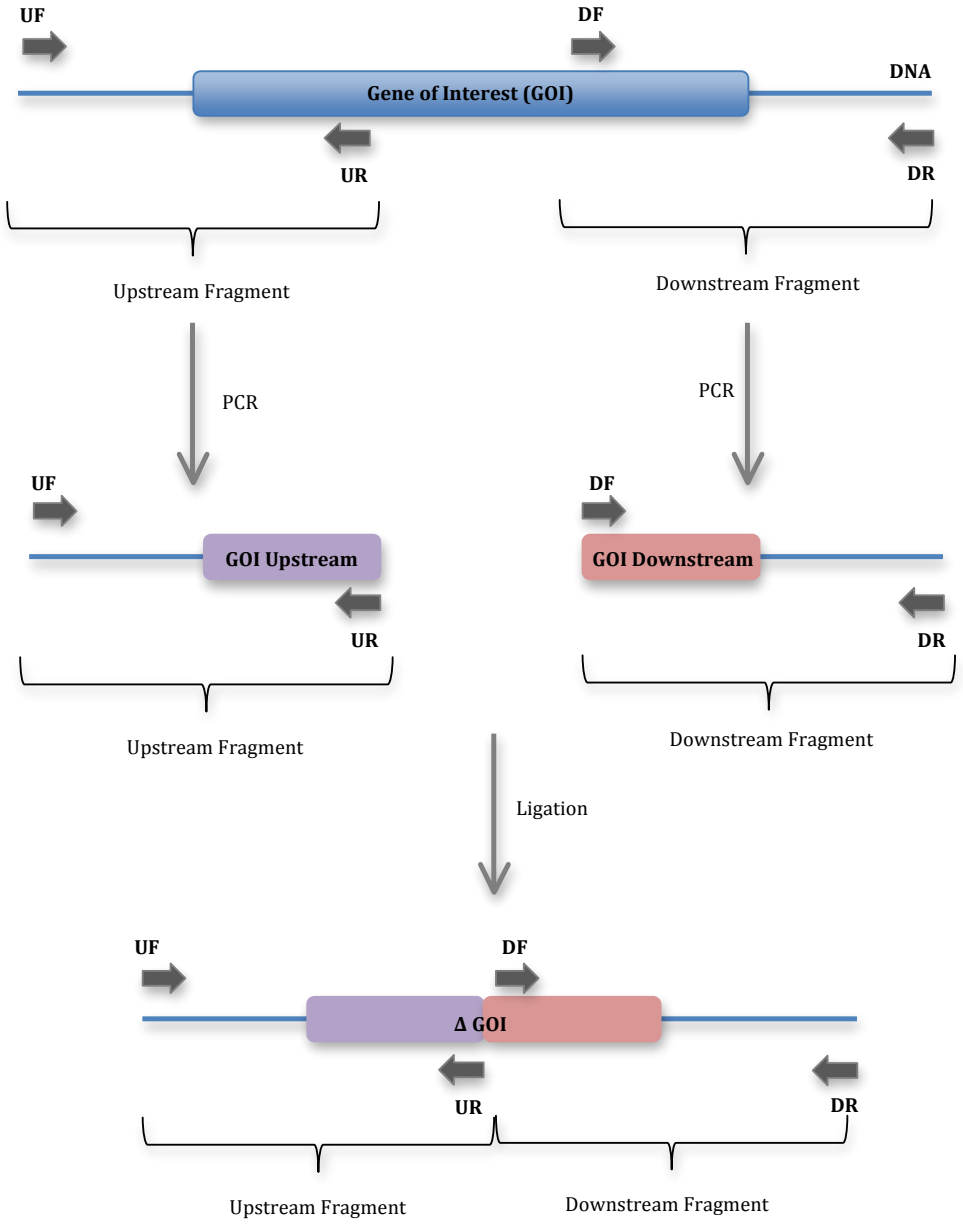


Figure 3.3 Mutagenesis by In-Frame Deletion. PCR products are generated for the upstream and downstream regions of gene of interest and flanking sequences, omitting the central region of the gene. These PCR products are ligated to produce a mutant gene possessing start and stop codons, but carrying an in-frame deletion. UF-upstream forward; UR-upstream reverse; DF-downstream forward; DR-downstream reverse.

Cloning of the Δ*oprI* and Δ*pilQ* constructs (described in detail in Section 2.3.1) was carried out in the pBluescript vector as shown in Figure 3.4. Each of the upstream and downstream fragments were initially cloned into individual vectors before the upstream and downstream fragments for each gene were ligated together within a single vector, creating pRB203 (carrying Δ*pilQ*) and pRB303 (carrying Δ*oprI*).

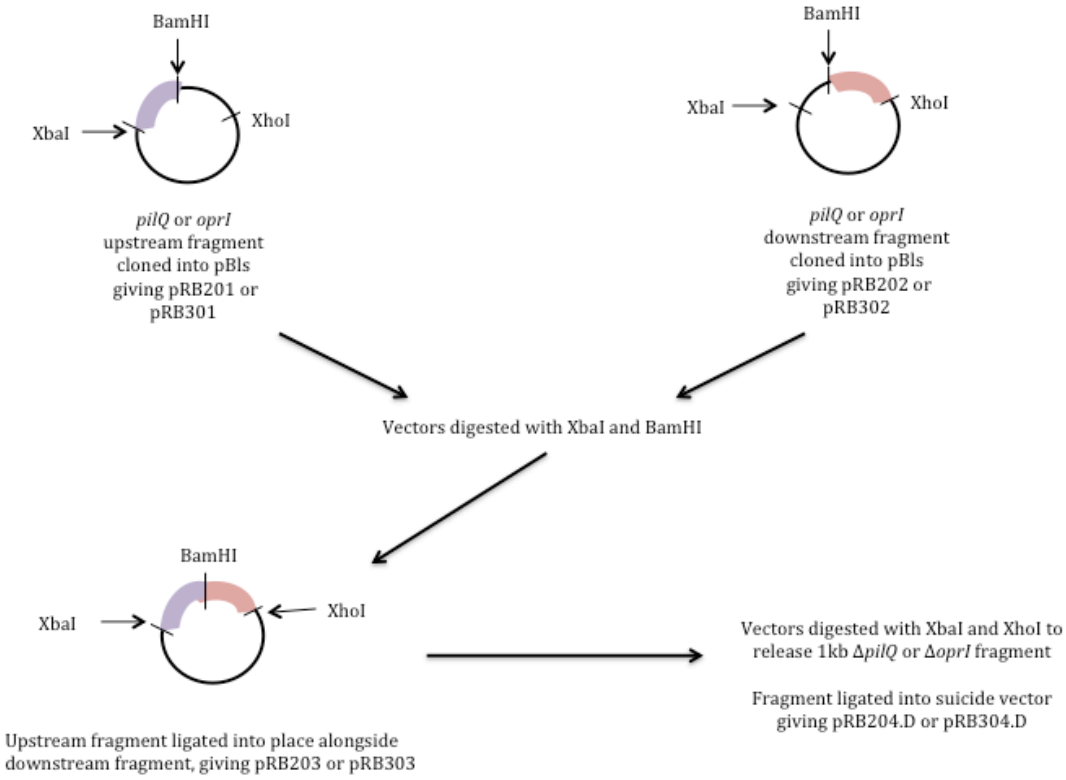


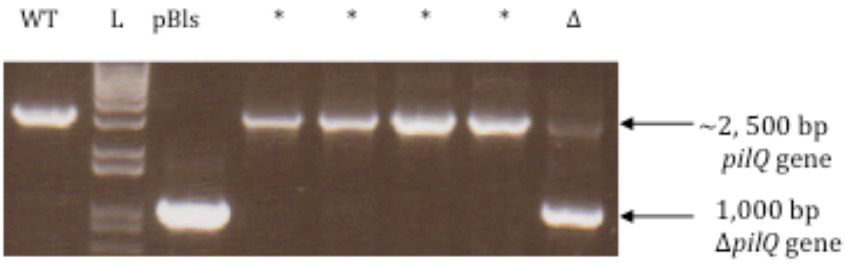
Figure 3.4: Creation of the Δ*oprI* and Δ*pilQ* deletion constructs. In order to complete the construction of the deletion constructs, the upstream fragment of each gene was transferred into the vector carrying the downstream fragment. Both vectors were digested with the same restriction endonucleases to allow generation of complementary overhangs and facilitating correct orientation of the gene fragments. The completed 1 kb in-frame deletion construct was then transferred to the MCS of a suicide plasmid. The arrows indicate sites of digestion by restriction endonucleases.

pRB203 and pRB303 were verified by DNA sequencing using the generic T7 forward and M13 reverse primer sites flanking the MCS within pBluescript. The deletion constructs were then released from their respective vectors by restriction digest, yielding Δ*pilQ* and Δ*oprI* fragments approximately 1 kb in size. These were cloned into pDM4, resulting in the suicide vectors pRB204.D (Δ*pilQ*) and pRB304.D (Δ*oprI*).

The suicide vectors were subsequently transformed into *E. coli* S17-1 λpir to create donor strains that were then used in conjugal mating experiments with the recipient, *P. aeruginosa* PAO1-L. Transconjugants from these mating experiments were selected on chloramphenicol, with nalidixic acid to counter select against the *E. coli* donor strain. *P. aeruginosa* clones carrying the mutant copy of the gene were identified by colony PCR using the primer pairs PilQ.uf/PilQ_ur and OprI.uf/OprI.dr as appropriate (results not shown).

P. aeruginosa colonies in which the first cross-over event between the suicide vector and the chromosome had occurred were then subjected to sucrose counter-selection. The pDM4 suicide vector contains the *sacB* gene, which renders sucrose toxic to any host that retains the plasmid. Thus, in the presence of sucrose, only those clones in which a second homologous recombination event has occurred will persist, since these no longer carry the plasmid.

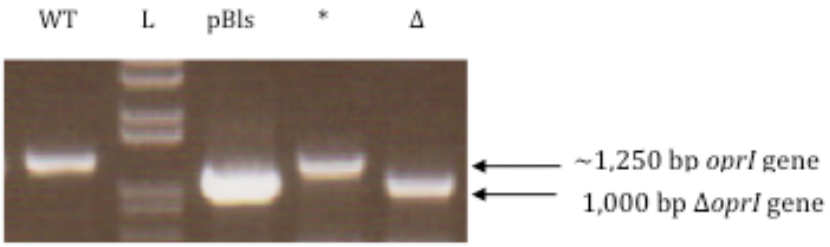
Colonies that grew on LB containing 10% w/v sucrose were replica plated on both LB agar and LB containing chloramphenicol in order to identify those that were sensitive to the antibiotic and therefore confirm that the clone had lost the plasmid, and with it both the *sacB* and *cat* (chloramphenicol acetyltransferase) genes. Chloramphenicol-sensitive colonies were subjected to PCR screening once more to confirm the presence of the Δ*pilQ* and Δ*oprI* genes, replacing the wild type genes. The results of these final screening experiments are shown in Figure 3.5 and Figure 3.6. The genes of interest were amplified by PCR from the genomic DNA of the mutants and verified by DNA sequencing to ensure the in-frame deletion had been successful.



In-Frame Deletion of *pilQ*:

ATG	AAC	AGT	GGC	GGA	TCC	ATC	GGT	CGC	TGA
M	N	S	G	G	S	I	G	R	*

Figure 3.5 Screening of the PAO1-L Δ*pilQ* Strain. The Δ*pilQ* gene is present in one of the screened colonies (Δ), as shown by the shift in the size of the PCR product from approximately 2,500 bp to 1000 bp. Some colonies retained the wild type 1,500 bp gene (*), screening negative for the desired mutation. Due to problems with the intrinsic chloramphenicol resistance of the PAO1-L strain, the use of the pDM4 vector led to the isolation of mixed clones carrying both the wild type and mutant *pilQ* genes, as is evident for Δ, and therefore an uncontaminated ΔpilQ mutant could not be isolated. Genomic DNA from PAO1-L (WT) was screened as a negative control, and pRB204 (pBls::Δ*pilQ;* pBls) was screened as a positive control. L denotes DNA ladder. DNA sequencing and peptide sequence analysis revealed that the ATG start and TGA stop (*) codons are intact in the Δ*pilQ* construct, despite the insertion of the BamHI restriction site, shown in green.



In-Frame Deletion of *oprI:*

ATG	AAC	AAC	GTT	GGA	TCC	AGC	CGC	AAG	TAA
M	N	N	V	G	S	S	R	K	*

Figure 3.6. Screening of the PAO1-L Δ*oprI* Strain. The Δ*oprI* gene is present in one of the screened colonies (Δ), as shown by the shift in the size of the PCR product from approximately 1,250 bp to 1000 bp. Some colonies retained the wild type 250 bp gene, screening negative for the desired mutation (*). Genomic DNA from PAO1-L (WT) was screened as a negative control, and pRB304 (pBls::Δ *oprI*; pBls) was screened as a positive control. L denotes DNA ladder. DNA sequencing and peptide sequence analysis revealed that the ATG start and TAA stop (*) codons are intact in the Δ*oprI* construct, despite the insertion of the BamHI restriction site, shown in green.

A *P. aeruginosa* PAO1-L double mutant was also generated with in-frame deletions in both the *pilQ* gene and the *oprI* gene. In this case, the pDM4 vector carrying the mutant *pilQ* construct was conjugated into the PAO1-L Δ*oprI* background. The gel electrophoresis results confirming the generation of this double mutant are shown in Figure 3.7; the mutant was also confirmed by DNA sequencing (data not shown).

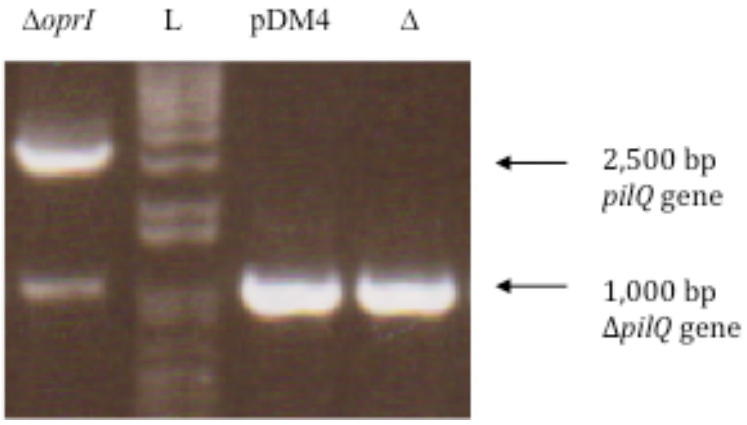


Figure 3.7. Screening of the PAO1-L Δ*oprI*Δ*pilQ* Double Mutant. Following the introduction of the *pilQ* deletion into the PAO1-L Δ*oprI* background, a shift was seen in the size of the *pilQ* PCR product in the mutant colony (Δ) to 1,000 bp, compared with the wild type *pilQ* PCR product (~2,500 bp) in the PAO1-L Δ*oprI* negative control strain (Δ*oprI*). pRB204D (pDM4::Δ*pilQ*; pDM4)was used as a positive control. L denotes DNA ladder.

In practice, creation of the in-frame deletion mutants using the pDM4 vector was problematic due to the high intrinsic resistance of *P. aeruginosa* PAO1-L to chloramphenicol. Due to this, mixed colonies carrying both wild type and mutant genes were obtained, as shown in Figure 3.5. In order to overcome this, the Δ*pilQ* and Δ*oprI* constructs were excised from pRB203 and pRB303 respectively and cloned into to pME3087, a suicide vector carrying a tetracycline resistance marker, to create pRB204.M (Δ*oprI*) and pRB304.M (Δ*pilQ*).

P. aeruginosa transconjugants carrying the pME3087-derived suicide vectors were subjected to three enrichment cycles to select for the second crossover. Cultures were grown with a bacteriostatic concentration of tetracycline to stop cell division in those cells sensitive to tetracycline, before the addition of carbenicillin to kill tetracycline-resistant cells that were still undergoing cell division. Following enrichments, tetracycline-sensitive colonies were screened for the presence of Δ*pilQ* or Δ*oprI* and the results are shown in Figure 3.8. The PAO1–L Δ*oprI*Δ*pilQ* double mutant was then made again as previously described, using the new suicide vector pRB204.M to create an in-frame deletion in the *pilQ* gene within the PAO1-L Δ*oprI* background (data not shown).

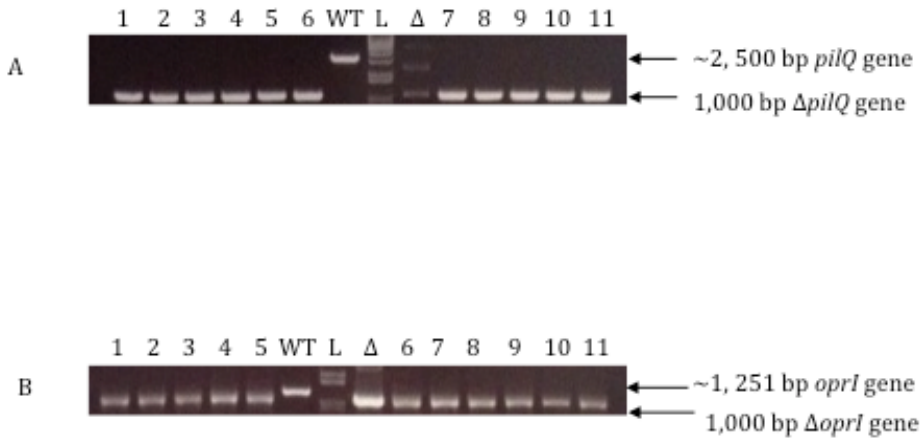


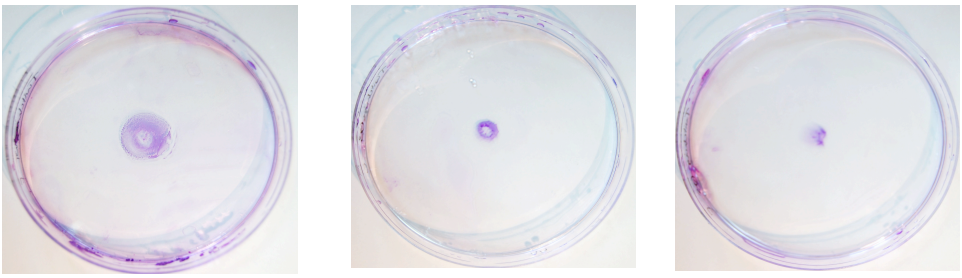
Figure 3.8. Screening of PAO1-L Δ*pilQ* and PAO1-L Δ*oprI* Strains Following Reconstruction Using pME3067. Of 11 colonies screened for each mutant, all showed the presence of either the 1 kb Δ*pilQ* gene (A) or the 1kb Δ*oprI* gene, as shown by the shift in the size of the PCR product from approximately 2,500 bp and 1,251 bp respectively to 1000 bp. Note the cleaner deletions with no wild type band contamination, particularly for the PAO1-L Δ*pilQ* strain. Genomic DNA from PAO1-L (WT) was screened as a negative control, and the suicide vectors pRB204M (pME3087::Δ*pilQ*) and pRB304M (pME3087::Δ*oprI*) were screened as positive controls (Δ) in A and B respectively. L denotes DNA ladder.

3.2.2. The Impact of the Δ*pilQ* Mutation on Motility.

The PAO1-L Δ*pilQ* strain was used in a series of twitching motility assays and compared against wild type (Figure 3.9). As expected, the strain lacking the *pilQ* gene displayed a reduced zone of twitching.

For the wild type strain, the zone measured was 1.4 cm on average, whereas for the Δ*pilQ* mutant this was reduced to an average of 0.7 cm. Type IV pili are most commonly associated with twitching motility, which occurs via pilus extension and retraction through the PilQ outer membrane secretin. Therefore, mutation of the *pilQ* gene reduces the ability of the bacteria to utilise this form of motility effectively, since transport of the pilus components to the cell surface is abrogated[19]. This experiment confirmed the results of the genotypic typing experiments.

A B C



Strain	Diameter of twitching zone (cm)		Average
	1	2	
PAO1-L	1.1	1.7	1.4
PAO1-L Δ*pilQ*	0.8	0.6	0.7

Figure 3.9. Twitching Motility Assays. (A) shows a representative example of wild type *P. aeruginosa* PAO1-L. (B) and (C) denote two repeats (1 and 2) using the PAO1—L Δ*pilQ* strain. As shown in the Table, there is a clear difference of 7 mm between the average diameters of the zones of twitching of the wild type and Δ*pilQ* strains, confirming the mutant phenotype.

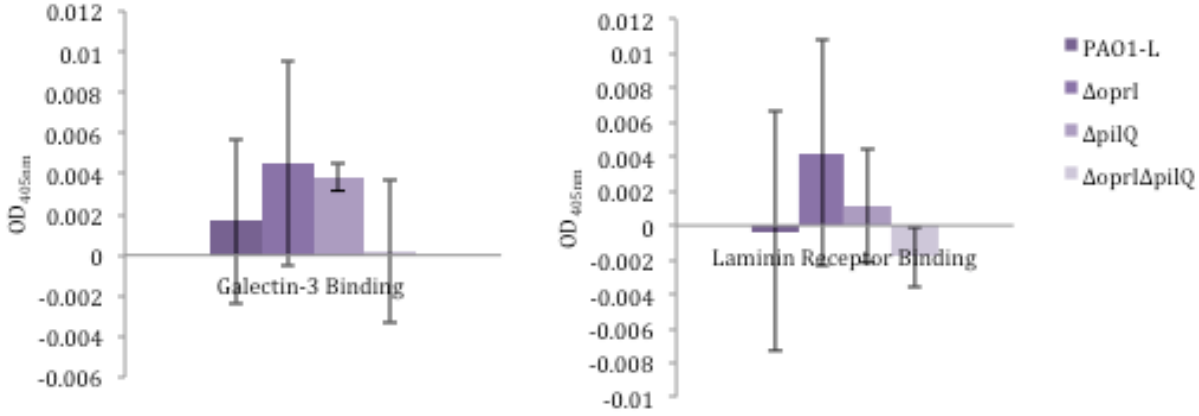
3.2.3. Use of ELISA to Investigate Bacterial Binding to the 37 kDa Laminin Receptor Precursor and Galectin 3.

Since this was a new application, the ELISA experiments adapted from Orihuela *et al.*[232] required optimisation for the *P. aeruginosa* PAO1-L strain. Mahdavi *et al.* (personal communication) used the *P. aeruginosa* reference strain NCTC 12934 in their initial retagging experiments and ELISA assays. Although it was deemed inappropriate for mutagenesis, the wild type NCTC 12934 strain displayed strong binding properties and was therefore included in ELISA experiments for comparison.

96 well plates were coated with either recombinant 37 kDa laminin receptor precursor, or galectin-3 whilst control wells were coated with 1% w/v BSA in PBST. Digoxygenin-labelled bacteria were added to each well and incubated at room temperature before the plate was washed and enzyme-linked anti-digoxygenin antibody was used to detect any bound bacteria. Upon addition of colourimetric substrate, a colour change was observed proportional to the level of bacterial binding in the well. Colour change was observed even in negative control wells coated with 1% w/v BSA, indicating background binding of *P. aeruginosa* to BSA. The OD_{405nm} values for the control wells were therefore subtracted from all values for 37 kDa laminin receptor precursor and galectin-3 coated wells, since 1% w/v BSA had been used as a blocking agent following the coating step. This experimental protocol remained fundamentally the same, although alterations were made throughout to many variables in an effort to optimise the assay and improve reproducibility. However, Figure 3.10 shows that, irrespective of experimental conditions such as the plate coating method, the bacterial optical density used, incubation times and the type of antibody used, binding of *P. aeruginosa* strains to the 37 kDa laminin receptor precursor and galectin-3 remained low.

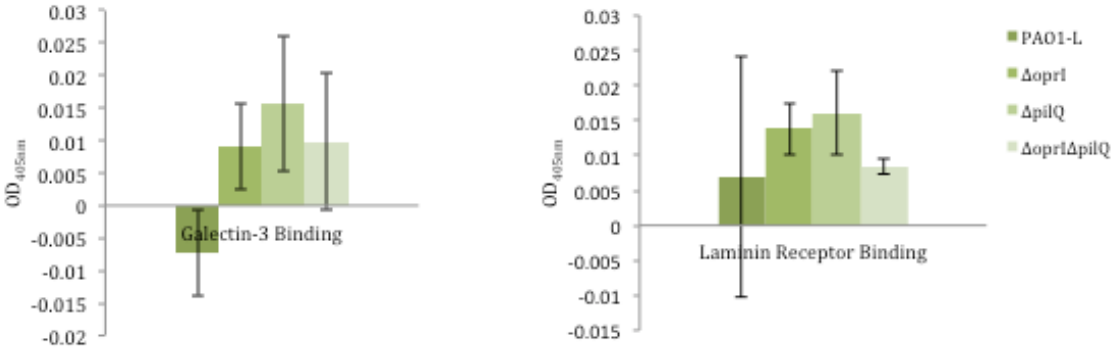
Initially, the experiment was carried out according to the original conditions used in the pilot experiment (Figure 3.1, Mahdavi *et al.*,

Figure 3.10. Results of 37 kDa Laminin Receptor Precursor and Galectin-3 ELISA Experiments. To optimise ELISA for *P. aeruginosa*, repeats with various changes to the protocol (described in individual legends) were carried out. Data were analysed to reduce the effect of background binding of *P. aeruginosa* to BSA by subtracting values for BSA control wells (not shown) from the values for experimental wells. Error bars were applied by calculating the 2x standard error of the optical density data, which was obtained in triplicate. PAO1-L, PA14 and NCTC 12934 are wild type *P. aeruginosa* strains; Δ*oprI* and Δ*pilQ* are the PAO1-L single mutant strains; Δ*oprI*Δ*pilQ* is the PAO1-L double mutant strain. The NCTC 12934 strain was not available when experiments (A) and (B) were carried out, so is not included in those Figures. Rd KW20 is a wild type *H. influenzae* strain. LR denotes 37 kDa laminin receptor precursor; gal-3 denotes galectin -3. Figures are continued on the subsequent pages.



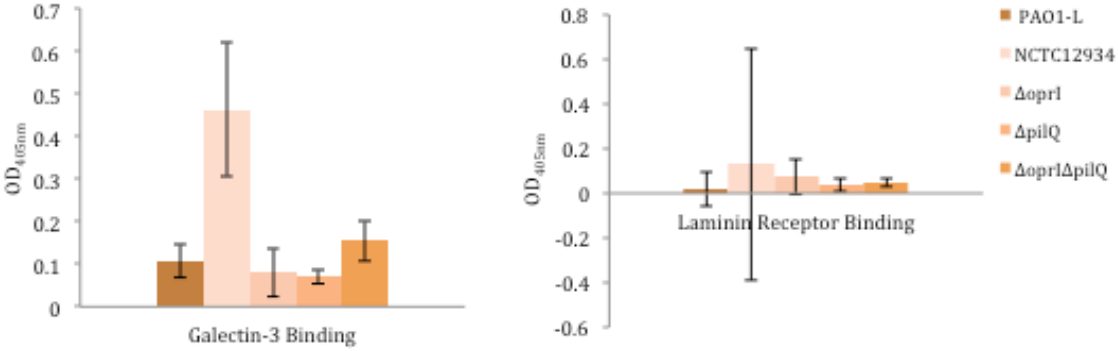
A:

- Coating of wells for 2 h in PBS at room temperature (RT) then overnight (o/n) at 4°C
- Bacterial inoculum OD_{600nm}= 0.02
- Bacteria digoxygenin-labelled in PBS
- Microtitre plate incubated with bacterial inoculum for 2 h RT, then o/n at 4˚C
- Antibody-substrate = HRP/ABTS, colour change observed after 1 h incubation



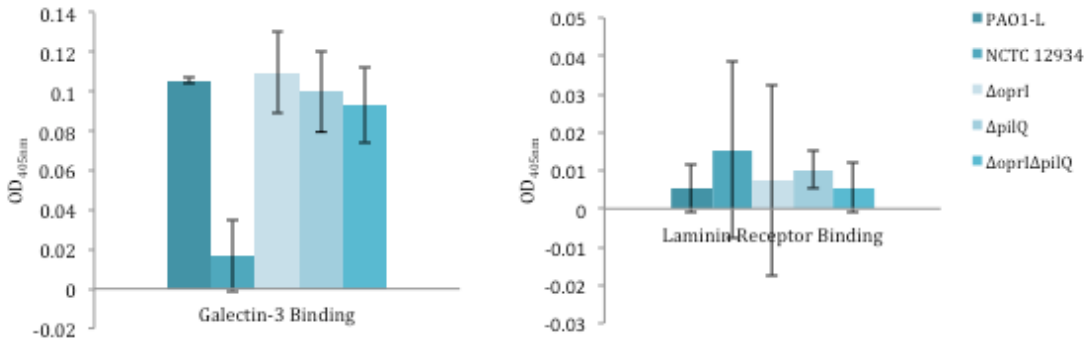
B:

- Coating of wells in PBS, 2 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.05
- Bacteria digoxygenin-labelled in carbonate buffer
- Microtitre plate incubated with bacterial inoculum for 4 h RT
- Antibody-substrate = HRP/ABTS, colour change observed after o/n incubation



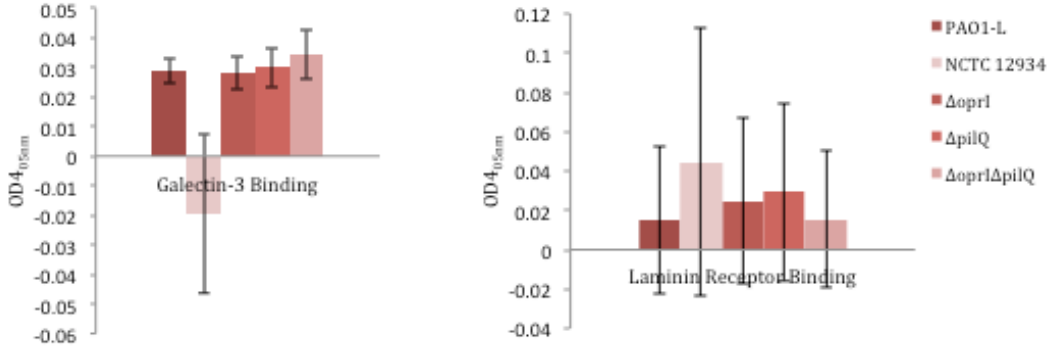
C:

- Coating of wells in PBS, 3 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.05
- Bacteria digoxygenin-labelled in carbonate buffer
- Microtitre plate incubated with bacterial inoculum for 4 h RT, then 4°C o/n
- Antibody-substrate = AP/NBT-BCIP, colour change observed after o/n incubation



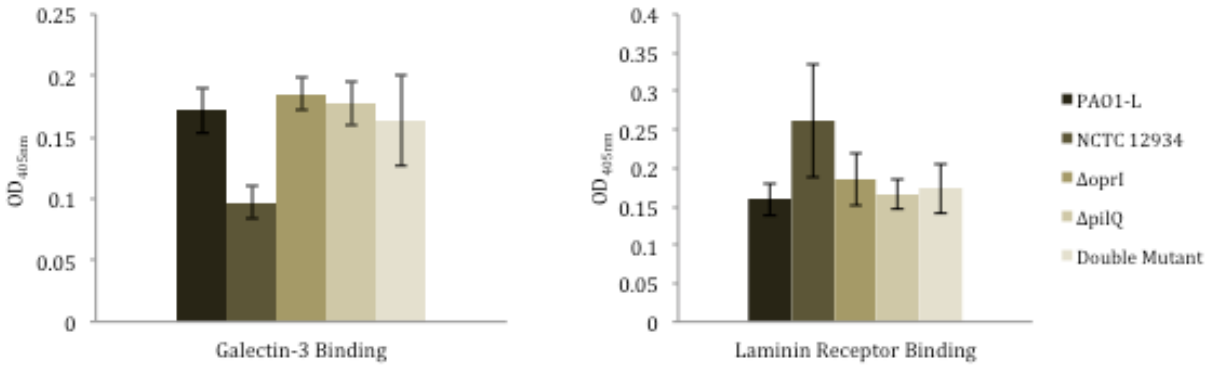
D:

- Coating of wells in PBS, 3 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.03
- Bacteria digoxygenin-labelled in carbonate buffer
- Microtitre plate incubated with bacterial inoculum for 4 h RT, then 4°C o/n
- Antibody-substrate = AP/NBT-BCIP, colour change observed after o/n incubation



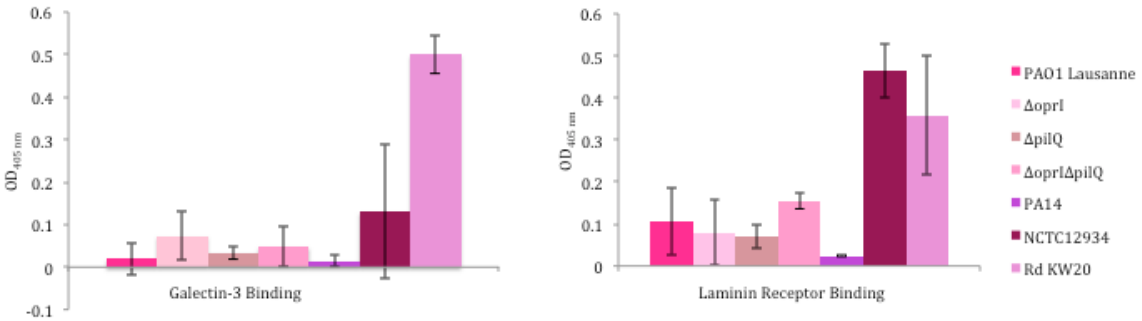
E:

- Coating of wells: Gal-3 in PBS, LR in carbonate buffer, 3 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.02
- Bacteria digoxygenin-labelled in carbonate buffer
- Microtitre plate incubated with bacterial inoculum for 5 h RT, then 4°C o/n
- Antibody-substrate = AP/NBT-BCIP, colour change observed after 48 h incubation



F:

- Coating of wells: Gal-3 in PBS/LR in carbonate buffer, 3 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.02
- Bacteria digoxygenin-labelled in carbonate buffer
- Microtitre plate incubated with bacterial inoculum for 4 h RT, then 4°C o/n
- Antibody-substrate = HRP-ABTS, colour change observed after 15 min incubation



G:

- Coating of wells: Gal-3 in PBS/LR in carbonate buffer, 2 h at room temperature (RT) then o/n 4°C
- Bacterial inoculum OD_{600nm}= 0.02
- Bacteria digoxygenin-labelled in PBS
- Microtitre plate incubated with bacterial inoculum for 2 hours RT, then 4°C o/n
- Antibody-substrate = HRP-ABTS, colour change observed after 30 min incubation

personal communication). However, the levels of binding were extremely low, corresponding to an OD_{405nm} 0.004 at their highest (Figure 3.10(A)).

In order to rectify this, the bacterial inoculum used in the assay was increased to OD_{600nm} 0.05, as was the incubation time following addition of bacteria to the plates (Figure 3.10(B)). As expected, the level of binding increased across all strains tested, but the general trends differed from those observed in Figure 3.10(A).

Subsequently, the experiment was repeated, again with a bacterial inoculum of OD_{600nm} 0.05, as in Figure 3.10(B), but this time the plate was incubated with bacteria overnight (Figure 3.10(C)). Overall levels of binding increased slightly, but tended to be similar across the PAO1-L wild type and mutant strains. The NCTC 12934 strain was also tested in this experiment, showing slightly elevated levels of binding to galectin-3 compared with PAO1-L, a result that contrasts with the pilot experiment, where NCTC 1293 and PAO1-L binding levels for both the 37 kDa laminin receptor precursor and galectin-3 were similar.

With an increased bacterial inoculum and increased incubation time, increased BSA background binding levels were observed as indicated by the intensity of colour change across all wells (data not shown). To ensure this non-specific binding would not interfere with or occlude specific interactions between PilQ or OprI with the target receptors, the bacterial numbers were again reduced to an OD_{600nm} of 0.02, and the incubation time shortened (Figures 3.10(D) and 3.10(E)). The results of these two experiments were similar in terms of trends; however, they differed from previous experiments in the respect that binding of the NCTC 12934 strain was almost non-existent where previously it had been the strain with highest levels of binding to both galectin-3 and the 37 kDa laminin receptor precursor.

The behaviour of the NCTC 12934 strain was verified under the original conditions used in Figure 3.10(A), the only exception being the use of carbonate buffer in the place of PBS during the laminin receptor coating step. The results of this assay (Figure 3.10(F)) showed higher

levels of binding than those seen in (Figure 3.10(G)). However, these levels were still low, and similar across all the strains tested.

Across all of the experiments, binding levels remained very low, and the original pilot experiments (Figure 3.1) were not reproducible, despite many attempts at optimisation. In order to ascertain whether this was due to experimental error or simply due to the binding properties of *P. aeruginosa*, the experiment was repeated using the original conditions in combination with the *H. influenzae* Rd KW20 strain for which specific affinity to the 37 kDa laminin receptor precursor and galectin-3 had already been demonstrated[232]. The results (Figure 3.10(G)) clearly demonstrated specific binding of the *H. influenzae* strain to both the laminin receptor and galectin-3. On the other hand, binding of the *P. aeruginosa* strains, both wild type and mutant, is negligible in comparison, although of similar levels to those seen in Figure 3.10(A). The PA14 strain was also tested in this repeat, and showed even lower levels of binding than the other wild type *P. aeruginosa* strains; this is consistent with the results of the pilot experiment (Figure 3.1).

The low binding levels of *P. aeruginosa* throughout these experiments, coupled with the high standard error across the data strongly suggests that *P. aeruginosa* might interact non-specifically with the 37 kDa laminin receptor precursor and galectin-3, rather than specifically via PilQ or OprI. It is also worth noting that the *P. aeruginosa* NCTC 12934 strain, suggested to have the highest level of binding to the 37 kDa laminin receptor precursor by Mahdavi *et al.* (Figure 3.1), displays high levels of binding even in BSA control wells lacking laminin receptor or galectin-3 (data not shown). During the washing steps of bacterial digoxygenin labelling, the NCTC 12934 strain formed clumps of cells that were difficult to disperse in liquid, whereas the strains of the PAO1-L background did not. This might suggest that NCTC 12934 might adhere differently to the bottom of the wells. However, attempts to construct Δ*oprI* and Δ*pilQ* mutations in the NCTC 12934 background proved unsuccessful because high levels of background resistance to chloramphenicol (when using pDM4) and tetracycline (when using

pME3087) meant that it was not possible to isolate potential mutants following conjugations. This makes it difficult to draw conclusions regarding the behaviour of the strain in the experiment.

3.2.4. Use of Laminin Receptor – Cell Pulldowns to Study Laminin Receptor Binding.

Further experiments were carried out in order to investigate whether the interaction between *P. aeruginosa* and the 37 kDa laminin receptor precursor truly existed. Recombinant laminin receptor was incubated with *P. aeruginosa* cell suspensions, which, following harvest by centrifugation, produced whole cell pellets that were analysed by SDS-PAGE and western blotting.

The laminin receptor-cell pulldown revealed that the pure laminin receptor positive control runs as two polypeptide bands of approximately 44 kDa and 120 kDa as shown in Figures 3.11(A) and 3.11(B). In agreement with Buto *et al*.[292], the 37 kDa laminin receptor precursor bound by *P. aeruginosa* is observed as a 44 kDa band. Although the intensity of 44 kDa bands for the cell pulldown experiments are much fainter than the corresponding band in the positive laminin receptor only control, the wild type PAO1-L and NCTC 12934 strains appear to bind free laminin receptor at slightly higher levels than do the PAO1-L deletion mutants, particularly the Δ*oprI* and Δo*prI*Δ*pilQ* strains (Figure 3.11(B)). The intensities of the bands present at 120 kDa do not differ remarkably across the pulldown samples, confirming that any differences in the 44 kDa bands are unlikely to be due to uneven protein loading. The implication of this is that OprI, and to a lesser extent, PilQ, might be mediating specific interactions between PAO1-L and NCTC 12934 and the 37 kDa laminin receptor precursor; however, this binding is most likely occurring at a very low level and additional mediators may be involved since none of the mutant strains show abolished binding to the 37 kDa laminin receptor precursor.

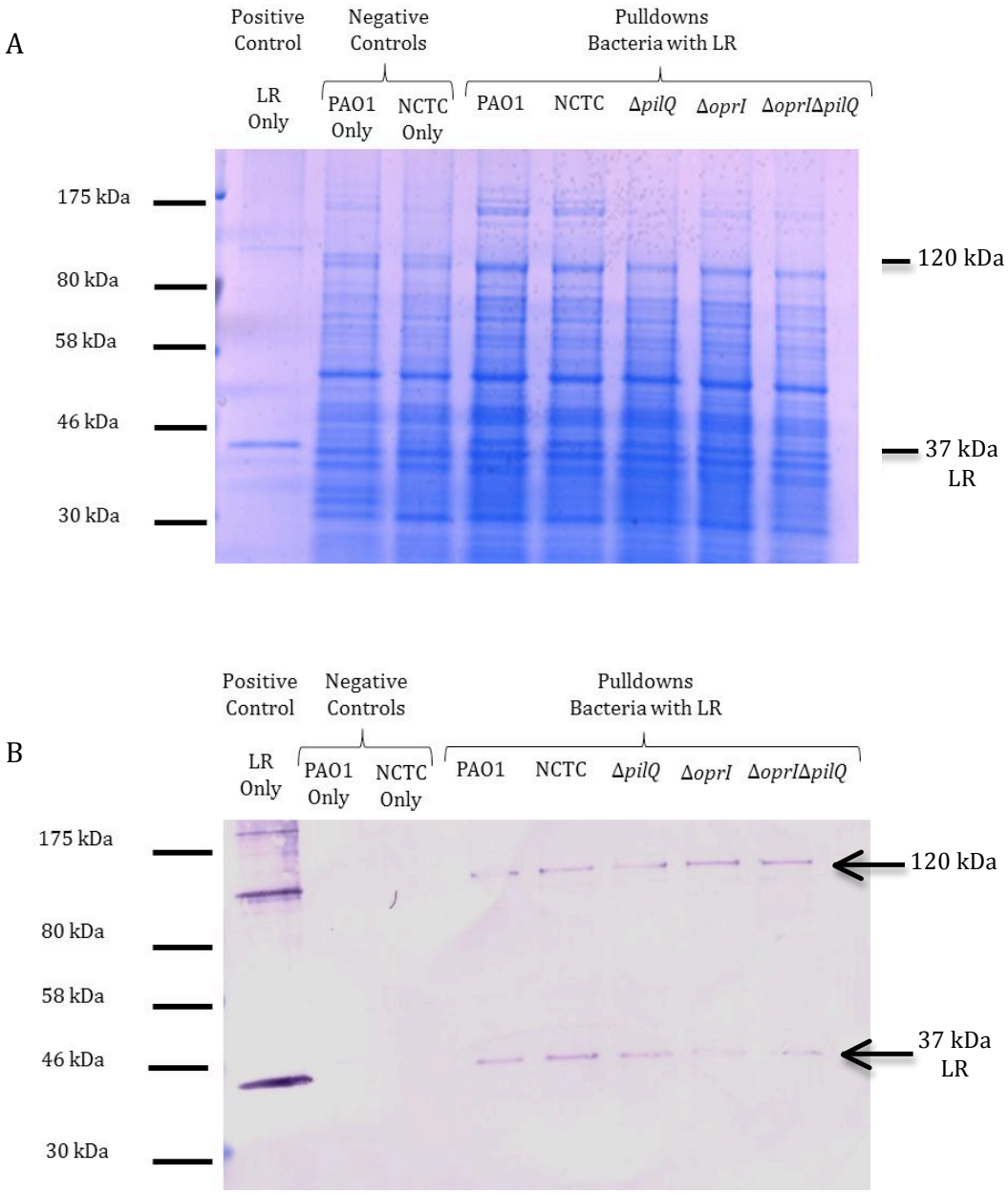


Figure 3.11. 37 kDa Laminin Receptor Precursor–Whole Cell Pulldown Experiments. Wild type PAO1-L (PAO1), wild type NCTC 12934 (NCTC), PAO1—L mutant strains Δ*oprI* and Δ*pilQ* and the Δ*oprI*Δ*pilQ* double mutant were incubated with free laminin receptor (LR) in a pulldown assay to determine whether PilQ and OprI are able to bind LR. Following washing of cells, the cell pellets were sonicated and the cell extracts analysed by SDS-PAGE (A) and western blot (B). Pure 37 kDa laminin receptor precursor was included as a positive control and resulted in 2 bands corresponding to the 37 kDa precursor and an unknown 120 kDa protein (A and B). In (B), low levels of 37 kDa laminin receptor binding were observed for all strains, but binding was slightly reduced in the PAO1-L deletion mutants, particularly those lacking *oprI*. The experiment was repeated twice, with similar results. Representative images are shown.

The detection of a 120 kDa polypeptide band, however is slightly more cryptic. In eukaryotic cells, the 37 kDa laminin receptor undergoes post-translational modification to form the mature 67 kDa laminin receptor, most likely involving either hetero- or homodimerisation. This 67 kDa isoform is able to undergo further modification, possibly involving interaction with galectin-3, to form a 120 kDa isoform. However, the recombinant 37 kDa laminin receptor used in the experiments in this Chapter was purified from *E. coli* carrying a vector expressing the 37/67 kDa laminin receptor gene, and the product obtained does not undergo translational modification in *E coli*; therefore, no other isoforms would be expected to be observed in this assay other than the 37 kDa precursor. Fatehullah *et al.*[293] described the propensity of the 37 kDa protein to form aggregates during expression in *E. coli*, a characteristic that complicates its purification. Therefore, the 120 kDa band may represent such aggregates.

3.3. Discussion.

This work examining the interaction between the 37 kDa laminin receptor precursor and the *P. aeruginosa* proteins OprI and PilQ was based on findings by Orihuela *et al.*[232] that elucidated the interactions between several human pathogens and the laminin receptor via various bacterial proteins, including *N. meningitidis* PilQ. Mahdavi *et al.* (personal communication) found in retagging experiments that *P. aeruginosa* PilQ and OprI were able to bind recombinant 37 kDa laminin receptor, and further hypothesised that since homology exists between *N. meningitidis* PilQ and *P. aeruginosa* PilQ, it is reasonable to assume that the proteins might have a similar function as adhesins during infection. The surface exposed C-terminal region of *N. meningitidis* PilQ has a degree of identity with both *P. aeruginosa* XcpQ and PilQ C-terminal region[282], and PilQ is indeed required for type IV pilus biogenesis in both species. However, despite these similarities, it must be acknowledged that the two species utilise the PilQ protein in different ways. *N. meningitidis* encodes only this one secretin for type IV pilus biogenesis, and produces it at high levels; *P. aeruginosa* on the other hand, encodes at least five secretins[305]. The expression of *P. aeruginosa pilQ* is also dependent on approximately 30 other genes, often in response to environmental cues[306]. *N. meningitidis*, *S. pneumoniae* and *H. influenzae* are very different to *P. aeruginosa*, not least in the mechanisms through which they cause infection and the types of infection they cause at different sites. These differences may offer an explanation as to the outcome of this study. The observation that a non-capsulated, non-piliated *N. meningitidis* strain that is still able to produce PilQ does not interact with endothelial cells is also of significance, since it suggests that the PilQ secretin alone may not be sufficient to mediate interactions with host cells in a physiologically relevant manner[271].

In order to investigate the hypothesis, mutants with in-frame deletions in one or both of the candidate genes, *pilQ* and *oprI*, were constructed in the PAO1-L background. The mutants were confirmed by

DNA sequencing. The genotypic typing for the PAO1-L Δ*pilQ* strain was further confirmed by twitching motility assays. These showed that the strain lacks the twitching phenotype possessed by the wild type strain, as expected; *pilQ* encodes a secretin required for biogenesis of type IV pili in *P. aeruginosa*, therefore the loss of twitching motility in this strain suggests that the product of the Δ*pilQ* gene is either functionally defective or not produced at all, due to the large deletion in the central region of the gene.

Although the PAO1-L Δ*oprI* strain was also genotypically confirmed, it was not possible to confirm the phenotype of the strain. OprI, as an outer membrane lipoprotein, has no easily quantifiable function; although western blot would allow detection, OprI-specific antibodies were not commercially available despite many studies investigating the use of OprI as a component of vaccines (as reviewed in [307]).

The behaviour of the PAO1-L Δ*pilQ*, Δ*oprI* and *ΔoprIΔpilQ* strains was compared with the wild type strains in ELISA and cell pulldown experiments which suggested that *P. aeruginosa* PilQ and OprI do not specifically bind the 37 kDa laminin receptor precursor or galectin-3 at high levels. However, non-specific interactions seem likely according to the ELISA results. Non-specific interactions would not be considered unusual – indeed, they are often a mechanism of adhesion and contribute to bacterial colonisation. Gupta *et al.*[266] observed that *P. aeruginosa* is able to bind galectin-3 via LPS, and this might explain the slightly elevated binding levels the *P. aeruginosa* strains displayed for galectin-3 compared with the 37 kDa laminin receptor precursor in the ELISA experiments. *In vivo*, this characteristic might translate into indirect bacterial interaction with the mature 67 kDa laminin receptor, since it has been postulated to heterodimerise with galectin-3[292].

Furthermore, the ability of *P. aeruginosa* to adhere to plastics has been well documented, since this feature allows colonisation of plastic catheters in the clinical environment, for example[308]. As a result, non-specific binding of *P. aeruginosa* to the bottom of the wells during ELISA

cannot be discounted. In studies examining the interaction of *P. aeruginosa* with mucin, Vishwanath *et al.*[309] coated wells of a microtitre plate with silicone prior to coating the wells with mucin in order to prevent the non-specific binding of bacteria; this could be one way of overcoming the problem.

The use of ELISA with *P. aeruginosa* may not be the best experimental approach due the high capacity of the strains for non-specific interactions with abiotic surfaces. Whole cell pulldowns using free 37 kDa laminin receptor precursor as bait removed this factor and examined the interactions mediated by *P. aeruginosa* PilQ and OprI qualitatively. Interestingly, the results of the cell pulldown suggested that specific interactions between the 37 kDa laminin receptor precursor and the wild type *P. aeruginosa* strains via PilQ and OprI appear to occur, since binding of the laminin receptor precursor by PAO1-L *oprI, pilQ* and *oprIpilQ* deletion mutants is reduced. This reduction is slightly more evident for the *oprI* deletion. Nonetheless, binding remains at a very low level, and may still represent non-specific interaction mediated by positive or negative charges, for example.

Whilst the use of recombinant 37 kDa laminin receptor precursor in the experiments allowed the study of the interaction with *P. aeruginosa in vitro*, the fact that *E. coli* is not able to post-translationally modify the protein means that the role of the mature 67 kDa laminin receptor has not been determined. Although Fatehullah *et al.*[293] showed that the laminin receptor precursor shares some of the same ligands as the mature receptor, it was also shown that interactions with those ligands occurred differently depending upon the laminin receptor maturation state; therefore, the maturation process might either potentiate interactions between the mature 67 kDa laminin receptor and PAO1-L PilQ or OprI, or abolish them completely. The 37 kDa precursor is a cytoplasmic protein, but has also been found within plasma membrane fractions[277], therefore, together with the mature 67 kDa isoform, the 37 kDa laminin receptor is likely to be surface expressed in a modified

form, and it is these modifications that may lead to altered PilQ or OprI affinity for the receptor. Thus, use of the recombinant 37 kDa precursor alone may not recreate the most physiologically relevant conditions, particularly since cell surface receptor interactions can be complex. A better initial approach might be to co-culture the *P. aeruginosa* PAO1-L wild type, PAO1-L Δ*oprI* and PAO1-L Δ*pilQ* strains *in vitro* with mammalian cell lines producing the 37/67 kDa laminin receptor and use immunofluorescence techniques to determine whether *P. aeruginosa* colocalises with the laminin receptor.

The original pilot experiments that revealed high levels of binding for the wild type *P. aeruginosa* PAO1-L and NCTC 12934 strains with both galectin-3 and the 37 kDa laminin receptor precursor could not be reproduced even under conditions identical to those used in the pilot experiment. The reasons for this are unclear. However, the variable levels of binding demonstrated by the NCTC 12934 strain between ELISA experiments suggest that levels of binding may vary from one experiment to the next, perhaps dependent on the extent of the non-specific interactions occurring. The *P. aeruginosa* PA14 strain, however, seems to display the lowest levels of binding to both galectin-3 and the 37 kDa laminin receptor precursor in a reproducible manner. This may be due to the natural mutation present within the *ladS* gene in the PA14 strain, which has downstream affects on the expression of additional genes. This might suggest that the PA14 strain shows reduced expression of a determinant involved in interactions with the 37 kDa laminin receptor and galectin-3.

In all, this study aimed to elucidate the interactions occurring between *P. aeruginosa* proteins PilQ and OprI, and the human 37/67 kDa laminin receptor in order to further understand *P. aeruginosa* virulence and pathogenesis. Tentative assumptions have been made regarding the ability of *P. aeruginosa* to interact with recombinant 37 kDa laminin receptor precursor; however, these interactions occur at a very low level and the idea that they may be non-specific or involve additional bacterial

cell surface determinants cannot be discounted by the work carried out in this Chapter. With regard to galectin-3, the ability of *P. aeruginosa* LPS to bind this host lectin complicated the ELISA experiments, but might also allow *P. aeruginosa* to bind the mature 67 kDa laminin receptor indirectly via galectin-3 *in vivo*. The ability of *P. aeruginosa* to non-specifically adhere to abiotic surfaces was also problematic during ELISA experiments, highlighting the fact that experiments successfully designed for use with one species may not necessarily be successful with another.

Although it is difficult to draw any clear conclusions from the work that has been carried out, it is most likely that if interactions do occur between the 37/67 kDa laminin receptor, they will be complex, and perhaps dependent upon the maturation state of the laminin receptor, the stage of bacterial growth or subject to regulation by quorum sensing, for example. Due to this, a different approach might be required to take into account the complexity of *P. aeruginosa* as a microorganism. The interactions demonstrated in the context of this study suggest the binding affinity of *P. aeruginosa* for the 37 kDa laminin receptor precursor may be too low to be physiologically relevant, implying that the increase in laminin within remodelling tissues - and in turn the possible increase in 37/67 kDa laminin receptor expression on host cell surfaces – is not a pre-requisite for *P. aeruginosa* colonisation following tissue injury.

3.4. Conclusions.

The work carried out in this Chapter aimed to investigate whether *P. aeruginosa* is able to interact with the human 37 kDa laminin receptor precursor and galectin-3 during pathogenesis, via the bacterial outer membrane proteins PilQ and OprI. The techniques used here found that OprI and PilQ are unlikely to use the 37 kDa laminin receptor or galectin-3 as ligands, although very low levels of binding were observed that may represent non-specific interactions. However, the use of ELISA during this work to study *P. aeruginosa* binding characteristics was problematic, due

to the ability of this species to adhere to abiotic surfaces such as plastics, and the use of recombinant 37 kDa laminin receptor precursor rather than the mature 67 kDa isoform may not have resulted in physiologically relevant results. Any future work should take this into account.

Chapter Four

The Regulation of the *Pseudomonas aeruginosa* Galactophilic Lectin, LecA.

4.1. Overview.

In Chapter 1, the ability of *P. aeruginosa* to produce an array of virulence factors during the course of infection was outlined, and the extensive regulatory pathways that determine the expression of these virulence factors were discussed. One such virulence factor, LecA, is an archetypal *P. aeruginosa* quorum sensing-controlled determinant of infection.

4.1.1. The Role of LecA in the Pathogenesis of *P. aeruginosa*.

The importance of LecA in pathogenesis has been well documented. Although it is believed to be predominantly intracellular, LecA is also found in small amounts at the bacterial cell surface. Since it is a multivalent carbohydrate-binding lectin with specificity for terminal galactose residues, its ability to effectively mediate interactions with exopolysaccharides and surface determinants on both bacterial and host cells has made it a prime target for novel therapeutics[27,310]. The wealth of research carried out into the role of LecA in biofilm maturation and the drive to find inhibitors that abrogate the LecA-dependent development of biofilms demonstrates the significance of this virulence factor, particularly in the cystic fibrosis lung, where LecA has been detected in sputum samples. This demonstrates that LecA is produced *in vivo* during infection[26,27,207,208].

Besides its role in biofilms, LecA is also able to bind a specific set of human blood group antigens on host cell surfaces that are distinct from those recognised by LecB; it has been postulated that these functional differences account for the ability of *P. aeruginosa* to infect such a range of different tissues. Binding of LecA to these host determinants can lead to

adhesion to host tissues as well as agglutination of erythrocytes[310,311]. More recently, Kirkeby *et al*.[312] demonstrated that LecA binds terminal galactose residues on host endothelial cell membranes in mouse infection models, suggesting that LecA may be an important factor in bacteraemia during invasive *P. aeruginosa* infection. The cytotoxic properties of LecA have also been described in several studies - the lectin is able to cause defects in airway epithelial barriers, inhibits the growth of respiratory epithelial cells, and abrogates the ciliary beat mechanism of immune clearance in a dose-dependent manner[26,313]. Laughlin *et al.*[314] further demonstrated that in an immunocompromised mouse model of gut-derived sepsis, caecal injection with both LecA and exotoxin A caused similar levels of mortality to that of treatment with intact *P. aeruginosa*. These levels of mortality were markedly higher than those seen following treatment with either LecA or exotoxin A alone. This suggests that the permeability-inducing effects of LecA on epithelial tissues serve to potentiate the effects of other bacterial cytotoxins.

4.1.2. The Quorum Sensing Control of *P. aeruginosa lecA* Expression.

LecA is a tetrameric protein consisting of 12.75 kDa subunits encoded by the 369 bp *lecA* gene[163,315]. It was identified as a quorum sensing-controlled gene by Winzer *et al*.[163], who established that LecA could not be detected by western blot in *P. aeruginosa* cultures prior to stationary phase. Furthermore, LecA production was abolished in a *rhlRI* double mutant unable to produce C4-HSL, whilst in a *rhlI* single mutant it could be restored by the addition of exogenous C4-HSL to the culture. In a *lasR* mutant however, LecA production was merely delayed, reflecting the ability of the *rhl* system to modulate gene expression in a LasR-independent manner as later confirmed by Dekimpe *et al.*[135].

Additional investigations using a bioreporter construct containing the *lecA-luxCDABE* promoter fusion revealed that expression of the *lux* operon could be induced from the *lecA* promoter in an *E. coli* background in the presence of RhlRI, establishing RhlR as a direct regulator of the *lecA*

promoter. This C4-HSL induction led to a dose-dependent increase in *lecA-lux* expression in *E. coli* in a RhlRI-dependent manner[163].

Despite this, in the wild type *P. aeruginosa* background carrying a chromosomal *lecA-lux* reporter fusion, addition of C4-HSL increased the activity of the *lecA* promoter but did not advance it to a time point earlier in growth, as might be expected. This suggested that the *lecA* promoter was under additional regulatory control that acts to preserve the growth phase-dependent expression of the *lecA* gene*,* preventing its induction prior to onset of stationary phase regardless of the levels of autoinducer present in the extracellular environment[163]. Indeed, Whiteley *et al.*[316] also identified a number of quorum sensing-controlled genes that could not be induced early in response to exogenous 3-oxo-C12-HSL and C4-HSL.

Winzer *et al.*[163] subsequently identified a consensus sequence for the stationary phase sigma factor RpoS upstream of the *lecA* promoter, and therefore characterised a *P. aeruginosa rpoS* mutant for *lecA* expression. The *rpoS* gene is expressed only from the onset of stationary phase and controls the expression of numerous virulence factors genes, including *rhlI*[165,317]. The *lecA-lux* promoter fusion was not expressed in the *rpoS* mutant background, thus, whilst the *rhl* system is clearly able to directly regulate *lecA* expression independently of RpoS, RpoS is required to induce the expression of *lecA* upon entry into stationary phase[163]. Given the controversy surrounding whether RpoS regulates RhlI[165], or whether the *rhl* system regulates *rpoS*[127], two hypotheses are possible. RpoS may regulate *lecA* indirectly via *rhl* at the onset of stationary phase, or it could also directly regulate *lecA* expression following activation by RhlR.

4.1.3. Growth Phase-Dependent Expression of the *lecA* Gene.

Given the dependence of *lecA* expression on super-regulation by mediators that could override quorum sensing signals, Diggle *et al.*[118] set out to elucidate the growth phase-dependent regulatory mechanisms that control the quorum sensing-controlled activation of virulence gene expression. After showing that stationary phase transcription of *rhlR,*

rpoS, lecA and *lasB* cannot be advanced by the addition of exogenous 3-oxo-C12-HSL and C4-HSL to wild type *P. aeruginosa* cultures, the *P. aeruginosa lecA-lux* reporter strain was subjected to transposon mutagenesis and *lecA-lux* expression analysed in transposon mutants. A number of mutants showed altered expression of this gene, suggesting that the mutations were within regulators of the *lecA* promoter. The mutations within the *rhlR* and *lasR* genes reduced *lecA-lux* expression, confirming LecA as a quorum sensing-controlled virulence factor. Furthermore, *lecA–lux* expression was also decreased in an *rpoS* mutant, confirming the results of Winzer *et al.*[163], and in PA4341, PA5265 and *mexI* mutants. PA4341 is a predicted regulator, whilst PA5265 is a hypothetical uncharacterised protein. The *mexI* gene encodes a component of the MexGHI-OpmD efflux system[318]. In contrast, *lecA-lux* expression was increased in two mutants; an *mvaT* mutant, and a *clpA* mutant, suggesting that the MvaT and ClpA proteins are involved in the negative regulation of *lecA* expression. ClpA is likely to be a post-translational regulator[319]; indeed, Clp proteases have been shown to post-translationally regulate RpoS in *Pseudomonas putida*[320].

Further experiments revealed that the mutants were altered in the production of additional quorum sensing-controlled virulence factors, such as pyocyanin, elastase, and LasA, and demonstrated that the *rpoS, mvaT, clpA* and *mexI* mutants produced higher levels of 3-oxo-C12-HSL and C4-HSL[118]. This confirmed the role of these gene products in the regulation of quorum sensing.

Interestingly, addition of exogenous 3-oxo-C12-HSL and C4-HSL to cultures allowed the induction of *lecA-lux* expression in the *mvaT* mutant prior to stationary phase, identifying MvaT as an important growth phase-dependent negative regulator of the quorum sensing system[118]. MvaT is believed to be an H-NS-like protein that exists *in vivo* as part of a higher order oligomer[321]; in this conformation, the MvaT protein complex is believed to bind specific DNA sequences in order to block other protein binding sites and silence gene expression[322–324]. It has been shown that MvaT is also able to form heteromultimeric complexes with MvaU, its

partner H-NS-like protein. Together, MvaU and MvaT are able to regulate genes synergistically; it has been shown that deletion of one protein can lead to an increase in the expression of the other. In addition, functional substitution has been observed between MvaT and MvaU[325,326]. MvaT has been implicated in the regulation of other virulence factors, such as *cup* fimbriae, exotoxin A and the MexEF-OprN efflux system, making it an important global regulator of virulence[119,225,327].

4.1.4. The Role of MexGHI-OpmD Efflux System in *lecA* Expression.

The impact of the *mexI* mutation upon *lecA-lux* transcription[118] was interesting, since MexI is an efflux protein functioning as part of the MexGHI-OpmD efflux complex and thus would not be expected to positively regulate *lecA* expression[318]. However, Aendekerk *et al*.[328] discovered that a *P. aeruginosa mexI* mutant and also an *opmD* mutant lacking the cognate outer membrane porin are in fact altered in their abilities to produce other virulence factors, such as elastase, rhamnolipids, and pyocyanin, or the signalling molecules 3-oxo-C12-HSL, C4-HSL or PQS, suggesting that this efflux pump plays a role in the expression of the full virulence phenotype.

Although neither the *opmD* mutant nor the *mexI* mutant were able to produce LecA, production of the lectin could be restored in the mutants by the addition of either exogenous PQS alone or a combination of PQS and C4-HSL, but not C4-HSL alone. The implication of this is that PQS on its own can bypass the effect of the *opmD* and *mexI* mutations on *lecA* expression in *P. aeruginosa* and that this quorum sensing signal molecule is key for the expression of *lecA*. This confirmed previous work by Diggle *et al.*[134], who showed that *lecA* is expressed in a PQS-dependent manner, regardless of the presence of a functional *rhl* system.

Following further investigations, it was determined that the mutations in *mexI* and *opmD* reduced the transcription of the PQS biosynthetic genes and increased the intracellular accumulation of anthranilate leading to bacterial growth defects. The *mexI* and *opmD*

mutants could be rescued from this defect by exogenous PQS, or by deletion of the *phnAB* genes, responsible for anthranilate biosynthesis. It was therefore concluded that in the absence of MexGHI-OpmD efflux pump activity, cytotoxic accumulation of intermediates from anthranilate synthesis may interfere with normal cellular processes, causing the repression of the *pqsA* promoter, and therefore inhibiting PQS biosynthesis[328]. This leads to additional downstream effects on virulence genes, since PQS has been shown to be required for virulence[150]. Indeed, the exogenous addition of PQS overcomes the effects of *mexI* and *opmD* mutation by activating *pqsABCD* expression in a PqsR-dependent manner and resuming conversion of anthranilate into HHQ, the precursor of PQS[328]. Thus, the MexGHI-OpmD efflux pump positively regulates *lecA* expression indirectly by maintaining the homeostasis of intracellular metabolic products.

4.1.5. The PQS-Dependent Expression of *lecA*.

Since the AQ system has been identified as an important determinant of *P. aeruginosa* virulence in numerous models of infection, Diggle *et al*.[134,143] sought to resolve the role of PQS in *lecA* expression. The production of LecA was abolished in *P. aeruginosa pqsA, pqsE* and *pqsR* deletion mutants, highlighting the importance of a functional PQS system even in the presence of functional *rhlRI* and *rpoS* genes. Methyl anthranilate-induced inhibition of PQS synthesis also prevented *lecA-lux* expression in a dose-dependent manner, whilst addition of excess PQS to cultures not only increased *lecA-lux* expression, but also increased production of C4-HSL, RpoS and RhlR. However, a PQS-mediated increase of *lecA-lux* expression was not accompanied by an advancement of expression to exponential phase, meaning that PQS is not able to overcome the growth phase-dependency of the LecA production phenotype[163].

Interestingly, addition of exogenous PQS, with or without C4-HSL, to cultures of *rpoS* and *rhlI* mutant strains was not able to significantly

restore *lecA* expression, demonstrating that LecA production is dependent upon the presence of RpoS, an intact *rhl* system, and an intact PQS system in order to be expressed. Furthermore, *lecA* expression could be induced in a *lasR* mutant by exogenous PQS and C4-HSL acting synergistically to activate the *rhl* system in a LasR-independent manner, and PQS was also able to override the negative regulation of *lecA* caused by *mvaT* and *rsmA* overexpression[118,134,177]. Taken together, these results reveal PQS to be a potent determinant of LecA production.

Given the importance of PqsE in the *P. aeruginosa* response to PQS, and the ability of PqsE to induce virulence factor expression independently of HHQ, PQS and PqsR[134,149,151], Rampioni *et al*.[152] subsequently set out to clarify the regulatory function of PqsE through the transcriptomic analysis of a mutant *pqsA* strain and a *P. aeruginosa* strain carrying an IPTG-inducible conditional mutation in the *pqsE* gene (*pqsEind*). The transcriptomic analysis was carried out at late exponential phase, and decreased *lecA* transcription was observed as expected in a *pqsA* mutant. However, no change in *lecA* transcription was observed in the *pqsEind* strain, regardless of whether *pqsE* expression had been induced by addition of IPTG.

In the same study, Rampioni *et al*.[152] also showed in western blot experiments that LecA is only produced in the presence of PqsE. When *pqsE* expression was not induced in the *pqsEind* strain, no LecA was detected, whilst overexpression of *pqsE* led to the overproduction of LecA at levels greater than were seen for the wild type strain. The western blots were carried out using cultures at early stationary phase of growth, which may account for the differences seen in *lecA* expression compared with the transcriptomic analysis. However, the differences in results may reflect a post-transcriptional role in regulation for PqsE that would not be detected during investigation of the transcriptome.

Interestingly, the transcriptional regulator SoxR was also found to be positively regulated by both PqsA and PqsE[152]. SoxR regulates the expression of the *mexGHI-opmD* operon[329], and the *mexI* and *opmD* genes were also found to be positively regulated by *pqsA* and *pqsE* in the

transcriptomic analysis[152], again demonstrating the way in which homeostasis of intracellular intermediates may be achieved during AQ signalling.

4.1.6. Other Regulators of *lecA* Expression.

The complexity of *lecA* expression increases with the discovery of additional regulators. Besides being subject to negative post-transcriptional regulation by RsmA[177], the *lecA* gene is also subject to indirect regulation via pathways that have not yet been elucidated.

OprF is a non-specific outer membrane porin of *P. aeruginosa*, with roles in maintaining normal cell physiology, antibiotic resistance and adhesion to tissues[330]. Wu *et al*.[331] showed that during infection, the host cytokine IFN-γ is able to bind OprF and induce the transcription of *rhlI* via an unknown pathway, in turn increasing the production of both the C4-HSL signal molecule and LecA. Fito-Boncompte *et al*.[332] investigated this further, showing that OprF is required for *P. aeruginosa* virulence due in part to its role as an adhesin, but also due to its ability to indirectly regulate the expression of virulence factors such as LecA, pyocyanin and elastase. As well as being diminished in virulence factor production, an *oprF* mutant produced lower amounts of 3-oxo-C12-HSL, and the accumulation of C4-HSL later on in growth occurred at a slower rate compared with that in the wild type strain. The *oprF* mutant also displayed an imbalance in AQs; higher concentrations of HHQ were accompanied by a delayed accumulation of PQS. Two hypotheses to explain the effect of the *oprF* mutation on the quorum sensing system were proposed. The loss of OprF from the outer membrane might induce membrane defects that in turn induce stress signals, leading to downstream effects on gene expression. Alternatively, interaction of OprF with the host IFN-γ or other ligands may lead to downstream signalling and induction of quorum sensing-controlled responses during infection. Although the pathway through which OprF-mediated LecA expression occurs has not been elucidated, these findings show how the regulation of

quorum sensing in *P. aeruginosa* is often determined by the extracellular milieu. Accordingly, OprF has also been shown to be required for *P. aeruginosa* biofilm formation under anaerobic conditions, such as those seen in the cystic fibrosis lung[333].

The complex regulation of virulence factors is particularly evident in *P. aeruginosa* within biofilms. AlgU (also known as AlgT) is a sigma factor that has been studied mainly within the context of the mucoid *P. aeruginosa* background[334]. In mucoidy, *algU* is overexpressed due to the loss of negative regulation and a concomitant increase in the transcription of the alginate biosynthetic genes occurs. However, AlgU is also found in wild type *P. aeruginosa* expressing the intact *mucA* gene, although it is sequestered to the inner membrane by MucA and therefore inhibited in its activity as a transcriptional regulator[61,335].

Bazire *et al*.[334] studied the role of AlgU in non-mucoid *P. aeruginosa* and found that *algU* mutants were severely affected in their ability to successfully form robust biofilms. The *algU* mutant biofilms were easily dispersed by shear forces and produced reduced amounts of exopolysaccharide; accordingly, it was determined that mutation of *algU* reduced the synthesis of the Psl polysaccharide. However, the study also identified AlgU as a regulator of *lecA* and *lecB* expression, since the mRNA transcript levels of both genes was decreased in the absence of AlgU. Analysis of the *lecA* promoter sequence revealed that AlgU does not regulate *lecA* expression directly, thus there are likely to be other downstream effectors. However, these results illustrated the convergent regulation of virulence factors involved in biofilm formation such as LecA and Psl, and identified AlgU as a key determinant of biofilm development even in non-mucoid strains.

The global regulator AmpR has also been identified as an indirect regulator of *lecA* expression; however, the regulon of AmpR has been found to include at least 313 genes and its effect on the *lecA* promoter is most likely transduced by additional regulators[336]. Intriguingly, Crabbe *et al.*[337] recently characterised the transcriptome of *P. aeruginosa* under oxygen-limited low shear flow conditions in spaceflight and found that

compared with *P. aeruginosa* grown under ground conditions, spaceflight conditions increased expression of the *lecA* gene, coupled with reduced transcription of the *rhlI* gene. Although *lecA* has been shown to be upregulated with *rhlI* in response to anaerobic conditions during infection as a result of increased quorum sensing system activity[338], the upregulation of *lecA* transcription independent of *rhlI* expression[337] contradicts the majority of research that shows the expression of *lecA* to be dependent on the *rhl* system and C4-HSL, and may indicate alternative pathways through which *lecA* is regulated in response to environmental changes .

As more indirect regulators of *lecA* are identified, the regulatory pathways that govern LecA production become more complex, particularly given the growth phase-dependency of its expression profile. Although *lecA* expression is understood at a general level, few transcriptional regulators that interact directly with the *lecA* promoter have been identified, as shown in Figure 4.1. Clarification of the regulatory events at the *lecA* promoter may lead to better understanding of the intricacies of the quorum sensing controlled and growth phase-dependent manner of LecA production.

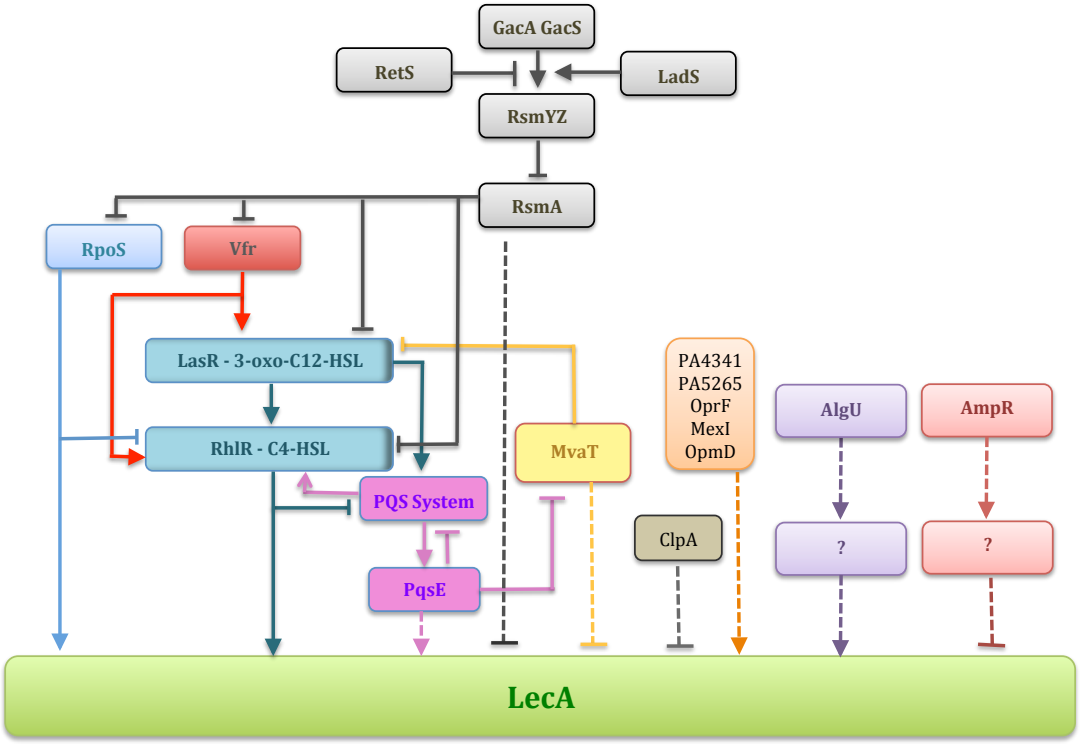


Figure 4.1. The Regulation of *lecA* Expression. Several regulatory pathways converge at the *lecA* promoter. LecA production is RhlR- and RpoS-dependent. These regulators are believed to directly bind the promoter. Both RhlR and RpoS are subject to regulation themselves by the Gac-Rsm, Las and PQS systems. PqsE is also required for *lecA* expression through an unknown pathway. For clarity, the *las, rhl* and PQS systems have been simplified. Direct *lecA* regulation is represented by solid lines. Indirect *lecA* regulation via an unknown mechanism is represented by broken lines. Arrows - Positive regulation; Blunt-ended lines – Negative regulation [118,134,152,163,177,334,336].

4.1.7. Aims of this Chapter.

The aim of the work presented in this Chapter was to identify novel direct regulators of *P. aeruginosa lecA* expression using promoter pulldown techniques. Using the *lecA* promoter region as bait, proteins that were able to bind the DNA sequence were isolated from *P. aeruginosa* cell extracts and identified by LCMS/MS. A number of potential regulatory proteins were chosen as candidates, and a set of *P. aeruginosa* mutant strains constructed. Characterisation of LecA production in these mutants revealed that *lecA* expression is subject to a large number of regulators that have not previously been associated with this virulence factor. This work also reinforces the PqsE-dependent nature of *lecA* expression, since *pqsE* overexpression can overcome the effects of most mutations on LecA production. Furthermore, a mechanism for the indirect regulation of *lecA* expression by AmpR and AlgU is proposed.

4.2. Results.

4.2.1. The *lecA* Promoter Region.

The *lecA* promoter region has previously been mapped by Winzer *et al.*[163], as illustrated in Figure 4.2. The promoter contains two overlapping RpoS consensus binding sequences through which RpoS may directly regulate *lecA* expression, and a 20 bp *lux* box upstream of the ATG start site of the *lecA* gene. *lux* box-type sequences are inverted repeat motifs within promoter regions to which *luxR*-type regulators such as LasR and RhlR are able to bind[339]. In the *lecA* promoter, the *lux* box may

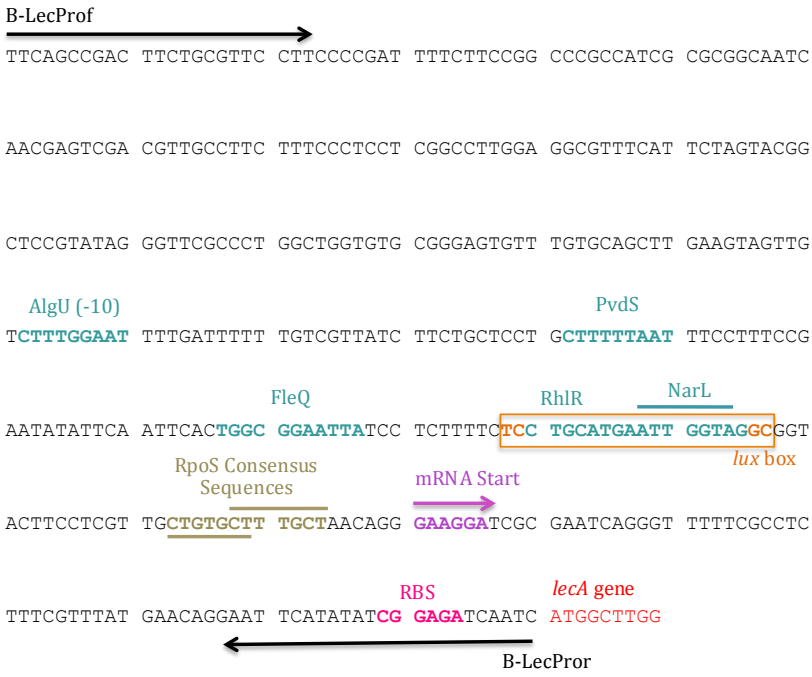


Figure 4.2. The *lecA* Promoter Region. The 400 bp DNA sequence immediately upstream of the *lecA* start codon was amplified using the primers B-LecProf and B-LecPror. The resulting sequence contains the proposed *lecA* promoter, including the ribosomal binding site (RBS), transcriptional start site (mRNA start), and two predicted RpoS consensus sequences, which overlap as indicated. A *lux* box (outlined in orange) is also present, which was confirmed by subsequent analysis to identify additional regulatory motifs. Motifs identified are indicated in green and include those for the binding of NarL and RhlR within the *lux* box and FleQ. A partial consensus sequence was identified for AlgU binding, but the -35 AlgU element was not found. Black arrows show the primer annealing sites; the beginning of the coding sequence of the *lecA* gene is highlighted in red. Adapted from [163,340].

allow the direct regulation of *lecA* expression by RhlR. The transcriptional start site occurs at the -70 position relative to the beginning of the *lecA* gene, whilst a ribosomal binding site (RBS) has also been identified 7 bp upstream of the start codon. The RBS allows initiation of *lecA* mRNA translation.

To study the direct interactions of regulatory proteins with the *lecA* promoter, the 400 bp region immediately upstream of the *lecA* gene was amplified by PCR using the biotinylated primers B-LecProf and B-LecPror. Fragment sizes of 300 bp or more had previously been shown to provide the best results in previous experiments (G. Rampioni, personal communication).

As a preliminary investigation, the 400 bp sequence containing the *lecA* promoter was analysed using the Virtual Footprint promoter analysis tool[340] to identify any potential binding sites of interest (Figure 4.2) by comparison with a database of previously published consensus sequences. Whilst the database did not contain the RpoS consensus sequence, the analysis confirmed the presence of the *lux* box by assigning consensus sequences for the binding of RhlR and LasR. Also identified within the *lecA* promoter was the NarL consensus binding sequence, overlapping the *lux* box. NarL is a transcriptional regulator within the two component system NarXL, which is involved in nitrate sensing, biofilm formation and the regulation of virulence[341]. Consensus sequences were also indicated for the binding of FleQ and the iron starvation sigma factor PvdS[342]. In agreement with Bazire *et al*.[334], the complete AlgU consensus binding sequence was not found in the *lecA* promoter region, although the -10 element was observed. However, the lack of the conserved -35 element and the distance of the sequence from the transcriptional start site means the presence of the -10 element is probably not significant.

4.2.2. Events at the *lecA* Promoter During Peak *lecA* Expression.

Previously, the promoter pulldown technique had been successfully used in our laboratory using cell extracts obtained from

P. aeruginosa overnight cultures. However, in order to use this technique to determine the events occurring at the *lecA* promoter, some optimisation was required.

LecA is produced only at onset of stationary phase[163]. It is assumed then, that many of the protein-DNA interactions responsible for *lecA* expression may only be observed at specific time points in growth, rather than continuously throughout all growth phases.

Accordingly, PAO1-L cytoplasmic cell extracts were initially harvested at the peak of *lecA* expression, OD_{600nm} 1.0. This time point was chosen based on previous work by Winzer *et al.*[163], in which *lecA* expression patterns were determined through western blots and *lecA-lux* reporter experiments. For the promoter pulldown, the 400 bp region upstream of the *lecA* gene (Figure 4.2) was amplified by PCR from PAO1-L genomic DNA using the 5' biotinylated primers B-LecProf and B-LecPror. The promoter fragments were purified and on average, 100 µg of promoter DNA was coupled to streptavidin-coated magnetic beads, which were then mixed with the PAO1-L cell extract. To obtain the final sample containing only promoter-protein complexes, the beads were resuspended in elution buffer containing Triton X-100 detergent. This resulted in denaturation of the proteins bound to the promoter DNA, releasing them into solution. Two controls were included; a DNA-only control, in which promoter-coupled beads were incubated only with lysis buffer, and a cell extract-only control, in which cell extract was incubated with uncoupled magnetic beads.

The proteins that had bound the *lecA* promoter were subsequently resolved by SDS-PAGE (Figure 4.3). Seven polypeptide bands of interest were excised from the gels and sent for LC-MS/MS analysis. This identified within each band a mixture of proteins, including both proteins likely to interact with DNA, and other proteins that would generally have other functions elsewhere in the cell. Table 4.1 shows all the proteins identified in each band, along with their relative abundance values based on the normalised unweighted LCMS/MS spectra for the proteins within a specific protein band; a protein with a higher value was assumed to be

more abundant within the band than one with a lower value. Each distinct band identifiable on the SDS-PAGE gels is referred to as a single band for the purposes of this study. However, it should be noted that a single polypeptide band might actually be made up of multiple fainter polypeptide bands. Clearly, a lot of proteins tended to bind non-specifically to the *lecA* promoter DNA, and it is relatively easy to identify these. For example, many biosynthetic proteins such as succinate dehydrogenase, SdhB in band 4 or outer membrane proteins such as OprH, OprG and OprQ, found in bands 1, 2 and 5 respectively would not be expected to bind DNA in an intact cell (Table 4.1). Furthermore, there were also a number of nucleotide-binding proteins that belong to the general transcriptional and translational machinery that would not necessarily be involved in the transcriptional regulation of a specific gene. Of the 132 proteins identified (including numerous 30S and 50S ribosomal proteins not elaborated on in Table 4.1), 14 proteins of interest were identified. Amongst these was RhlR, a known transcriptional regulator involved in quorum sensing. Since *lecA* expression is directly dependent on RhlR[163], the presence of this protein in the pulldown serves to validate the technique and also suggests RhlR regulates the *lecA* promoter by binding to it directly. In band 2, MvaT was identified. This negative regulator has also been shown to have a role in regulating growth phase dependent *lecA* expression[118], again validating the technique; however, RpoS was not found in this experiment. Taken at face value, this might suggest that RpoS regulates *lecA* expression indirectly via other mediators or that this regulator was sequestered by other regulatory proteins at the time point under study. It may also reflect the detection limit of this assay. In band 3, Vfr and RoxR, both characterised transcriptional regulators, were present.

The protein sequencing results also revealed a number of uncharacterised proteins. Amongst these is PA3697, which has recently been shown by Koskenkorva *et al*.[226] to be involved in the regulation of nitric oxide sensing and detoxification.

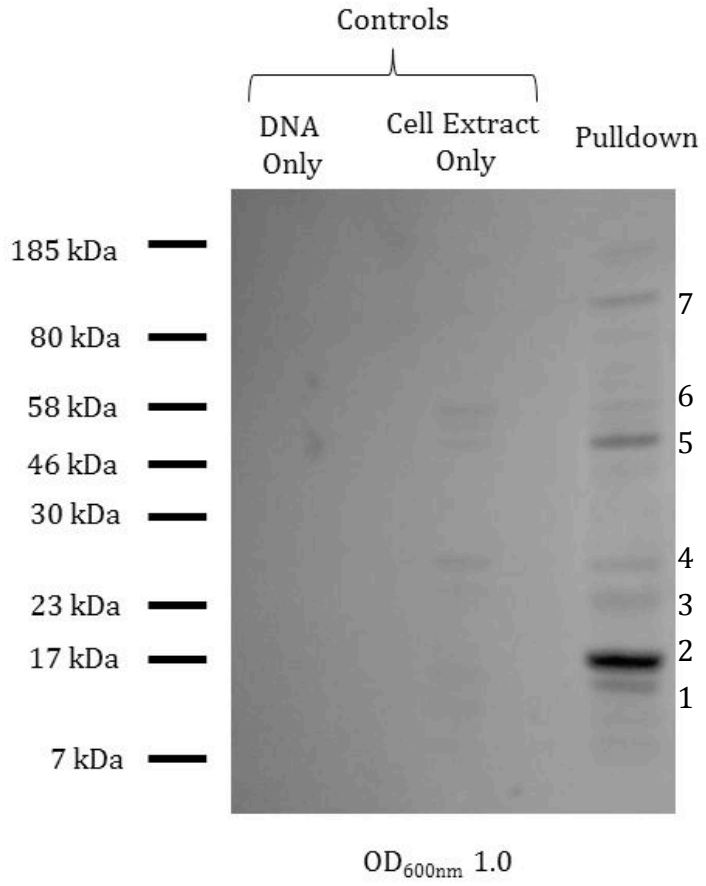


Figure 4.3: The *lecA* Promoter Pulldown. PAO1-L cell extract harvested at OD_{600nm} 1.0 was used in promoter pulldown experiments to identify proteins at this timepoint that bound to the promoter. Controls were carried out with either *lecA* promoter DNA only, or cell extract only. Protein samples were resolved on 4-12% Bis-Tris gradient gels and individual bands excised. The experiment was repeated three times to ensure reproducibility of the band migration pattern, and a representative gel is shown. 'Pulldown' denotes the *lecA* promoter pulldown experiment. 1 - 7 indicates the bands that were analysed by LCMS/MS to identify the protein content. These numbers correspond to those for the bands in Table 4.1.

Table 4.1. LCMS/MS Analysis of OD_{600nm} 1.0 *lecA* Promoter Pulldown Bands. Bands 1-7 were excised from the SDS-PAGE gel as shown in Figure 4.3, and the protein content of each band determined by LCMS/MS analysis. A mixture of proteins was identified in each band, and included a mixture of characterised and uncharacterised proteins. Each protein was assigned a relative abundance value according to its unweighted spectrum count. Proteins of interest that may bind the *lecA* promoter specifically are highlighted in red, along with proteins of uncharacterised function. LCMS/MS analysis of the bands was carried out only once per band. The Table is continued on subsequent pages.

Band	Band Size	Protein	Relative Abundance	Function
1	14 kDa	**MvaU**	**17**	**Transcriptional regulator**
		Ribosomal proteins	From 8 to 3	Protein synthesis machinery
		OprH	3	Outer membrane protein
		PA1053	3	Putative lipoprotein
2	16 kDa	**MvaT**	**36**	**Transcriptional regulator**
		Ribosomal proteins	From 8 to 3	Protein synthesis machinery
		Ndk	6	Nucleoside disphosphate kinase
		PscR	5	Bacteriohemerythrin
		Dps	5	DNA-binding stress protein
		PilA	4	Fimbrial protein
		OprG	3	Outer membrane protein
		Fur	**3**	**Ferric uptake regulator**
3	23 kDa	InfC	21	Translation initiation factor 3
		Ssb	15	Single stranded DNA-binding protein
		Ribosomal proteins	From 14 to 3	Protein synthesis machinery
		AhpC	12	Alkyl hydroperoxide reductase subunit
		OprH	6	Outer membrane protein
		Vfr	**6**	**Transcriptional regulator**
		PA2575	5	Putative nitroreductase
		Frr	5	Ribosome recycling factor
		PasP	4	Secreted protease

Band	Band Size	Protein	Relative Abundance	Function
3 (cont)	23 kDa	**PA4473**	**4**	**Uncharacterised protein**
		AccB	4	Biotin carboxyl carrier protein
		PA1913	**3**	**Uncharacterised protein**
		RoxR	**3**	**Transcriptional regulator**
4	25 kDa	Ribosomal proteins	From 19 to 3	Protein synthesis machinery
		PA0937	**12**	**Uncharacterised protein**
		Adk	7	Adenylate kinase
		PA4010	6	Putative 3-methyladenine DNA glycosylase
		PdxH	5	Pyridoxine/pyridoxamine 5'-phosphate oxidase
		AotJ	4	Arginine/Ornithine binding protein
		SdhB	4	Succinate dehydrogenase
		RhlR	**4**	**Transcriptional regulator**
		HisA	4	Phosphoribosylformimino-5-aminoimidazole carboxamide
		PA4429	3	Probable cytochrome C1
		PA0943	**3**	**Uncharacterised protein**
		PA2441	**3**	**Uncharacterised protein**
		SpeA	3	Arginine decarboxylase
		PA2990	3	Probable phosphodiesterase
5	50 kDa	TufA	30	Elongation factor Tu
		PhaF	**28**	**Polyhydroxyalkanoate granule-associated protein**
		Icd	13	Isocitrate dehydrogenase
		PA3697	**12**	**Predicted transcriptional regulator**
		GltA	8	Citrate synthase
		PA5046	8	Malic enzyme
		DnaN	8	DNA polymerase

Band	Band Size	Protein	Relative Abundance	Function
5 (cont)	50 kDa	OprD	6	Outer membrane protein
		FtsA	6	Cell division protein
		GabT	6	4-Aminobutyrate aminotransferase
		MetK	6	S-adenosylmethionine synthase
		SucB	5	Dihydrolipoamide succinyl transferase
		IscS	5	Cysteine desulphurase
		HemL	3	Glutamate-1-semialdehyde 2,1-aminomutase
		PA4131	3	Probable iron-sulphur protein
		PrfB	3	Peptide chain release factor 2
		GlyA2	3	Serine hydroxymethyltransferase 3
		DnaJ	3	Chaperone protein
		SerA	3	D-3-phosphoglycerate dehydrogenase
		FabF1	3	3-oxoacyl acyl carrier protein synthase 2
		OprQ	3	Outer membrane protein
		MurD	3	UDP-N-acetylmuramylalanine-D-glutamate ligase
		MurA	3	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
		Pfm	3	Proton motive force protein
6	58 kDa	PepA	22	Leucine aminopetidase
		FliC	20	Flagellin type B
		IpdG	13	Dihydrolipoamide dehydrogenase
		ArcA	12	Arginine deaminase
		AtpD	11	ATP synthase subunit β
		Tig	11	Trigger factor
		AtpA	8	ATP synthase subunit α
		PA3001	8	Probable glyceraldehyde-3-phosphate dehydrogenase

Band	Band Size	Protein	Relative Abundance	Function
6 (cont)	58 kDa	GuaB	6	Inosine-5'-monophosphate dehydrogenase
		PA3455	**5**	**Uncharacterised protein**
		HsiC2	5	Component of type VI secretion system
		Eno	4	Enolase
		NrdB	4	Ribonucleoside disphosphate reductase subunit β
		SahH	4	Adenosyl homocysteinase
		FumC2	4	Fumarate hydratase class II 2
		PA3047	4	Probable D-alanyl-D-alanine carboxypeptidase
		WpbA	4	UDP-N-acetyl-D-glucosamine 6-dehydrogenase
		PA0421	4	Uncharacterised protein
		BetB	3	Betaine aldehyde dehydrogenase
		Acc	3	Biotin carboxylase
		Rho	3	Transcription termination factor
7	100 kDa	TopA	88	DNA topoisomerase
		AceE	24	Pyruvate dehydrogenase E1 subunit
		PA0459	23	Probable ClpA/B protease ATP-binding subunit
		InfB	16	Translation initiation factor 2
		AcnB	14	Aconitate hydratase 2
		NrdA	14	Ribonucleoside diphosphate reductase
		AcnA	13	Aconitate hydratase 1
		FusA	12	Translation elongation factor G1
		SucA	8	2-oxoglutarate dehydrogenase, E1 subunit
		Rnr	7	Ribonuclease R
		GyrA	7	DNA gyrase subunit A
		PpsA	7	Phosphoenolpyruvate synthase

Band	Band Size	Protein	Relative Abundance	Function
7 (cont)	100 kDa	MutS	6	DNA mismatch repair protein
		Lon	6	Protease
		SecA	6	Protein translocase
		ClpB	5	Chaperone protein
		IleS	5	Isoleucine tRNA ligase
		PA2735	5	Probable restriction modification protein
		AlaS	4	Alanine tRNA ligase
		LeuS	3	Leucine tRNA ligase
		PheT	3	Phenylalanine tRNA ligase β subunit

4.2.3. The Protein Profile at the *lecA* Promoter Over Time.

To investigate further the regulatory events taking place at the *lecA* promoter, further pulldown experiments were carried out with this promoter using cell lysates prepared at different points in growth with a view to identifying proteins binding to it in a growth phase-dependent manner, and also to validate the reproducibility of the results shown in Figure 4.3 and Table 4.1. Cell extracts were therefore harvested at OD_{600nm} 0.8 (mid exponential phase), 1.0 (entry to stationary phase) and 2.0 (late stationary phase) and used in pulldown experiments with the *lecA* promoter. The resulting protein samples were resolved by SDS-PAGE and the bands analysed by LCMS/MS. The results are shown in Figure 4.4 and Table 4.2 respectively.

A large number of proteins were identified within the bands shown in Figure 4.4, most of which were deemed to be binding the *lecA* promoter non-specifically. For clarity, Table 4.2 shows only those proteins that were deemed proteins of interest, likely to have specific DNA binding or regulatory functions based on information given by the *Pseudomonas* Community Annotation Project (PseudoCap)[230]; also included are hitherto uncharacterised proteins and those with predicted functions.

Comparison of *lecA* promoter binding proteins identified in cell extracts harvested at OD_{600nm} 1.0 as shown in Figures 4.3 and 4.4(B), and Tables 4.1 and 4.2(B) revealed that there is some variation in both band distribution and the proteins identified. An increased number of polypeptide bands are evident using extracts harvested at OD_{600nm} 1.0 in Figure 4.4(B) compared with Figure 4.3, although the intensity of band 2 at this time point in each gel suggests that the protein concentrations of the two samples was similar. With regard to the protein sequencing results, the only proteins common to both *lecA* pulldowns at OD_{600nm} 1.0 were MvaU, MvaT and PA0937 (Tables 4.1 and 4.2(B)).

However, when comparing the LCMS/MS results across the three time points in Table 4.2, some patterns emerge. PhaF, although no

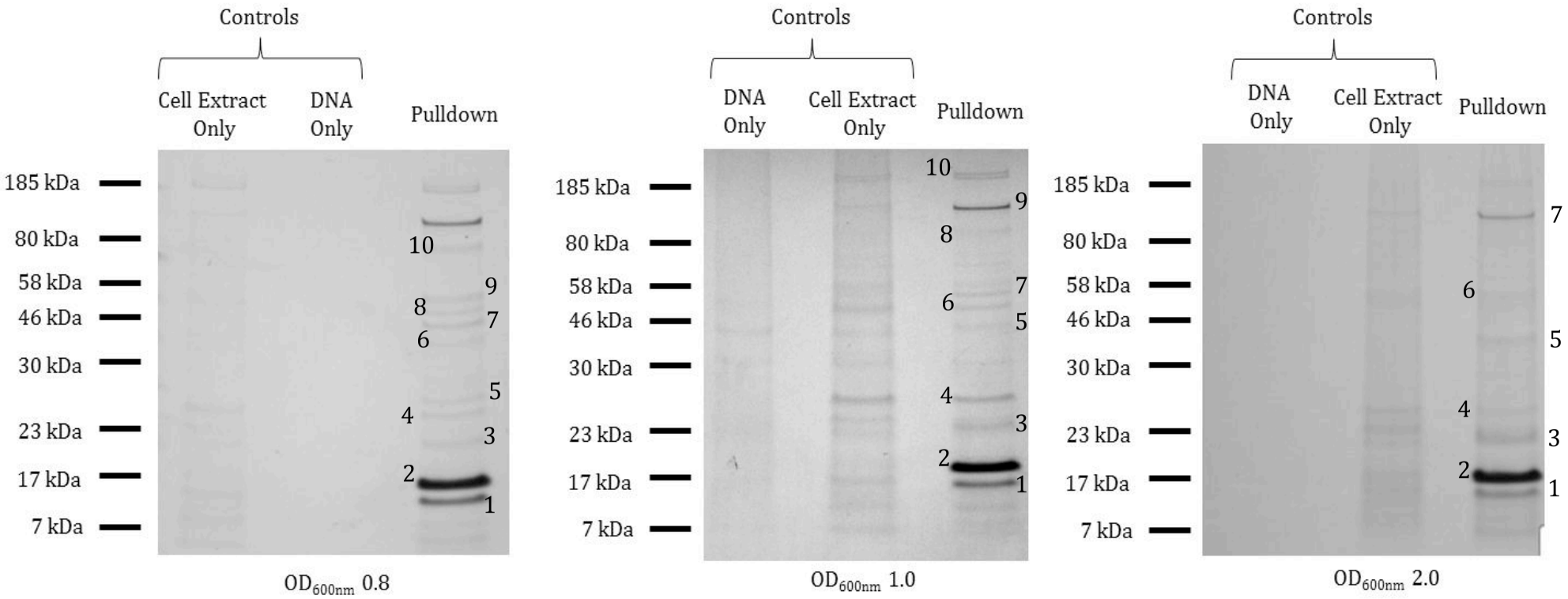


Figure 4.4. *lecA* Promoter Pulldowns Using Cell Lysates Harvested at Different Stages of Growth. Promoter pulldowns were repeated using cell extracts harvested at different time points; early in *lecA* expression (OD_{600nm} 0.8; A), at the peak of *lecA* expression (OD_{600nm} 1.0; B) and later on in *lecA* expression (OD_{600nm} 2.0; C). Control promoter pulldowns were carried out using either *lecA* promoter DNA only, or cell extract only. 'Pulldown' denotes the *lecA* promoter pulldown experiment. Bands were excised as indicated numerically and sequenced by LCMS/MS. The experiment was repeated three times to ensure reproducibility of the band migration patterns, and representative gels are shown. The number of each band corresponds to the LCMS/MS results for the appropriate time point shown in Table 4.2.

Table 4.2. The Protein Binding Profile at the *lecA* Promoter at Different Stages of Growth. Bands excised from the SDS-PAGE gels shown in Figure 4.4 were analysed for protein content, identifying a mixture of proteins of diverse function. The proteins were sorted by PseudoCap function to leave only those of interest, either uncharacterised in function, or characterised as having a regulatory role in gene expression. Individual proteins were assigned a relative abundance value according to the unweighted LCMS/MS spectrum counts; values in brackets represent the highest protein abundance value within the band to give an idea of scale. LCMS/MS analysis was carried out only once per band. Tables A-C correspond to Figures 4.4(A-C). The tables are continued on subsequent pages. A – OD_{600nm} 0.8; B - OD_{600nm} 1.0; C - OD_{600nm} 2.0.

A

OD_{600nm} 0.8					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	14 kDa	MvaU	32	(32)	Transcriptional regulator
2	15 kDa	MvaT	52	(52)	Transcriptional regulator
3	23 kDa	No proteins of interest			
4	25 kDa	PA0937	4	(25)	Uncharacterised protein
5	28 kDa	GlpR	34	(34)	Transcriptional regulator
		GbuR	4		Transcriptional regulator
6	40 kDa	PA2117	19	(19)	Uncharacterised protein
7	46 kDa	PhaF	30	(30)	Polyhydroxyalkanoate granule-associated protein
		PA3697	14		Predicted transcriptional regulator
		PA0141	5		Uncharacterised protein
8	48 kDa	AlgP	18	(18)	Transcriptional regulator
9	55 kDa	PA3455	10	(66)	Uncharacterised protein
10	80 kDa	No proteins of interest			

B

OD_{600nm} 1.0					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	14 kDa	MvaU	47	(47)	Transcriptional regulator
2	15 kDa	MvaT	36	(36)	Transcriptional regulator
3	23 kDa	PA4352	3	(27)	Probable stress protein
		PA1768	3		Probable stress protein
4	25 kDa	PA0937	7	(21)	Uncharacterised protein
5	40 kDa	PA2117	2	(20)	Uncharacterised protein
6	46 kDa	AlgP	4	(14)	Transcriptional regulator
7	50 kDa	No proteins of interest			
8	80 kDa	PA1372	3	(17)	Probable DNA repair protein
9	100 kDa	No proteins of interest			
10	185 kDa	No proteins of interest			

C

OD_{600nm} 2.0					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	14 kDa	MvaU	33	(33)	Transcriptional regulator
2	15 kDa	MvaT	116	(116)	Transcriptional regulator
		Fur	3		Transcriptional regulator
3	23 kDa	PA4352	7	(62)	Probable stress protein
		PA3309	3		Probable stress protein
		PA4495	3		Probable stress protein
4	25 kDa	PA0937	4	(10)	Uncharacterised protein
		PA5208	4		Predicted transcriptional regulator
		PhoP	3		Transcriptional regulator
		PA3988	3		Probable lipoprotein
5	40 kDa	PA2117	25	(25)	Transcriptional regulator
		AlgP	12		Transcriptional regulator
		PhaF	4		Polyhydroxyalkanoate granule-associated protein
		PA4842	4		Uncharacterised protein
		PA3481	3		Uncharacterised protein
6	50 kDa	PA2734	4	(18)	Probable restriction modification protein
		FleQ	3		Transcriptional regulator
		DctD	33		Transcriptional regulator
7	100 kDa	PA0588	13	(125)	Putative serine threonine kinase

observed in promoter pulldown experiments using cell extracts harvested at OD_{600nm} 1.0, was identified at OD_{600nm} 0.8 and 2.0. Nonetheless, this protein is unlikely to be binding specifically to the *lecA* promoter as a regulator since it has a histone-like C-terminal region that non-specifically binds DNA during chromosome segregation[343,344]. PA3697 was also observed bound to the *lecA* promoter when using cell lysates harvested at OD_{600nm} 0.8 (Figure 4.4); this is in contrast to the results shown in Figure 4.3, where PA3697 is observed bound to the promoter when using cell extracts harvested at OD_{600nm} 1.0. Similarly, the ferric uptake regulator Fur appears to bind the *lecA* promoter when using cell extracts harvested at OD_{600nm} 2.0 (Figure 4.4), a later timepoint than that shown in Figure 4.3, where Fur was also seen bound to the *lecA* promoter. Conversely, PA0937, an uncharacterised protein 57% similar to *E. coli* YaiL, was seen bound to the *lecA* promoter when using cell extracts harvested at all three time points. Since YaiL is a predicted nucleoprotein or polynucleotide-associated enzyme[345], again, PA0937 is unlikely to be binding in a regulatory manner.

A number of new proteins were also identified in the second *lecA* promoter pulldown (Table 4.2). These include stress proteins and proteins involved in DNA modification and repair, but also some characterised transcriptional regulators. For example, GlpR, the glycerol 3-phosphate regulon repressor[346], and GbuR, the transcriptional activator of GbuA in the arginine dehydrogenase pathway[347] were found binding the promoter only at the earlier stage of growth tested, whilst AlgP is seen across all time points. AlgP (also known as AlgR3) is a positive transcriptional activator of AlgD, which is involved in alginate biosynthesis[348,349]; since alginate production in mucoid cells is an important determinant of biofilm formation[350] - in which LecA also plays a role – the presence of AlgP is intriguing.

The uncharacterised protein PA2117 was also observed binding the *lecA* promoter at high levels when using cell extracts harvested across all three time points, while at OD_{600nm} 0.8, another uncharacterised protein, PA0141, was identified; this protein has predicted polyphosphate

kinase activity and is 74% similar to the predicted transcriptional regulator PvdS in *M. tuberculosis.*

Later on in growth, as *lecA* expression occurs, 4 more proteins appear to bind the *lecA* promoter. DctD is a response regulator in the DctB-DctD two component system, in which it activates expression of the DctA gene to facilitate transport of C4-dicarboxylates such as succinate[351]. PA0588, however, is another largely uncharacterised protein that has been identified as a serine-threonine kinase. The gene for PA0588 is part of the PA0588-PA0584 gene cluster, which has a positive effect on swarming, pyocyanin production and *rsmY/Z* expression[352].

Finally, the transcriptional regulator FleQ was found to bind when using cell extract harvested at OD_{600nm} 2.0. FleQ has been shown to be a major regulator of flagellar expression[353], and is involved in regulating the switch between planktonic growth and formation of biofilms in response to changes in the level of intracellular c-di-GMP[116]. FleQ also regulates the expression of the *pel* exopolysaccharide biosynthesis genes[115], which is again interesting with respect to the role of LecA in biofilms.

The negative regulators MvaT and MvaU were also observed binding the *lecA* promoter when using cell extracts harvested across all three time points. This was unexpected since Diggle *et al.*[118] had previously suggested that MvaT is involved in growth phase dependent control of *lecA* expression, implying that when *lecA* is expressed, MvaT repression has been reversed.

4.2.4. The Interaction Between MvaT and the *lecA* Promoter.

Given the fact that MvaT forms large multimeric structures that occlude promoter binding sites[324], *lecA* promoter pulldowns were carried out using protein extracts across different points in the growth of a PAO1—N Δ*mvaT* strain[118] to ascertain whether the removal of MvaT allows other proteins to then bind the *lecA* promoter at sites previously inaccessible to them. The results are shown in Figure 4.5.

As expected, the *lecA* promoter pulldowns from Δ*mvaT* cell extracts did not show the 16 kDa band corresponding to MvaT. Interestingly, when MvaT is depleted, the 14 kDa band corresponding to MvaU appears to increase in density. This supports the findings of Vallet-Gely *et al.*[325], who observed that MvaU is able to complement the function of MvaT in an MvaT mutant, and that when one is depleted, the amount of the other in the cell increases.

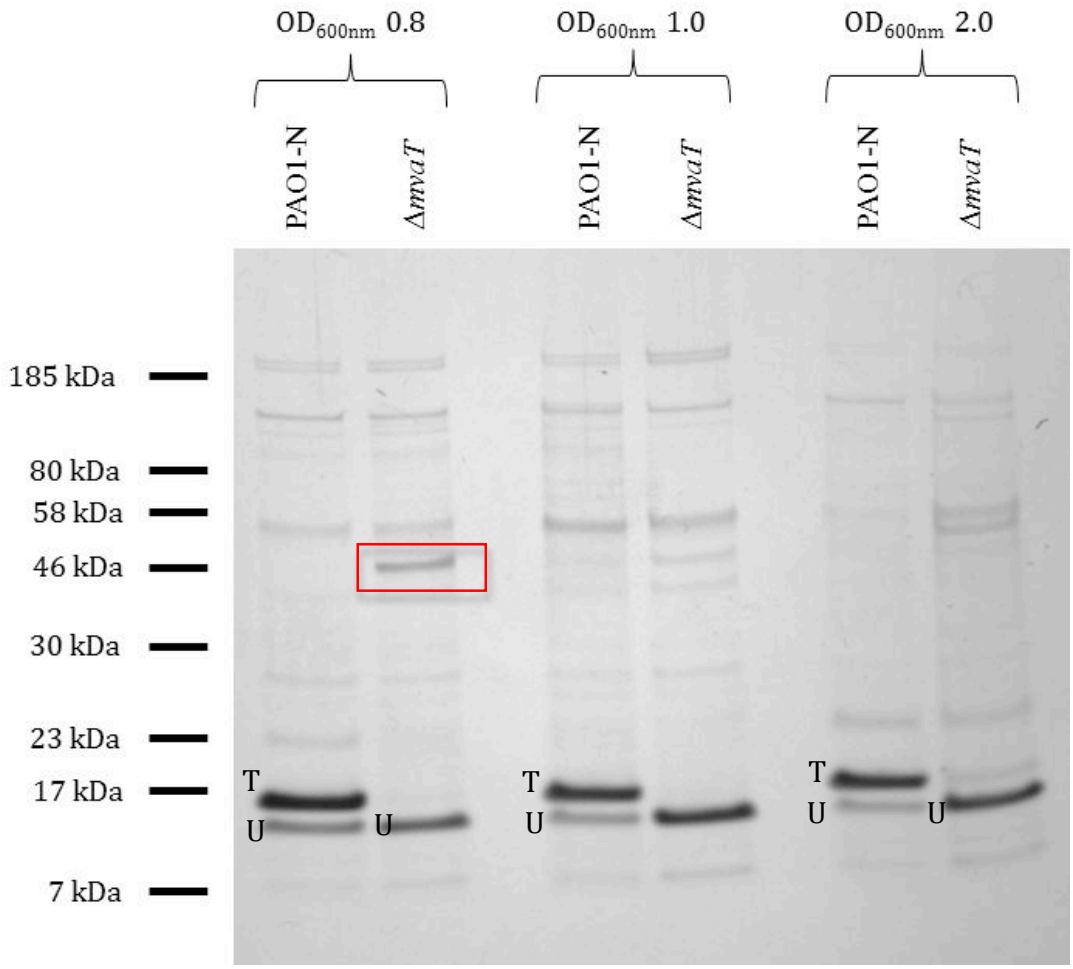


Figure 4.5: The Effect of MvaT on the Protein Binding Profile at the *lecA* Promoter. Promoter pulldowns were carried out across three time points to ascertain whether the negative regulator MvaT is able to block binding of other proteins to the promoter region of *lecA*. Promoter pulldowns carried out using cell extracts harvested from wild type PAO1 Nottingham (PAO1-N) and the PAO1-N *mvaT* mutant (Δ*mvaT*) were compared for the presence of additional protein bands in the absence of MvaT. In the Δ*mvaT* samples, the band corresponding to MvaT (T) is clearly absent, whilst the band corresponding to MvaU (U) increases in strength. Only one band, indicated on the gel at 46 kDa, changes remarkably upon depletion of MvaT, most notably at OD_{600nm} 0.8. LCMS/MS analysis of this band revealed it to consist mainly of PhaF. SDS-PAGE was repeated three times to ensure reproducibility of the band migration pattern, and a representative gel is shown.

Furthermore, it has been shown that in both Δ*mvaT* and Δ*mvaU* strains, expression of *lecA* is increased[119]; this further demonstrates a role for both proteins in the regulation of LecA. MvaU is proposed to behave in the same way as MvaT, forming homomeric or heteromeric_oligomers with MvaT[325], therefore it is likely that MvaU would be equally capable of blocking protein binding sites on the *lecA* promoter during the experiment. Nonetheless, one pronounced difference between the wild type PAO1-N and the Δ*mvaT* strains can be seen at OD_{600nm} 0.8 in Figure 4.4; this 46 kDa band was excised and sequenced by LCMS/MS and identified as predominantly PhaF, thus unlikely to be of interest in this study, given its aforementioned role in chromosome segregation during cell division.

4.2.5. The Effect of PqsE on the *lecA* Promoter.

Given that PqsE is required for LecA production[134,152], albeit most likely at the post-transcriptional level, experiments were carried out to determine whether PqsE could also affect the protein binding profile at the *lecA* promoter. Cell extracts were harvested from wild type PAO1-N and PAO1-N *pqsEind* cultures at OD_{600nm} 1.0 and used in *lecA* promoter pulldowns using the *lecA* promoter fragment amplified from the PAO1-N strain. The PAO1-N *pqsEind* strain has a conditional mutation in *pqsE*, the expression of which is controlled from the *tac* promoter under the control of the LacIQ repressor. Thus, expression of the *pqsE* gene only occurs in the presence of IPTG[152,354].

The SDS-PAGE results from these experiments are shown in Figure 4.6, where it is clear that the protein profile at the *lecA* promoter differed markedly between strains dependent on the expression of *pqsE*. The MvaT and MvaU bands remained constant for both strains regardless of *pqsE* expression; however, the number of bands increases when no PqsE is produced, and increases further when *pqsE* is overexpressed. From this gel, 19 bands were sequenced by LCMS/MS; the sequencing results yielded 203 proteins that bound the *lecA* promoter sequence non-

specifically, including PhaF. These were again classified by function to obtain only those considered to be of interest, as summarised in Table 4.3.

In this experiment, many regulators were observed that had also previously been identified in earlier *lecA* promoter pulldowns. FleQ, which was found bound to the *lecA* promoter using the PAO1-L cell extracts

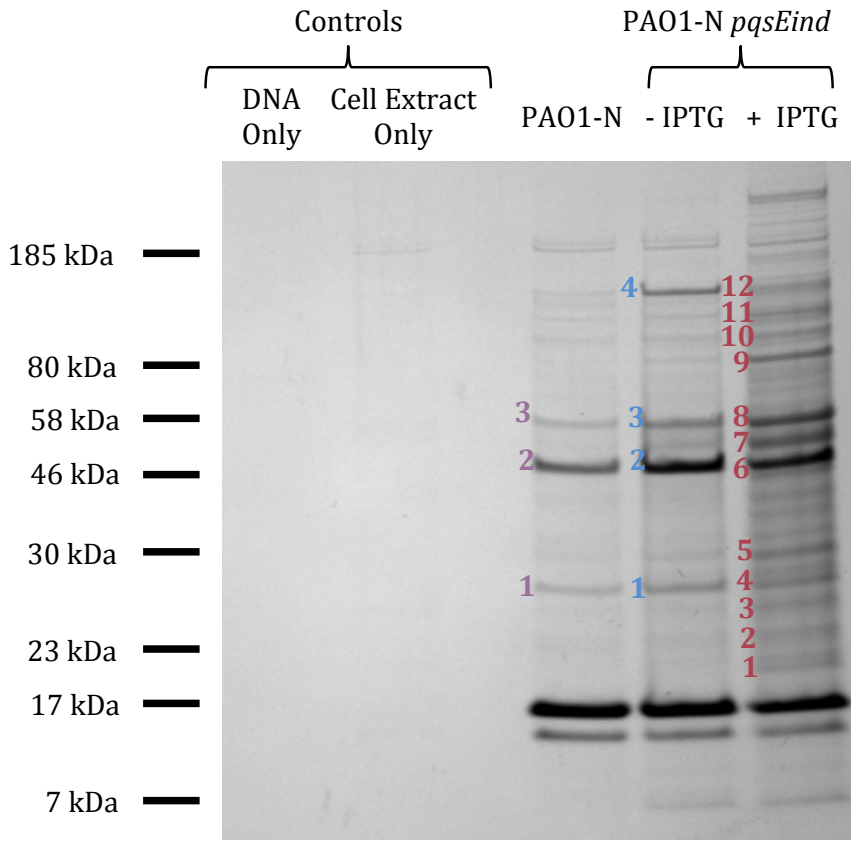


Figure 4.6: The Effect of Variation of PqsE Expression in *lecA* Promoter Pulldowns. Promoter pulldowns were carried out using the *lecA* promoter region in combination with cell extracts harvested from wild type PAO1 Nottingham (PAO1-N) and a PAO1-N with an IPTG-inducible *pqsE* conditional mutant (PAO1-N *pqsEind*) to determine the effect of normal *pqsE* expression (PAO1-N), *pqsE* overexpression (+IPTG), and absence of PqsE (-IPTG) on the protein profile at the *lecA* promoter. *pqsE* overexpression was induced by the addition of 1mM IPTG to PAO1-N *pqsEind* cultures; all cell extracts were harvested at OD_{600nm} 1.0. Control experiments were carried out using either *lecA* promoter DNA only or cell extract only, as previously. The experiment was repeated three times to ensure reproducibility of the band migration pattern, and a representative gel is shown. Bands excised for LCMS/MS analysis are indicated on the gel; colour coded numbers correspond to the LCMS/MS results shown in Table 4.3. Purple - PAO1-N; blue – PAO1-N *pqsEind* -IPTG; red – PAO1-N *pqsEind* +IPTG.

Table 4.3. The Protein Binding Profile at the *lecA* Promoter in the Presence of Different Levels of *pqsE* Expression. The protein bands illustrated in Figure 4.6 were analysed by LCMS/MS to identify a mixture of proteins within each band. Proteins were sorted according to function as previously. For clarity, only uncharacterised proteins and proteins of confirmed or predicted regulatory functions are shown here. Individual proteins were assigned a relative abundance value according to the unweighted LCMS/MS spectrum counts; values in brackets represent the highest protein abundance value within the band to give an idea of scale. Colour coded numbers correspond to the protein bands shown in Figure 4.6. Purple - PAO1-N (A); blue – PAO1-N *pqsEind* –IPTG (B); red – PAO1-N *pqsEind* +IPTG (C). LCMS-MS analysis was carried out only once per band.

A

PAO1-N					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	29 kDa	GlpR	54	(54)	Transcriptional Regulator
2	46 kDa	No proteins of interest			
3	56 kDa	FleQ	10	(54)	Transcriptional regulator

B

PAO1-N *pqsEind* - IPTG					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	29 kDa	GlpR	47	(47)	Transcriptional Regulator
2	46 kDa	PA3697	7	(50)	Predicted transcriptional regulator
3	56 kDa	FleQ PA4132	14 8	(56)	Transcriptional regulator Predicted transcriptional regulator
4	130 kDa	No proteins of interest			

C

PAO1-N *pqsEind* + IPTG					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	20 kDa	DadR	6	(12)	Transcriptional regulator
		PA5115	5		Predicted transcriptional regulator
2	23 kDa	Vfr	8	(8)	Transcriptional regulator
		GacA	7		Transcriptional regulator
		RoxR	7		Transcriptional regulator
3	25 kDa	RhlR	26	(26)	Transcriptional regulator
		OmpR	10		Transcriptional regulator
		LasR	7		Transcriptional regulator
		Anr	7		Transcriptional regulator
		BfmR	6		Transcriptional regulator
		PA0236	5		Predicted transcriptional regulator
4	29 kDa	GlpR	39	(39)	Transcriptional regulator
		AlgR	7		Transcriptional regulator
		PqsE	7		Quinolone signal response protein
		AmgR	5		Transcriptional regulator
5	30 kDa	PA0123	6	(22)	Predicted transcriptional regulator
		MexT	6		Transcriptional regulator
6	46 kDa	PA2705	10	(34)	Uncharacterised protein
		PA4781	9		Predicted transcriptional regulator
7	50 kDa	DctD	15	(25)	Transcriptional regulator
		AlgB	14		Transcriptional regulator
		AlgP	13		Transcriptional regulator
		MifR	7		Transcriptional regulator

PAO1-N *pqsEind* + IPTG					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
7 (cont)	50 kDa	PA2572 PA5209	6 5		Predicted transcriptional regulator Uncharacterised protein
8	56 kDa	FleQ CbrB	16 7	(89)	Transcriptional regulator Transcriptional regulator
9	80 kDa	PA0588 PA0178	47 7	(47)	Putative serine threonine kinase Predicted two component system sensor
10	100 kDa	PA3729	18	(35)	Uncharacterised protein
11	120 kDa	No proteins of interest			
12	130 kDa	No proteins of interest			

prepared at all three timepoints (Table 4.2), was again apparent in PAO1—L regardless of the PqsE phenotype, but appeared to be more abundant in the pulldowns using wild type PAO1-N and the *pqsE* mutant (Uninduced; no IPTG added) cell extracts than in the pulldowns using *pqsE* overexpressing (Induced; IPTG added) cell extracts (Table 4.3). On the contrary, AlgP, despite having been seen previously in the wild type PAO1—L pulldowns at all time points (Table 4.2), was in this instance only seen in the pulldown where *pqsE* was overexpressed (Table 4.3)

Furthermore, GlpR, which had previously been identified bound to the *lecA* promoter when using cell extracts harvested at OD_{600nm} 0.8 in the PAO1-L pulldowns (Table 4.2(A)), was this time seen binding the *lecA* promoter under all *pqsEind* expression conditions when using cell extracts harvested at OD_{600nm} 1.0, whilst PA3697 was observed binding only in the absence of PqsE (Table 4.3). The detection of PA3697 in these experiments has been variable, observed in pulldowns using cell extracts prepared at OD_{600nm} 1.0 in Table 4.1, OD_{600nm} 0.8 only in Table 4.2, and OD_{600nm} 1.0 only when *pqsE* was overexpressed in Table 4.3.

PA0588, on the other hand, was seen binding the *lecA* promoter in pulldowns using cell extracts harvested at OD_{600nm} 2.0 in earlier pulldowns (Table 4.2). However, when *pqsE* is overexpressed, the binding of this protein to the *lecA* promoter also occurs when using cell extracts harvested at OD_{600nm} 1.0 (Table 4.3).

Nonetheless, many proteins listed in Table 4.2 are not seen in Table 4.3. However, the majority of these were previously identified in pulldown experiments using cell extracts harvested at OD_{600nm} 0.8 and 2.0, therefore their absence in pulldowns carried out using extracts harvested at OD_{600nm} 1.0 is not surprising. One exception is PA2117, which was observed across all three time points in Table 4.2, but was not seen at all in the experiment investigating the effect of PqsE.

At the same time, several proteins listed in Table 4.1 (but not Table 4.2) can also be seen in Table 4.3. These proteins, Vfr, RhlR and RoxR, only bound the *lecA* promoter when *pqsE* was overexpressed. This occurred alongside a strikingly large number of other regulatory proteins binding

the *lecA* promoter at the same time, and this is reflected in the remarkably increased number and density of protein bands in the pulldown sample in which *pqsE* was overexpressed (Table 4.3). Amongst 28 proteins of interest identified in this sample, 3 were uncharacterised, and 2 unlikely to be transcriptional regulators; PA0588, the aforementioned serine-threonine kinase, and PA0178, a predicted two component system sensor protein. The remaining 23 proteins were largely characterised transcriptional regulators, along with 5 predicted transcriptional regulators. Besides RoxR, MexT, Anr and AmgR, the major regulators Vfr, RhlR, GacA and LasR also bound the *lecA* promoter region under these conditions. In addition, PqsE itself was identified, although this protein is not predicted to have a DNA-binding function.

Interestingly, amongst the many transcriptional regulators bound to the *lecA* promoter when *pqsE* was overexpressed, a number of proteins involved in the regulation of alginate production and biofilm development were observed. AlgP, AlgR and AlgB, which regulate mucoidy, were seen alongside BfmR and MifR. BfmSR and MifSR have been characterised by Petrova *et al.*[355] as novel two component systems that regulate specific stages during the creation of biofilm, specifically during biofilm maturation.

Moreover, PA0236, PA3729 and PA2572 were also bound to the *lecA* promoter when *pqsE* was overexpressed. These are all predicted transcriptional regulators, although PA2572 has been shown to be involved in biofilm formation and virulence[356]; in addition, PA3729 was identified. Although this protein is a predicted transmembrane protein, it is said to be part of the biofilm-associated cluster (*bac)* encompassing the loci PA3729-3732[357].

Finally, one new protein was identified that bound to the *lecA* promoter only when no PqsE was produced. Although uncharacterised, PA4132 is 64% similar to a putative transcriptional regulator in *E.coli* and contains a predicted helix-turn-helix domain typical of many DNA binding regulatory proteins.

4.2.6. Construction of In-Frame Deletions in Candidate Regulators of *lecA* Expression.

Early in the study, five candidate proteins that bound the *lecA* promoter were initially chosen on the basis of the findings of the first two *lecA* promoter pulldowns (Tables 4.1 and 4.2). These proteins, GbuR, FleQ, Vfr, PA2117 and PA3697 were identified across the range of time points analysed and deemed to be of interest considering the functional information available. In-frame deletions were created in the genes encoding these proteins within the *P. aeruginosa* PAO1-L background using a different method to that used in Chapter 3 in order to overcome problems encountered in finding appropriate restriction sites that would allow cloning into both pBluescript and pME3087 without also digesting within the genes themselves.

The method used was overlap extension PCR, which was first described by Ho *et al.*[358] and is summarised in Figure 4.7. Primers are designed to amplify two regions; upstream forward (uf) and upstream reverse (ur) primers amplify the sequence including the beginning of the gene of interest and its flanking upstream DNA sequence, whilst the downstream forward (df) and downstream reverse (dr) primers amplify the sequence including the end of the gene of interest and its flanking downstream DNA sequence. The central portion of the gene is omitted, creating the deletion.

This method requires two PCR steps. The first step amplifies the two upstream and downstream fragments separately to produce two PCR products. The upstream forward and downstream primers are the overlap extension primers, and are designed with an additional sequence at the 5' ends (creating a 5' overhang. This sequence makes the primers complementary to each other. In the case of overlap extension PCR to create an in-frame deletion, the DNA sequence 5' of the deleted region is chosen to be the 5' overhang sequence. This ensures that after the first PCR step, each of the two fragments has 3' overhangs complementary to

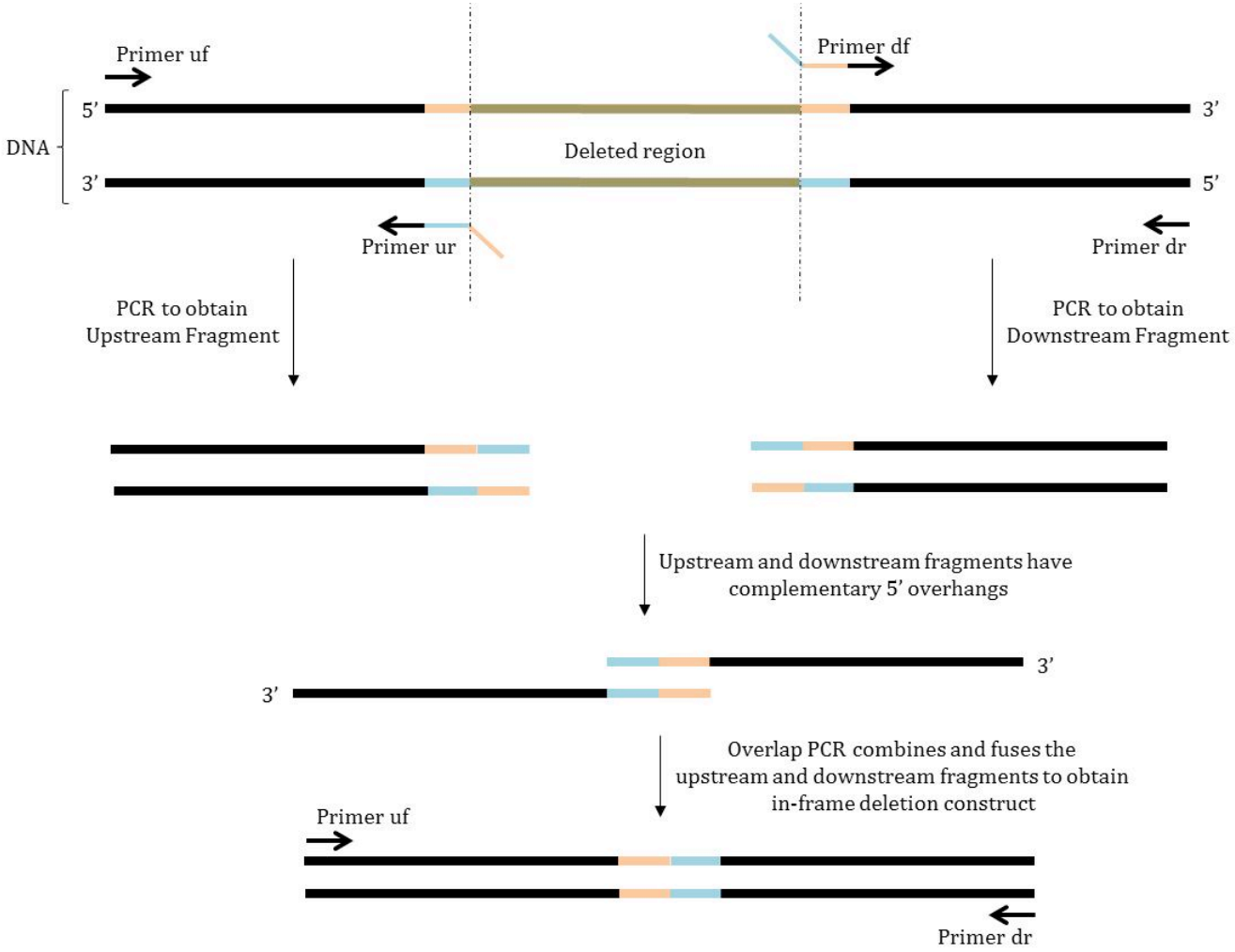


Figure 4.7. Overlap Extension PCR. In-frame deletion mutants were created using overlap extension PCR. Two PCR products, upstream and downstream, are generated by two primer pairs respectively; the upstream forward (uf)/upstream reverse (ur) pair and the downstream forward (df)/downstream reverse(dr) pair. The ur and df primers contain 3' extension sequences which are amplified along with the template to create 5' overhangs within the PCR products. These are complementary between the upstream and downstream fragments, enabling the two fragments to be fused in a second PCR reaction using the uf/ dr primer pair. These primers contain restriction sites to allow the resulting deletion construct to subsequently be cloned into vectors.

each other (introduced by the overlap extension primers), which in the second PCR step allows the two fragments to be fused together and amplified as one template strand by the outermost flanking upstream forward and downstream reverse primers, resulting in the final product. This method was used to create in-frame deletion constructs for the PA3697, *gbuR*, *fleQ* and PA2117 genes, and has been described in detail in Chapter 2, Section 2.3.1. The construction of the 897 bp ΔPA3697 fragment is shown in Figure 4.8, and involved optimisation to identify the most appropriate annealing temperatures for amplification of the upstream and downstream fragments (Figure 4.8(A)) and the overlap PCR step (Figure 4.8(B)). Figure 4.9 shows the construction of the 1,013 bp Δ*gbuR*, 979 bp Δ*fleQ* and 1,029 bp ΔPA2117 fragments.

Once the deletion fragment had been obtained, ΔPA3697, Δ*fleQ*, and ΔPA2117 were digested using HindIII and BamHI before being ligated into similarly digested pBluescript to make the plasmids pRB501, pRB601 and pRB701 respectively; Δ*gbuR* was digested with BamHI and XbaI and then ligated into similarly digestion pBluescript to make pRB801. Inserts were confirmed by blue and white screening followed by colony PCR and DNA sequencing (results not shown), before being released from the vectors by double digestion with either BamHI/HindIII (pRB501) or XbaI/KpnI (pRB601, pRB701, pRB801). The resulting fragments containing the deletion constructs were ligated into similarly digested pME3087 to make the suicide vectors pRB502 (ΔPA3697), pRB602 (Δ*fleQ*), pRB702 (ΔPA2117) and pRB802 (Δ*gbuR*).

Donor *E. coli* S17-1 strains carrying the suicide vectors were then used in conjugal mating experiments with PAO1-L. Transconjugants resistant to tetracycline were screened by PCR for the presence of the mutant gene constructs and colonies screening positive were subjected to enrichments for the selection of tetracycline-sensitive cells in which the double cross-over event had occurred as described in Chapter 3, Section 3.2.1. Tetracycline-sensitive colonies were then confirmed by colony PCR for the presence of the mutant genes. Figure 4.10 shows the results, which were subsequently confirmed by DNA sequencing.

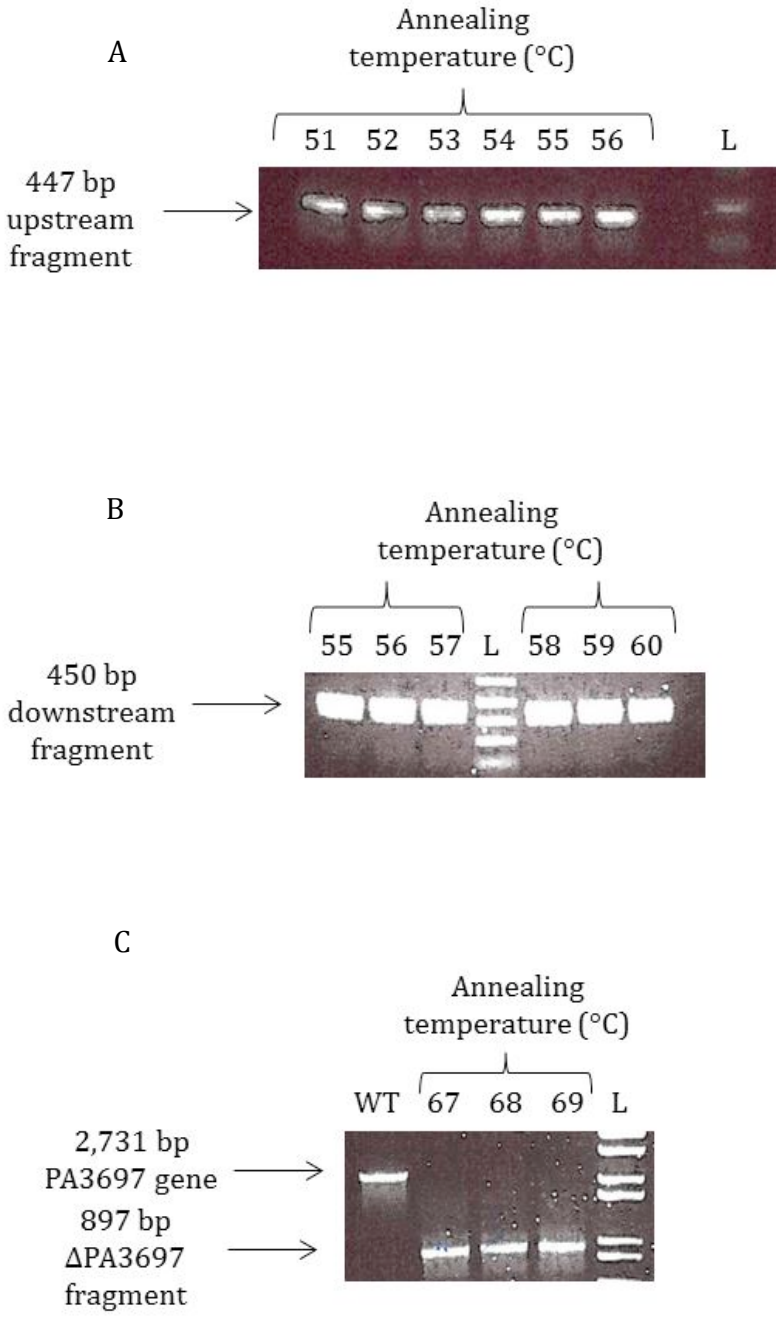


Figure 4.8. Generation of DNA Fragments for In-Frame Deletion of PA3697. The overlap extension PCR method was optimised using different annealing temperatures, as shown above. The upstream and downstream regions of the PA3697 gene were amplified giving specific PCR products approximately 450 bp in size (A and B). These were purified and combined, then used in the overlapping PCR reaction (C) to fuse the two fragments and create the ΔPA3697 construct, with a 1,266 bp deletion in the centre of the gene. L – DNA ladder, WT – wild type PAO1-L control.

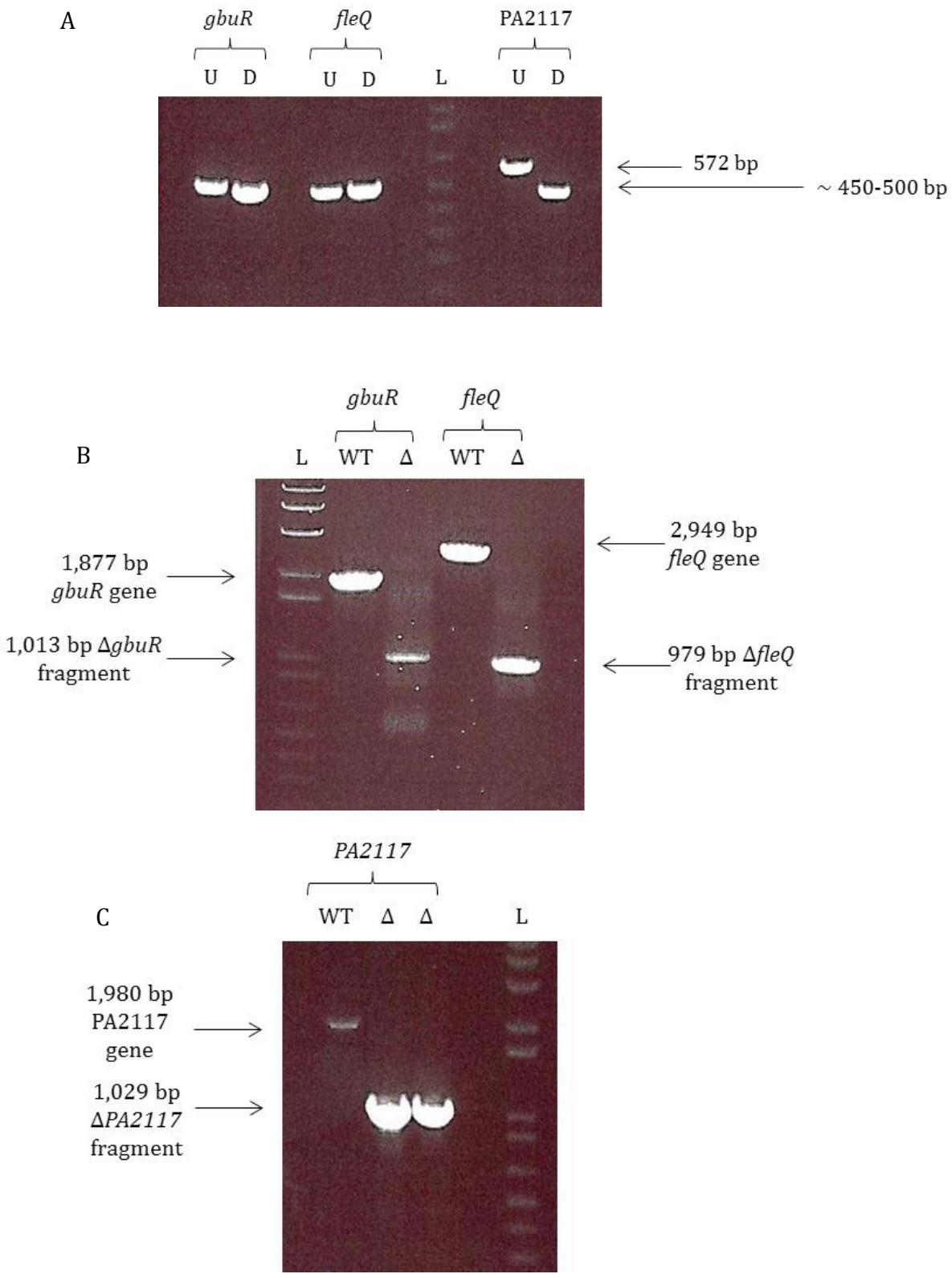


Figure 4.9. Generation of DNA Fragments for In-Frame Deletion of *gbuR*, *fleQ* and PA2117. Upstream and downstream fragments were obtained for *gbuR*, *fleQ* and PA2117 (A). These were purified, combined, and used in overlapping PCR reactions to fuse the appropriate upstream and downstream fragments to create the Δ*gbuR* (864 bp deletion), Δ*fleQ* (2,458 bp deletion) and ΔPA2117 (951 bp deletion) constructs (B and C). U – upstream fragment; D – downstream fragment; L – DNA ladder, WT – wild type PAO1-L control, Δ – overlap PCR to create deletion construct.

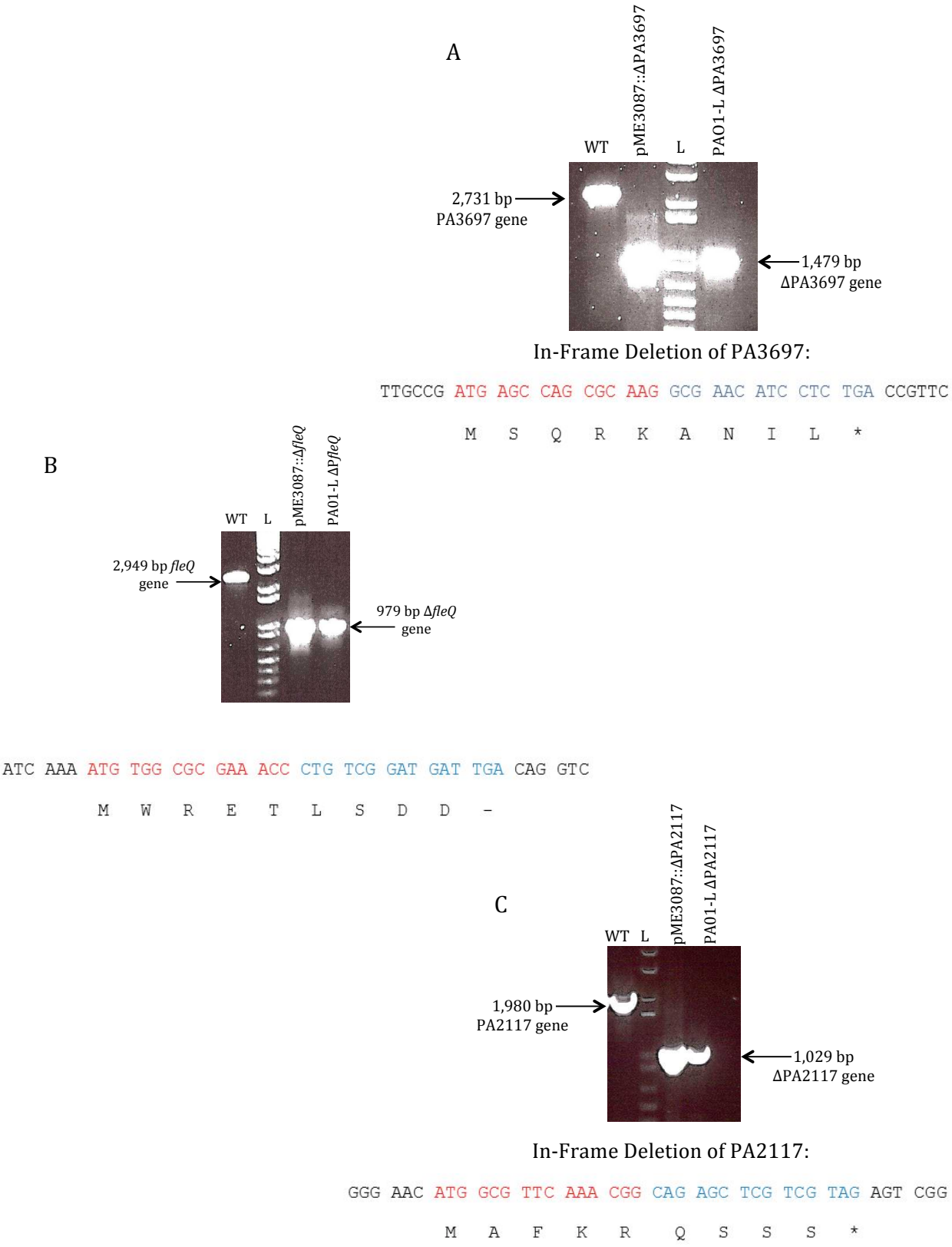


Figure 4.10. Screening of the PAO1-L ΔPA3697, Δ*fleQ* and ΔPA2117 Strains. After enrichments, tetracycline-sensitive colonies were screened by colony PCR for the presence of the mutant gene and the loss of the suicide plasmid, and amplicons compared against positive and negative controls; the wild type PAO1-L strain (WT) and the respective pME3087 suicide vectors. The DNA and amino acid sequences verified that deletions were in-frame, and no premature stop codons were added into the sequence during mutagenesis. The results confirm the ΔPA3697, (A) Δ*fleQ* (B) and ΔPA2117 (C) strains as being correct. L- DNA ladder; red DNA sequences denote upstream coding regions, blue DNA sequences denote downstream coding regions, black DNA sequences indicate the flanking regions. * denotes stop codon.

Although the Δ*gbuR* suicide vector pRB802 was made successfully and transconjugants obtained as shown in Figure 4.11, enrichments for tetracycline sensitive cells were consistently unsuccessful.

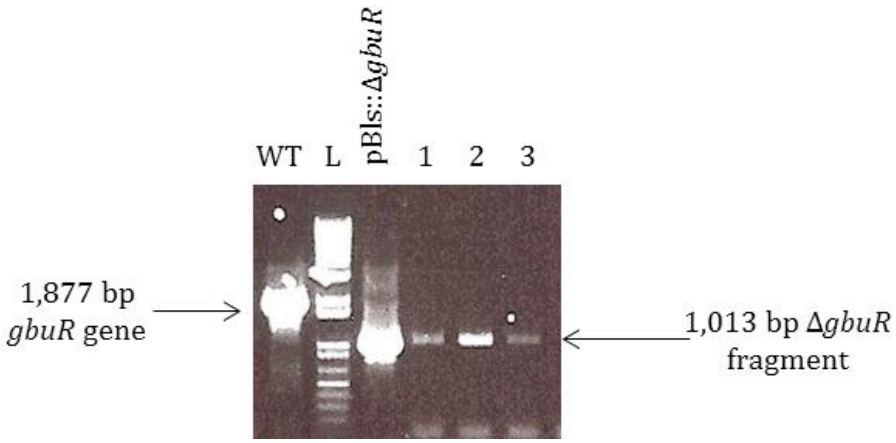
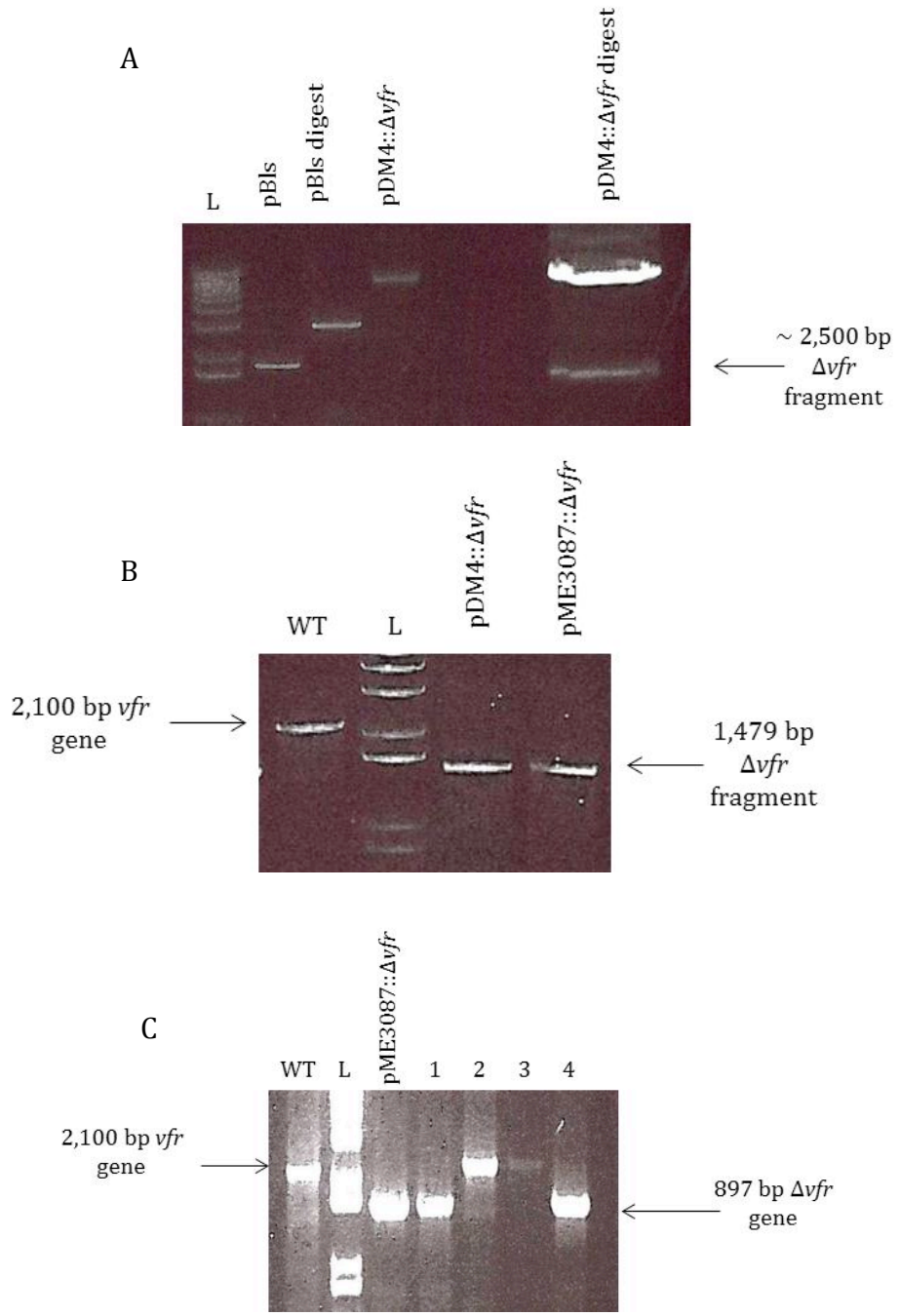


Figure 4.11: Screening of PAO1-L pRB802 Transconjugants. Colonies 1-3 obtained following the conjugation of pRB802 into the PAO1-L background were screened for the presence of the Δ*gbuR* construct and compared against the wild type PAO1-L strain (WT). All colonies contained the deletion construct, and were subjected to enrichments to obtain the double crossover, and subsequently the PAO1-L Δ*gbuR* strain; however, the enrichments were unsuccessful and ultimately, a tetracycline-sensitive mutant was not constructed for this study. L – DNA ladder.

The Δ*vfr* strain, on the other hand, was made using the pDM4::Δ*vfr* suicide vector that had previously been constructed in this laboratory (unpublished). However, the use of the pDM4 vector is problematic in PAO1 Lausanne due to the high background resistance of this strain to chloramphenicol as discussed in Chapter 3, Section 3.2.1. To overcome this, the 1,479 bp Δ*vfr* construct was digested from pDM4::Δ*vfr* using the restriction enzymes XbaI and SalI (Figure 4.12(A)) and ligated into similarly digested pBluescript to make pRB401; this was then digested with KpnI and XbaI to release the fragment for subsequent ligation into pME3087, also digested with KpnI and XbaI, to make pRB402 (Figure 4.12(B)). Mutagenesis was completed following enrichment for tetracycline-sensitive colonies and confirmed as shown in Figure 4.12(C).



In-Frame Deletion of *vfr*:

```
TCGGGC ATG GTA GCT ATT GGA TCC GGC ACC CGC TGA ACAGCA
       M   V   A   I   G   S   G   T   R   *
```

Figure 4.12. Construction of the PAO1-L Δ*vfr* Strain. To construct pRB402, a pre-existing pDM4::Δ*vfr* construct was digested with XbaI and SalI to release the Δ*vfr* fragment (A). This fragment was initially cloned into similarly digested pBluescript (pBls digest) to make pRB401; subsequent restriction of this plasmid with KpnI and XbaI released the Δ*vfr* fragment once more for cloning into pME3087 to make the final suicide vector (B). Following mutagenesis of PAO1-L using pRB402 and enrichments, tetracycline sensitive colonies were screened for the presence of the Δ*vfr* gene; of colonies 1-4, only 1 and 4 were correct, incorporating an in-frame deletion of 621 bp and a central BamHI restriction site (C). Wild type PAO1-L (WT) was used as a negative control; the Δ*vfr* suicide plasmids were used as positive controls. L – DNA ladder; pBls – intact pBluescript control; red DNA sequences denote upstream and downstream coding regions, whilst the BamHI recognition site is highlighted in blue. Black DNA sequence s indicate the flanking regions; * denotes stop codon.

4.2.7. Verification of PAO1-W Transposon Mutants.

After the *lecA* pulldown had been repeated using the PAO1-N *pqsEind* strain, further candidate proteins were chosen for investigation. However, considering the large number of proteins identified in the experiment (as summarised in Table 4.3), construction of in-frame deletion mutations in all the candidate regulators of *lecA* expression was not feasible due to time constraints.

Instead, transposon mutants were purchased from the University of Washington PAO1 transposon mutant library[217]. These mutants have either IS*phoA*/hah or IS*lacZ*/hah Tn5 elements inserted within the candidate gene in the *P. aeruginosa* PAO1 Washington background. The transposons confer tetracycline resistance, and also encode either alkaline phosphatase (*phoA*) or β-galactosidase (*lacZ*), which can create translational gene fusions if inserted into the target gene in the correct reading frame and orientation. The transposon mutants obtained for this study included Δ*gbuR*, for which the attempts at in-frame deletion mutagenesis were not successful. Each transposon mutant required confirmation by PCR in order to verify the genotype.

Two different methods of confirming the mutants by PCR were used. The first screened for the presence of the transposon within the gene of interest, using one primer specific for either the IS*lacZ/*hah or *ISphoA/*hah transposon paired with a flanking primer specific to the gene of interest. The flanking primer was chosen dependent on whether the transposon was in the forward or reverse orientation in that specific gene. If the transposon was present within the gene of interest, the transposon specific primer was able to anneal to the template DNA and a PCR product was observed. This PCR product was not observed in wild type controls.

The second PCR amplified the entire gene of interest using the pair of flanking primers specific to that particular gene. Transposon insertion increases the size of the target gene such that it cannot be effectively amplified by standard PCR; therefore, presence of the transposon within the gene of interest resulted in complete lack of PCR product when

compared with the same PCR reaction using wild type PAO1-W genomic DNA.

The results are summarised in Table 4.4, which confirms that all but two of the PAO1-W transposon mutants obtained for this study are correct, as determined firstly by the presence of a transposon-specific PCR product that is not present in the wild type control, and secondly by the absence of a PCR product for the full length gene compared with the wild type control.

Table 4.4. Verification of PAO1-W Transposon Mutants. For this study, 18 PAO1-W transposon mutants were obtained. To confirm that the Tn5 insertions were present within those genes encoding the candidate regulators of *lecA* expression, the transposon mutants were screened by PCR. Using primer pairs in which one primer was transposon-specific and the other was gene-specific, all 18 transposon mutants screened positive for the presence of the transposon in the correct position and orientation within the appropriate open reading frame (transposon present). The transposon mutants were also screened using primers flanking the gene of interest. Mutants were confirmed by the absence of PCR product (absence of gene). ΔPA3729 and ΔPA0236 displayed similar PCR products to their respective wild type controls, and so were not confirmed by this PCR. ✓- confirmed; ✗ - not confirmed.

PAO1-W Transposon Mutant	**PCR Confirmation**	
	Transposon Present	Wild Type Gene Absent
Δ*glpR*	✓	✓
Δ*dctD*	✓	✓
Δ*algB*	✓	✓
Δ*pilR*	✓	✓
Δ*mifR*	✓	✓
Δ*algP*	✓	✓
Δ*bfmR*	✓	✓
Δ*gbuR*	✓	✓
Δ*amgR*	✓	✓
Δ*cbrB*	✓	✓
ΔPA5208	✓	✓
ΔPA2572	✓	✓
ΔPA0141	✓	✓
ΔPA4132	✓	✓
ΔPA0588	✓	✓
ΔPA0178	✓	✓
ΔPA3729	✓	✗
ΔPA0236	✓	✗

The PA3729 and PA0236 transposon mutants produced full length gene products similar to the wild type PAO1-W controls; however, these mutants also screened positive for the presence of transposons in the correct positions, which were absent in the wild type controls as expected. Therefore, the PAO1-W PA3729 and PA0236 transposon mutants were assumed to be correct.

4.2.8. Construction of IPTG-Inducible *pqsE* Conditional Mutants.

Many of the candidate proteins in this study were identified bound to the *lecA* promoter in the PAO1-N *pqsEind* background. To validate the results of the promoter pulldowns carried out under conditions of altered *pqsE* expression, characterisation experiments for those particular candidate proteins would also have to be carried out in the presence of altered *pqsE* expression. With this in mind, IPTG-inducible *pqsE* conditional mutations were introduced into the PAO1-L and PAO1-W strains, as well as into each of the PAO1-L and PAO1–W based mutant strains as appropriate. The conditional mutation was not introduced into the PAO1-L ΔPA3697, PAO1-L ΔPA2117, PAO1-W ΔPA0141, PAO1-W ΔPA5208 and PAO1-W Δ*gbuR* strains, because the cognate candidate proteins were not observed under conditions of altered *pqsE* expression (Table 4.3).

Since the *pqsEind* conditional mutation construct had been previously cloned into the pDM4 suicide vector by Rampioni *et al*.[152], the conditional mutation was introduced into the required strains using this vector as previously described in Chapter 2, Section 2.3.4, by conjugative mating and selection of chloramphenicol resistant clones followed by sucrose selection. The *pqsEind* strains were identified on the basis of their ability to grow on streptomycin, and confirmed visually by their production of the blue-green pigment, pyocyanin, in broth cultures both in the presence and absence of IPTG. Successfully generated *pqsEind* strains overexpressing *pqsE* in the presence of IPTG also overexpress pyocyanin, whereas in the absence of IPTG, the strains produce no pyocyanin[151,152].

This correlates with marked colour differences between parental strain cultures, uninduced *pqsEind* cultures, and induced *pqsEind* cultures, as shown in Figure 4.13.

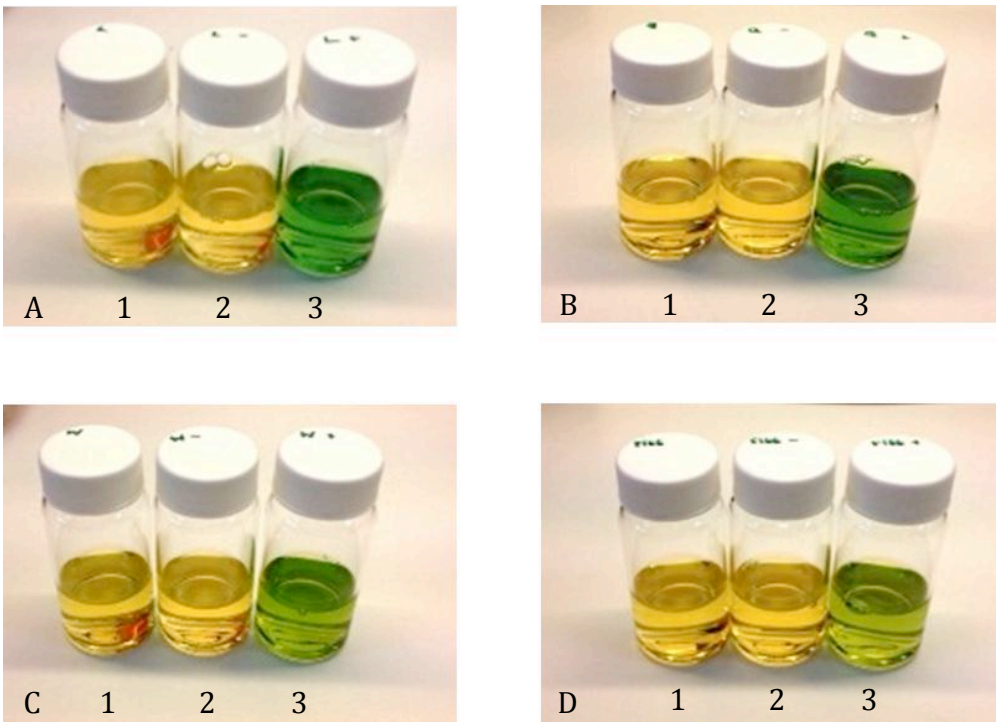


Figure 4.13: Pyocyanin Production in PAO1 *pqsEind* Strains. Strains in which the IPTG-inducible *pqsE* deletion (2 and 3) had been introduced successfully were grown in LB broth with 1mg/ml streptomycin, in the absence (2) or presence (3) of 1mM IPTG. Addition of IPTG causes the overexpression of *pqsE*, in turn causing upregulation of pyocyanin production and resulting in the bright green colour of the supernatants shown above (3). Parent strains with functional *pqsE* genes were grown in LB for comparison (1). The images show 4 representative strains; PAO1-L (A), PAO1-L Δ*fleQ* (B), PAO1 Washington (C) and PAO1-W ΔPA5166 (D).

In all, 19 *pqsEind* strains were made. Unfortunately, attempts to make a PA01-L ΔPA3697 *pqsEind* strain were unsuccessful; of 600 colonies screened, none of these showed resistance to streptomycin, indicating that the double crossover event required to integrate the inducible *pqsE* deletion into the chromosome had not occurred. The selection of colonies was also complicated by the intrinsic resistance of the PAO1-L ΔPA3697 strain to chloramphenicol.

4.2.9 Western Blot Determination of LecA Expression.

The four in-frame deletion mutants, 18 transposon mutants and 19 *pqsEind* strains, alongside the wild type strains PAO1-L and PAO1-W, were analysed by western blotting for the presence of the LecA protein in order to validate the results obtained in the *lecA* promoter pulldowns. Winzer *et al.*[163] had previously carried out LecA western blot experiments across a range of time points, finding LecA production to be only just detectable early in growth. Once *lecA* expression had begun to occur at 7 hours, the amount of protein detected in cell lysates steadily increased until approximately 11 to 13 hours, after which it remained constant. For this experiment then, cell lysates of each strain – with induced *pqsE* expression where appropriate - were harvested from overnight cultures standardised to an OD_{600nm} of 1.0. The qualitative results of this assay are shown in Figure 4.14.

Based on analysis of the western blots, mutation of the transcriptional regulators AlgB, AmgR and DctD, along with the predicted transcriptional regulator PA3697, have little or no effect on *lecA* expression. However, mutation of FleQ, PA2117 or PA0178 leads to overexpression of *lecA* compared with the wild type strain, which can be increased further by the overexpression of *pqsE* in the strains.

Mutations in Vfr, PilR, MifR, BfmR, CbrB, PA2572, PA0141, PA0588, PA3729, PA0236, PA5208 or PA4132, on the other hand, reduce *lecA* expression to varying degrees compared with the wild type. Where the *pqsE* inducible deletion has been constructed in these strains, *lecA*

expression can be restored by overexpression of *pqsE*, but if *pqsE* expression is not induced then generally, LecA production is reduced further. This suggests that in these mutant strains, the action of PqsE masks the effect of the mutations slightly.

One exception to this can be seen when looking at the LecA production of the PA0588 mutant. In the single mutant, LecA production is reduced, suggesting that PA0588 behaves as a positive regulator of *lecA* expression. In the ΔPA0588 *pqsEind* strain, induction of *pqsE* overexpression results in overexpression of LecA, again as expected. However, when *pqsE* expression is not induced in the ΔPA0588 *pqsEind* strain, LecA production is increased to a level greater than that that of the ΔPA0588 parent strain. This is an intriguing result, as it suggests that PA0588 may be involved in the PqsE-independent regulation of *lecA* via a potentially complex mechanism. However, it must also be considered that this finding could be an experimental anomaly, and therefore the experiment will need to be repeated in order to confirm this result.

In this study, LecA production is abolished when AlgP or GbuR are mutated, and is almost abolished in the *glpR* mutant. In the PAO1-W Δ*glpR* *pqsEind* strain not expressing *pqsE*, *lecA* expression is completely abolished, again suggesting that PqsE masks the effects of the GlpR mutation. For the GlpR and AlgP mutant strains, *pqsE* overexpression restores the production of LecA to higher than wild type levels.

However, it must be noted that the basal *pqsE* expression levels were not determined for the single mutants within this study, therefore it is not possible to know whether the single mutations themselves have an effect on *pqsE* expression, rather than vice versa.

In some instances where *pqsE* is overexpressed, two bands are observed on the LecA western blots. Whilst the lower band at approximately 13 kDa corresponds to the LecA monomer, the upper band at approximately 20 kDa is more difficult to explain. The polypeptide may have arisen due to the use of polyclonal anti-LecA antibody, which may recognise other bacterial proteins containing epitopes similar to those found in LecA. However, it is interesting that this second 20 kDa

polypeptide is only observed when *pqsE*, and therefore *lecA*, is overexpressed, although it is not present in the all *pqsE* overexpressing samples. Another explanation may be that the band represents the LecA dimer, which would be present at around 25 kDa. At around 20 kDa, the unknown protein may be slightly too small to be a LecA dimer; however, if this band does transpire to be a LecA dimer it would indicate that the levels of LecA in the samples displaying two bands are significantly higher than in those samples containing only the single 13 kDa band.

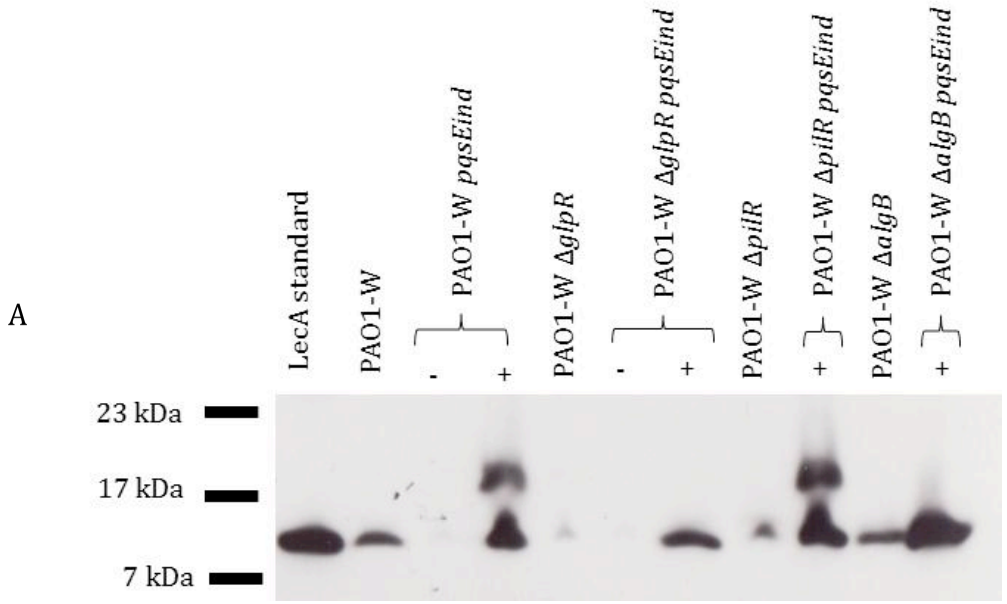
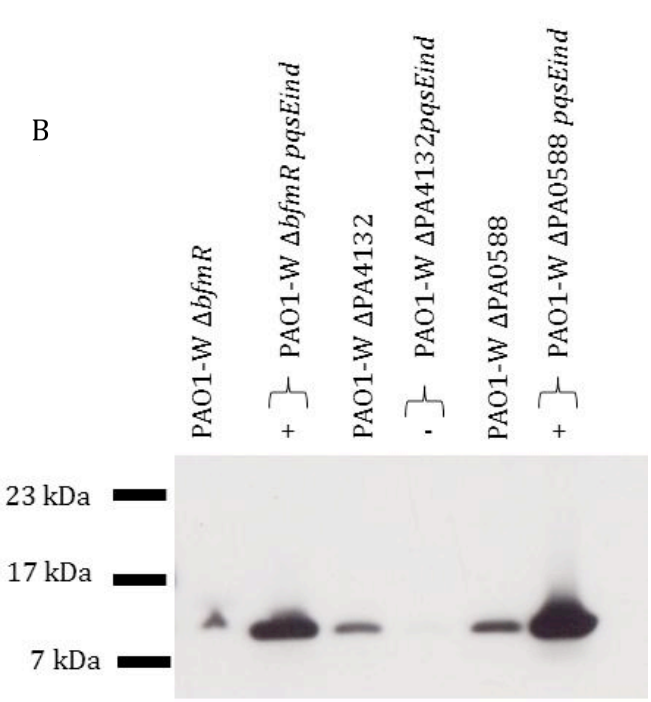
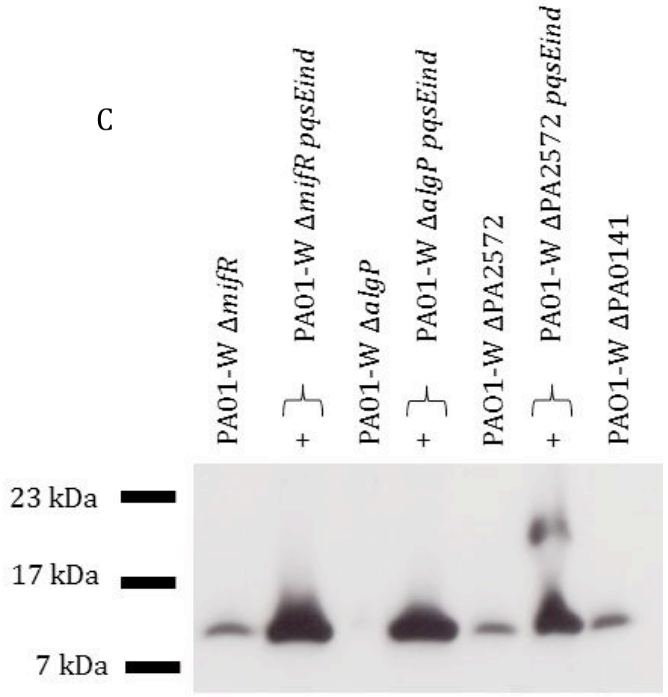


Figure 4.14: Determination of LecA Production. Cell lysates obtained from wild type PAO1 Lausanne (PAO1-L), wild type PAO1 Washington (PAO1-W), the PAO1-L in-frame deletion mutants, PAO1-W transposon mutants and *pqsEind* strains were analysed by western blot for the presence of LecA. Purified LecA standard was included as a positive control, and the protein was identified at approximately 13 kDa, as expected. A second band is observed in some samples at approximately 20 kDa, which may be due to the binding of the polyclonal antibody to other bacterial proteins, or possibly the presence of a LecA dimer. Using PAO1-W and PAO1-L as reference points, many of the mutant strains show altered LecA expression as a result of the in-frame deletion or transposon insertion. Uninduced *pqsEind* strains not expressing *pqsE* (-) generally produced LecA at very low levels, whilst IPTG-induced strains overexpressing *pqsE* (+) produced markedly increased LecA in comparison with their parent strains. The experiments were repeated twice with similar results, and representative blots are shown here. An example SDS-PAGE gel image has been included (J(ii)) corresponding to western blot J(i), to demonstrate equal protein loading across wells; this gel is representative of all those obtained during these experiments. The Figures are continued on subsequent pages.

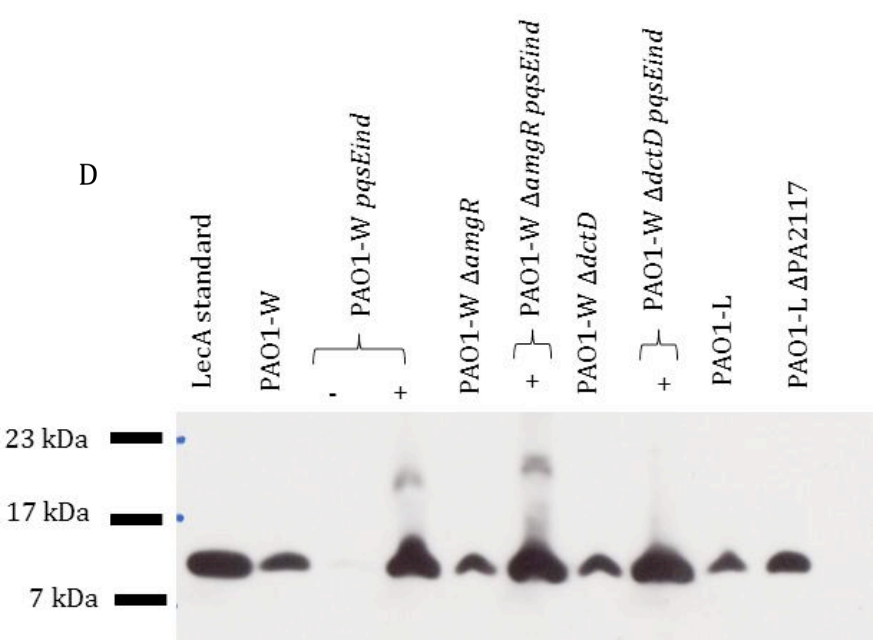
B


PAO1-W Δ*bfmR*
+
PAO1-W Δ*bfmR pqsEind*
PAO1-W ΔPA4132
-
PAO1-W ΔPA4132*pqsEind*
PAO1-W ΔPA0588
+
PAO1-W ΔPA0588 *pqsEind*
23 kDa
17 kDa
7 kDa

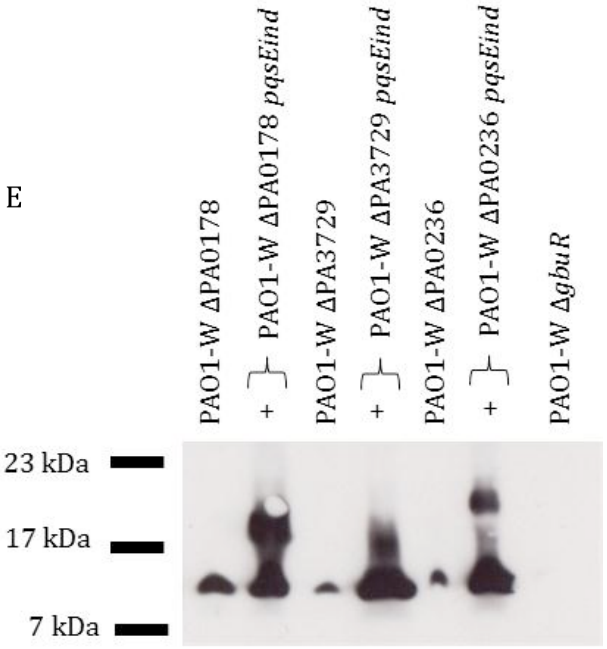
C


PA01-W Δ*mifR*
+
PA01-W Δ*mifR pqsEind*
PA01-W Δ*algP*
+
PA01-W Δ*algP pqsEind*
PA01-W ΔPA2572
+
PA01-W ΔPA2572 *pqsEind*
PA01-W ΔPA0141
23 kDa
17 kDa
7 kDa

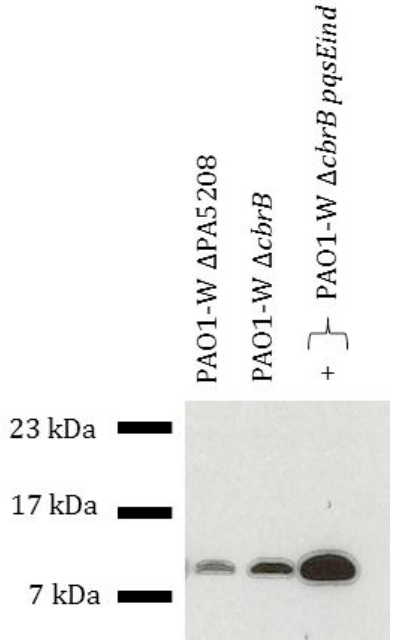
D


LecA standard
PAO1-W
PAO1-W pqsEind
-
+
PAO1-W ΔamgR
PAO1-W ΔamgR pqsEind
+
PAO1-W ΔdctD
PAO1-W ΔdctD pqsEind
+
PAO1-L
PAO1-L ΔPA2117
23 kDa
17 kDa
7 kDa

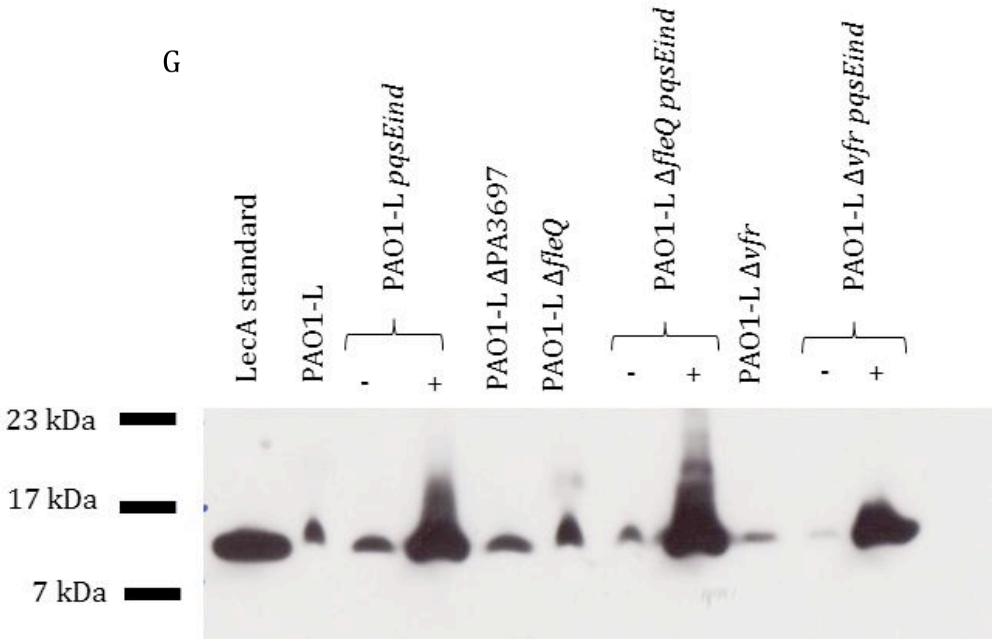
E


PAO1-W ΔPA0178
PAO1-W ΔPA0178 pqsEind
+
PAO1-W ΔPA3729
PAO1-W ΔPA3729 pqsEind
+
PAO1-W ΔPA0236
PAO1-W ΔPA0236 pqsEind
+
PAO1-W ΔgbuR
23 kDa
17 kDa
7 kDa

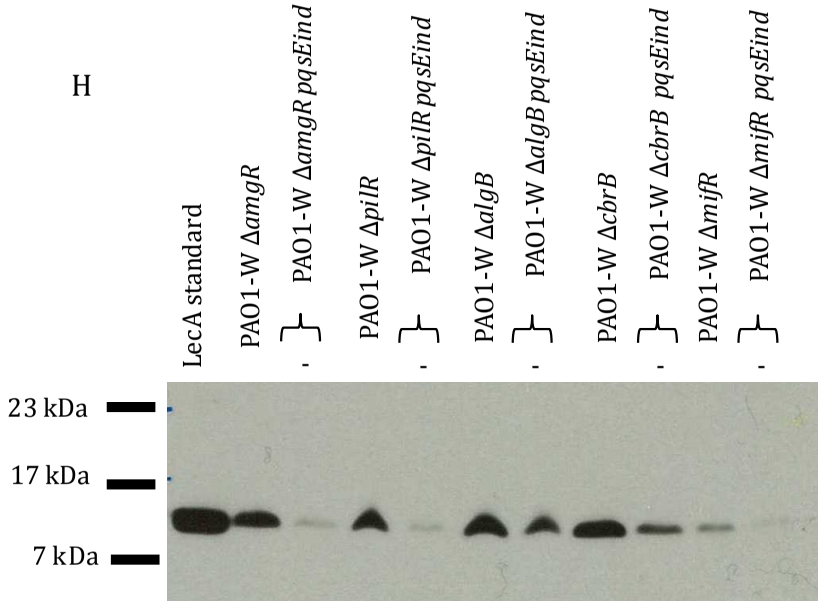
F


PAO1-W ΔPA5208
PAO1-W Δ*cbrB*
PAO1-W Δ*cbrB pqsEind*
+
23 kDa
17 kDa
7 kDa

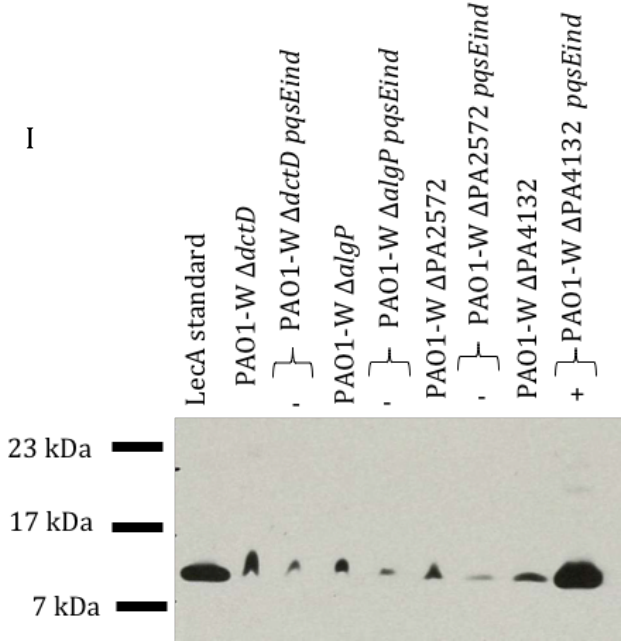
G


LecA standard
PAO1-L
PAO1-L *pqsEind*
-
+
PAO1-L ΔPA3697
PAO1-L Δ*fleQ*
PAO1-L Δ*fleQ pqsEind*
PAO1-L Δ*vfr*
PAO1-L Δ*vfr pqsEind*
23 kDa
17 kDa
7 kDa

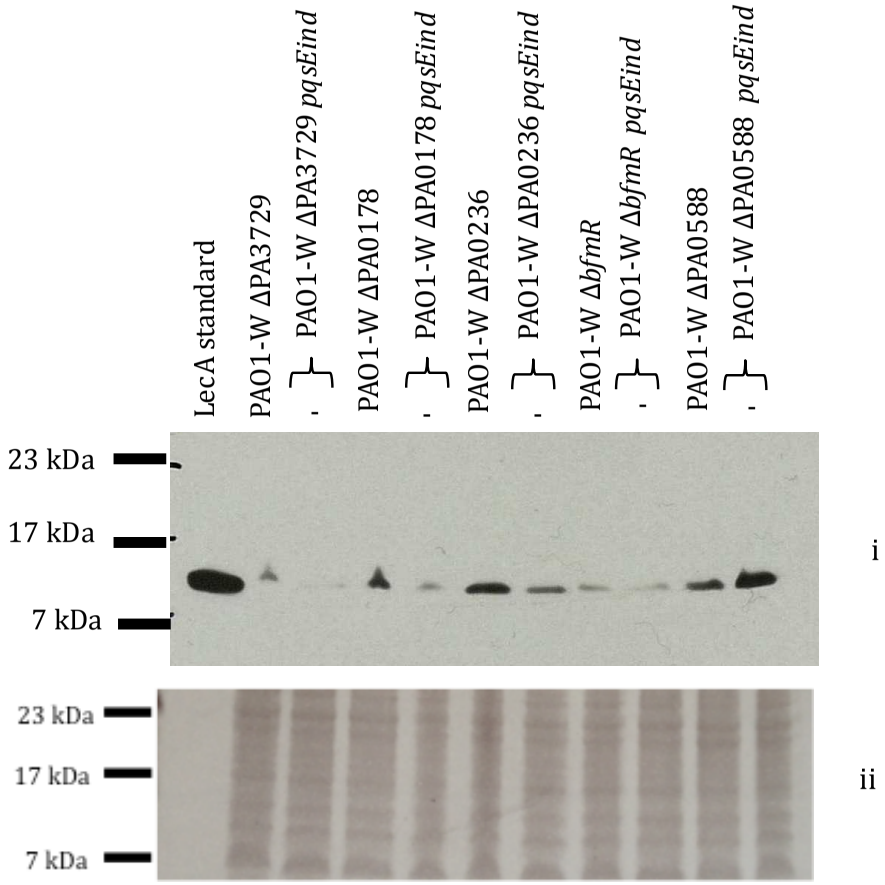
H


LecA standard
PAO1-W ΔamgR
PAO1-W ΔamgR pqsEind
-
PAO1-W ΔpilR
PAO1-W ΔpilR pqsEind
-
PAO1-W ΔalgB
PAO1-W ΔalgB pqsEind
-
PAO1-W ΔcbrB
PAO1-W ΔcbrB pqsEind
-
PAO1-W ΔmifR
PAO1-W ΔmifR pqsEind
-
23 kDa
17 kDa
7 kDa

I


LecA standard
PAO1-W ΔdctD
PAO1-W ΔdctD pqsEind
-
PAO1-W ΔalgP
PAO1-W ΔalgP pqsEind
-
PAO1-W ΔPA2572
PAO1-W ΔPA2572 pqsEind
-
PAO1-W ΔPA4132
PAO1-W ΔPA4132 pqsEind
+
23 kDa
17 kDa
7 kDa

J


LecA standard
PAO1-W ΔPA3729
PAO1-W ΔPA3729 pqsEind
PAO1-W ΔPA0178
PAO1-W ΔPA0178 pqsEind
PAO1-W ΔPA0236
PAO1-W ΔPA0236 pqsEind
PAO1-W ΔbfmR
PAO1-W ΔbfmR pqsEind
PAO1-W ΔPA0588
PAO1-W ΔPA0588 pqsEind
23 kDa
17 kDa
7 kDa
i
23 kDa
17 kDa
7 kDa
ii

The results of this experiment show that, of the 22 mutants tested, all but four show altered LecA production, as summarised in Table 4.5.

Table 4.5. Summary of LecA Western Blot Results. In this schematic, colours correspond to the relative amounts of LecA produced by each mutant strain compared with the either the PAO1-L or PAO1-W wild type strain. Pale green – *lecA* not expressed; bright green – reduced *lecA* expression; yellow – normal *lecA* expression similar to wild type; orange – increased *lecA* expression; red – highly increased *lecA* expression. Each PAO1 strain expressed either the *pqsE* gene at wild type levels (WT *pqsE*), or carried the *pqsEind* conditional mutation. *pqsEind* strains did not produce PqsE, while *pqsEind* strains grown with IPTG (*pqsEind* +IPTG) overexpressed *pqsE*. Some mutant strains were not analysed (✗) for LecA production under certain conditions because the cognate candidate protein was not observed under those conditions in the *lecA* promoter pulldown.

Strain \ PqsE phenotype	WT *pqsE*	*pqsEind*	*pqsEind* + IPTG
PAO1-L			
PAO1-W			
PAO1-L ΔPA3697		✗	✗
PAO1-W Δ*algB*			
PAO1-W Δ*amgR*			
PAO1-W Δ*dctD*			
PAO1-L Δ*fleQ*			
PAO1-L ΔPA2117		✗	✗
PAO1-W ΔPA0178			
PAO1-L Δ*vfr*			
PAO1-W Δ*glpR*			
PAO1-W Δ*pilR*			
PAO1-W Δ*mifR*			
PAO1-W ΔPA2572			
PAO1-W ΔPA0141		✗	✗
PAO1-W Δ*bfmR*			
PAO1-W ΔPA4132			
PAO1-W ΔPA0588			
PAO1-W ΔPA3729			
PA01-W ΔPA0236			
PAO1-W Δ*cbrB*			
PAO1-W ΔPA5208		✗	✗
PAO1-W Δ*algP*			
PAO1-W Δ*gbuR*		✗	✗

Highly increased LecA

Increased LecA

Wild type LecA

Reduced LecA

No LecA

✗ - Not tested

The identification of so many proteins involved in the regulation of *lecA* expression not only serves to validate the efficacy of the promoter pulldown technique in identifying novel regulators of gene expression, but it also provides new targets for further investigation. A number of these have previously been shown to be involved in biofilm development and regulation of mucoidy, important aspects of *P. aeruginosa* virulence.

That LecA production is increased so drastically when *pqsE* is overexpressed, but is diminished in the *pqsE* mutant is in line with the results previously described by Rampioni *et al.*[152]. The wild type PAO1–W strain and the PAO1-W *pqsEind* strain both behave as expected under the tested conditions (Figure 4.14(A)). The PAO1-L *pqsEind* strain, however, seems to produce LecA in the absence of PqsE, although this may be an experimental anomaly since, when *pqsE* expression in the strain is induced, the LecA production appears to be as expected (Figure 4.14(G)). Furthermore, the results also appear to demonstrate the different relationships that exist between the candidate proteins and PqsE, since the extent to which PqsE restores LecA expression in a mutant strain is dependent upon the particular mutation.

4.3. Discussion.

The expression of the *lecA* gene is under the control of a complex regulatory network spanning the *las* and *rhl* systems, the PQS system, and the Gac-Rsm signal transduction pathway[118,143,163,177]. The high level of regulation surrounding the transcription of the *lecA* gene serves to ensure the product of this gene, LecA, is only produced in a growth phase-dependent manner, upon onset of stationary phase[163], although the reason for this is not clear. Whilst a number of regulators involved in *lecA* expression are now known, this study set out to determine whether any of these directly regulate the *lecA* promoter, and, in the process, attempt to identify novel regulators.

Promoter pulldown experiments were used to study interactions between *P. aeruginosa* cellular proteins and the *lecA* promoter region *in vitro* at specific time points during growth in an isolated manner, focusing on the point at which *lecA* expression is at its highest, as well as time points prior to and after the peak of expression. During optimisation of the promoter pulldown technique, it became clear that the level of non-specific binding in the experiment is extremely high, creating a lot of 'noise' that must be discounted in order to effectively identify any potential regulatory proteins that might be binding the *lecA* promoter specifically. Nonetheless, experiments comparing the protein interactions with the *lecA* promoter across time points were encouraging, with some reproducibility over time and the detection of the binding of transcriptional regulators known to be involved in *lecA* regulation, such as RhlR and the negative regulator MvaT; however, RpoS was not observed bound to the *lecA* promoter under any conditions, despite its role in *lecA* expression and the presence of RpoS consensus sequences in the promoter region[118,163]. LasR was also found to bind the *lecA* promoter, although this only occurred when *pqsE* was overexpressed. Expression of the *lecA* gene is not LasR-dependent, therefore the binding of this protein to the promoter may be simply due to non-specific recognition of the *lux box*. However, it would be interesting to investigate further whether LasR

binds the *lecA* promoter in combination with RhlR only under specific conditions, and the impact this may have on *lecA* overexpression

Unexpectedly, MvaT, and its partner protein, MvaU, appeared to strongly bind the *lecA* promoter across all time points and under all conditions tested. This prompted the question as to whether this would occur *in vivo*, or whether it was an experimental artefact produced as a result of the artificial conditions *in vitro*. MvaT is able to form large multimers upon binding DNA, and in the process represses transcription[321,359], and so promoter pulldowns were carried out using an *mvaT* mutant strain to determine whether MvaT was blocking *lecA* promoter binding sites in this manner. The findings suggested that this was not the case, although the presence of MvaU, which is able to complement MvaT function to some extent, complicated matters somewhat. The mutation of both MvaT and MvaU would be required to properly determine whether they abrogate the binding of other proteins during *lecA* promoter pulldowns. However, previous studies investigating the roles of MvaU and MvaT have disagreed on whether it is possible to create a viable *P. aeruginosa mvaT mvaU* double mutant strain[321,326].

Despite the high levels of MvaT and MvaU binding to the *lecA* promoter, a large number of candidate proteins were nevertheless identified in interactions with the DNA fragment, and indeed this number increased further when the experiment was carried out using the *pqsEind* strain overexpressing *pqsE*. The fact that the binding of both characterised and predicted transcriptional regulators to the *lecA* promoter increased so significantly when a single variable - *pqsE* expression - was altered suggests that although a high proportion of proteins identified by LCMS/MS appear to be non-specific, there is also a smaller proportion of important specific interactions occurring. The increase in protein binding seen under conditions of increased *pqsE* expression ties in with what is already known about the global effects of PqsE on the cell; in microarray experiments using the PAO1-N *pqsEind* strain, induction of *pqsE* expression via the addition of IPTG affects the expression of 237 genes, including *lecA*, which was upregulated in the presence of PqsE[152]. This is

reflected in the promoter pulldown results; overexpression of *pqsE* causes a marked influx of proteins to the *lecA* promoter *in vitro*, although this may simply be due to the downstream effects of PqsE overexpression causing an overall increase in protein levels within the cell, and thus a proportional increase in the level of protein bound to the DNA fragment, potentially non-specifically. This might be demonstrated by the fact that PqsE only bound the *lecA* promoter when it was overexpressed, although the theory that PqsE could be involved in DNA-protein interactions cannot be discounted. One way to assess the specificity of protein-DNA binding in promoter pulldown experiments would be to repeat the experiment with negative control DNA, such as another promoter sequence unrelated to the *lecA* promoter.

Whilst many of the protein interactions with the *lecA* promoter in this experiment might not necessarily occur specifically, the presence of such a sheer number of transcriptional regulators is encouraging. This phenomenon, whereby the capacity of the *lecA* promoter to bind additional proteins increases so dramatically in the presence of PqsE overexpression, also hints that MvaT and MvaU may not be playing such a role in interfering with protein-DNA interactions as initially believed, since the intensity of the MvaT bands remained constant regardless of *pqsE* expression. Diggle *et al.*[134] previously showed that addition of exogenous PQS to *P. aeruginosa lecA-lux* cultures is able to overcome MvaT- and RsmA-mediated repression of *lecA* expression. Thus, it would be interesting to use the promoter pulldown technique to investigate whether the protein profile at the *lecA* promoter is altered in response to PQS.

The LCMS/MS protein identification results obtained for each of the promoter pulldown experiments in this Chapter included abundance values derived from the unweighted mass spectra in order to convey a general idea of how common a certain protein was in each band, compared with other proteins in the same band. According to these figures, the potential regulators bound to the *lecA* promoter were, for the most part, only present in very small amounts. However, in choosing the

candidate regulators of *lecA* expression to investigate further, these figures were disregarded, since they may have been slightly skewed by the presence of such large amounts of non-specific protein. It later became apparent that the abundance value of a particular protein has little relation to its effect on *lecA* expression; for example, the PA2117 protein was one of the most abundant at the *lecA* promoter throughout growth, but seemed to have little effect on LecA production compared with other less abundant candidate proteins.

From the protein identification results, 22 candidate proteins were chosen. These included known transcriptional regulators, some of which were well-characterised, and also a number of predicted, but uncharacterised, transcriptional regulators. In-frame deletion mutations were constructed within four of the candidate proteins in the PAO1-L background, whilst PAO1-W transposon mutants were obtained for the remainder. Since some candidate proteins were identified in the *pqsEind* background, an IPTG-inducible conditional mutation was also introduced into the *pqsE* genes of the appropriate strains.

The mutants were characterised by western blotting for the presence of LecA, in the presence or absence of IPTG to induce *pqsE* expression as required. PqsE is necessary for LecA production; since PQS acts as a coinducer of PqsR which then activates the *pqsABCDE* operon, it may be that the effect of PQS is transduced to the *lecA* promoter by PqsE via additional unknown regulators, particularly since PqsE is believed to mediate the cellular response to PQS[149,151]. However, when *pqsE* is overexpressed in the *pqsEind* strains, no HHQ or PQS is produced due to the PqsE-mediated repression of the *pqsA* promoter[152]. Despite this, LecA production in the induced *pqsEind* cell extracts analysed by western blot was markedly increased despite the lack of AQ production, confirming that *lecA* production is regulated in a PQS-independent manner by PqsE. Intriguingly, overproduction of PqsE was able to overcome the effect of every mutation tested in this study, while normal levels of *pqsE* expression masked the effects of the mutations such that they could only be fully observed in the absence of PqsE. This may suggest that the ability

of PQS to relieve MvaT repression of the *lecA* promoter[134] occurs due to the expression of *pqsE*. At the same time, the fact that MvaT remains bound to the *lecA* promoter *in vitro* despite PqsE overexpression implies that this may not be the case.

Of the candidate proteins investigated in this study, only four were found to have little or no effect on LecA production. These proteins were AlgB, AmgR and DctD, all characterised transcriptional regulators[351,360,361], and PA3697, a predicted transcriptional regulator recently found to regulate the response to nitric oxide[226]. Of the remaining candidate proteins, three appear to be negative regulators of *lecA* expression, whilst fifteen positively regulate *lecA*. This highlights the efficacy of the technique in the identification of novel regulators.

As expected, the PAO1-L *vfr* mutant produced lower levels of LecA than the wild type. However, since Vfr is a global regulator[156], this effect may be indirect via the quorum sensing system. Indeed, the Vfr consensus sequence was not identified in the *lecA* promoter during *in silico* analysis. PilR and CbrB were also found to positively regulate *lecA* expression. PilR is part of the PilSR two component system that regulates the expression of type IV pili[362], whilst CbrB, as part of the CbrA two component system, is proposed to indirectly regulate *P. aeruginosa* virulence via the small RNA *crcZ*[363]. In *Pseudomonas putida*, mutation of *cbrB* leads to the development of a highly aggregative phenotype and increased biofilm formation, along with an increase in the expression of genes encoding polysaccharide export and adhesive proteins[364]. This contradicts the results of the LecA western blot for the PAO1-W Δ*cbrB* strain, which showed reduced LecA production. However, inter-species differences may account for this contradiction; indeed, *P. putida* does not appear to possess any genes with significant similarity to *lecA* (data not shown).

MifR and BfmR are two of the proteins found to positively regulate *lecA* expression in this study. These transcriptional regulators have previously been characterised as two component response regulators of biofilm development. Interdependently with the GacA-GacS system, the MifSR and BfmSR two component systems regulate specific stages during

the creation of biofilms, forming a phosphorelay system with BfiSR. Each of the two component systems is responsible for a specific stage in biofilm development; after BfiSR has mediated irreversible attachment and initiation of biofilm formation, BfmSR and MifSR regulate the later stages, of biofilm maturation and microcolony formation respectively[355]. Considering that LecA has been shown to be involved in biofilm maturation, but not formation[27], and that BfmR and MifR, but not BfiR, were observed in the *lecA* promoter pulldown, the fact that mutation of *bfmR* and *mifR* leads to reduced production of LecA suggests that these proteins may play a role in the expression of LecA in biofilms.

A number of uncharacterised proteins were also investigated. LecA production was slightly reduced in the PA2117 mutant. Although this is a hypothetical protein, it has been shown to be downregulated in response to pyocyanin in *Pseudomonas chlororaphis*[365]. While pyocyanin has previously been shown to alter the expression of a small subset of genes in *P. aeruginosa*, including the *mexGHI-opmD* operon, the set of genes does not include PA2117[366]. Furthermore, a mutant in PA3729, part of the *bac* gene cluster, expresses reduced LecA despite this protein being a predicted outer membrane protein. Mace *et al*.[357] suggested that the *bac* genes might be important in *P. aeruginosa* virulence since deletion of PA3731 leaves the strain attenuated for virulence and highly defective in the formation of biofilms. Deletion of PA0588, a serine threonine kinase unlikely to be a direct transcriptional regulator[352], also shows similar results. However, the cryptic LecA production phenotype of the ΔPA0588 *pqsEind* strain overexpressing *pqsE* may hint at additional downstream events that add further complexity to what is already known. This study has also identified PA0236 and PA2572 as novel positive regulators of *lecA* expression, along with PA0141, PA0236, PA5208 and PA4132. However, whether these proteins truly regulate *lecA* through direct interaction with the promoter remains to be determined.

PA2572 is a two component response regulator believed to respond to signals from the orphan chemotaxis sensor PA2573[367]. However, McLaughlin *et al.*[367] found that mutation of PA2572 or PA2573

had no apparent affect on *lecA* expression, despite altered expression of other virulence factors. This contradicts the findings of this study. In addition, PA0236 is homologous to an *E. coli* repressor protein; in this study, however, PA0236 appears to positively regulate *lecA* expression. PA0236 is also downregulated in a *pqsA* mutant[152]. PA4132, a predicted transcriptional regulator containing a helix-turn-helix domain, has been identified as a core quorum-sensing controlled gene across most *P. aerginosa* isolates[368], therefore may transduce quorum sensing signals to the *lecA* promoter.

Mutation of the uncharacterised protein PA5208 has previously been shown to reduce swarming motility and biofilm formation[369]. In this study, PA5208 appears to positively regulate LecA suggesting that PA5208 is involved in *P. aeruginosa* virulence.

PA0178 has been identified as a negative regulator, although this is likely to be indirect since it is a predicted sensor kinase. PA0178 is part of the *P. aeruginosa che* I cluster, which encodes *E. coli*-like chemotaxis genes. In *P.* aeruginosa, the *che* I, II and V clusters have been shown to play a role in flagellar chemotaxis; disruption of this process leads to impaired flagellar chemotaxis and reduced surface attachment, thereby impairing the formation of biofilms. Chemotactic cues also mediate dispersion[370]. PA0178 may therefore be involved in the downregulation of LecA expression in order to reduce attachment during biofilm dispersion.

FleQ was identified as a candidate regulator early in the study, having been detected in pulldown experiments consistently, albeit with very low abundance values when compared with the values of the most abundant proteins in the same samples. A FleQ binding consensus sequence was also identified within the *lecA* promoter sequence. When LecA production by the PAO1-L *fleQ* mutant was investigated, it seemed apparent that FleQ is a negative regulator of *lecA*. FleQ also negatively regulates the *pel* operon in the absence of c-di-GMP[115], potentially making it an important determinant of *P. aeruginosa* biofilm formation. Interestingly, FleQ is negatively regulated by Vfr[158], and also by AmzR, which is itself positively regulated by AlgU[371]. This may explain the

positive effect of AlgU on *lecA* expression described by Bazire *et al.*[334], particularly since AlgU was not found to associate with the *lecA* promoter directly in this study. Furthermore, Balasubramanian *et al.*[336] found that the *algU* gene is negatively regulated by AmpR, potentially explaining the negative effect of AmpR on *LecA* production. AmpR has been shown to be regulated by the Gac-Rsm system[336], whilst in *Pseudomonas fluorescens*, FleQ has been shown to be negatively regulated by SadB as well as the Gac-Rsm system[372]. SadB has also been reported to inversely regulate biofilm formation and motility[373].

In this study, AlgP and GlpR, both identified as the most abundant proteins in their respective bands, and GbuR, are clearly the three most notable proteins. LecA production was abolished in PAO1−W *algP* or *gbuR* mutants, an effect that was not masked by PqsE expression. Coupled with the consistently high abundance of AlgP in the promoter pulldown LCMS/MS analysis results, the findings of the western blots for these proteins make for compelling evidence suggesting that the work presented here has identified potential novel regulators of *lecA,* opening new avenues for further investigation - particularly with respect to AlgP, given its role in mucoidy via the regulation of the *algD* promoter[348,349]. GbuR has been characterised as an RpoS-controlled gene; interestingly, swarming motility is also abolished when *gbuR* is mutated[166,374].

Mutation of the *glpR* gene reduced *lecA* expression significantly, such that it was barely detectable even in the PAO1-W background. GlpR has been shown to regulate the production of virulence factors, particularly in mucoid strains; mutation of *glpR* in a *P. aeruginosa* mucoid isolate led to a further increase in alginate production, and an increase in pyocyanin, pyoverdin and rhamnolipids[375]. Although the binding motif for GlpR has been characterised, it was not identified in the *lecA* promoter.

PvdS and NarL were not found to bind the *lecA* promoter, despite the presence of consensus sequences in the promoter for these proteins. This may be because PvdS and NarL are involved in regulating the response to iron and nitrate starvation respectively[341,342], and therefore

may not bind their target promoters under the culture conditions used in this study.

Thus, a revised hypothetical model for the regulation of *lecA* expression is presented in Figure 4.15, in which the *lecA* promoter is both positively and negatively regulated by additional mediators besides those previously described. These mediators involve BfmR and MifR, which positively regulate *lecA* expression during biofilm formation, and FleQ, which negatively regulates *lecA*. FleQ-mediated repression may be potentiated or relieved by the activities of AlgU, SadB, AmrZ and AmpR, interlinked with the Gac-Rsm system. Although the mechanism through which PqsE regulates *lecA* expression has not been elucidated, this study has found that the pathways governing the production of LecA by *P. aeruginosa* are likely to be more complex than was previously understood.

Further investigation would be required to confirm the effect of the transcriptional regulators identified on *lecA* expression. For example, gel shift assays would isolate the protein-DNA interactions further and allow the specificity of the interactions to be determined more accurately. The fact remains that the western blot results have validated the findings of the promoter pulldowns to a certain extent, since many of the mutants defective in the candidate proteins show altered LecA production phenotypes. Nonetheless, it would be interesting to elucidate the expression levels of *lecA* at a range of time points throughout growth in these mutants.

The easiest and most quantitative way to do this would be in *lecA-lux* bioreporter experiments, measuring the level of *lecA* expression as directly proportional to the level of light output over a period of time, and this would discount the effect of post-transcriptional regulation. However, *lecA-lux* bioreporter experiments were not feasible within the context of this study, because the miniCTX::*lecA-lux* bioreporter currently available is selectable only by tetracycline, which was problematic due to the fact that the transposon mutants also carry a tetracycline resistance marker as part of the IS*phoA*/hah and ISl*acZ*/hah transposons. Upon investigation, the insertion elements appeared to be removable using Cre-mediated

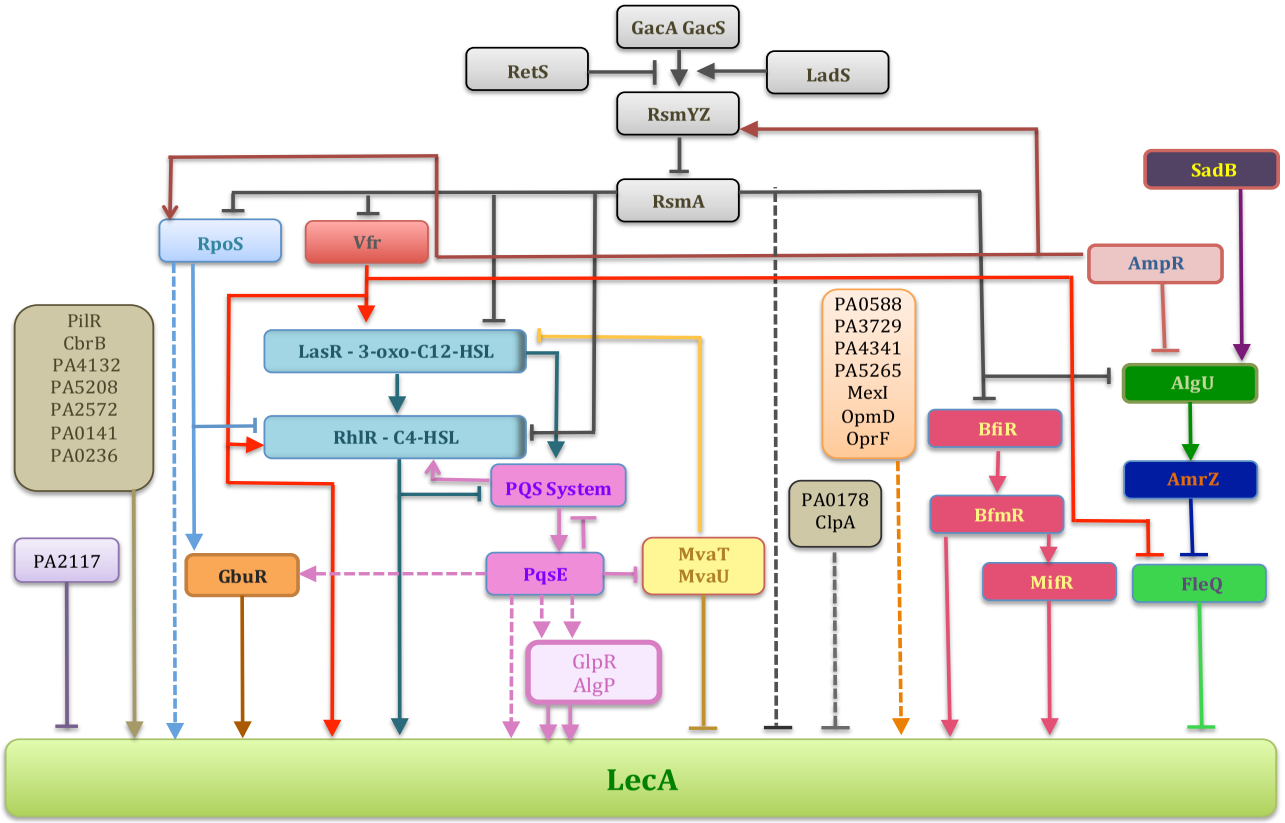


Figure 4.15. A Revised Model for the Regulation of *lecA* Expression. The *lecA* gene appears to be regulated directly and indirectly by numerous mediators, all under the ultimate control of the Gac-Rsm system. In this work, RpoS interaction with the *lecA* promoter was not observed, suggesting it may regulate *lecA* indirectly, perhaps via GbuR. PqsE has also been found to be a highly important determinant of *lecA* expression, though the mechanism through which this occurs is still unknown. LecA production may be inversely regulated with flagella by FleQ, itself under the negative control of Vfr and AlgU via AmrZ. AmpR may repress *lecA* expression in FleQ-dependent manner via repression of AlgU. BfmR and MifR may positively regulate the expression of *lecA,* particularly in biofilm development. Many regulators of lecA expression may bind the *lecA* promoter directly, including GlpR and AlgP. However, it is likely that PA0178 and PA0588, for example, regulate LecA production indirectly. Direct *lecA* regulation is represented by solid lines. Indirect *lecA* regulation via an unknown mechanism is represented by broken lines. Arrows - Positive regulation; Blunt-ended lines – Negative regulation [118,134,152,163,177,334,336,355,371,372]

recombination at the *loxP* sites at each end of the transposons; however, whilst this would remove the tetracycline resistance marker from the transposon strains, it would also leave behind a 63 bp codon sequence encoding a influenza haemaglutinin epitope and a hexa-histidine affinity purification tag within the target genes[217]. The genes would then be fully transcribed as full-length histidine-tagged proteins, most likely functional, with unknown downstream effects. Construction of a new *lecA*-*lux* reporter vector was not possible given the time constraints, and so western blot analysis was chosen as an alternative. Since the use of in-frame deletion mutants is preferable to the use of transposon mutants, future work would perhaps involve the construction of in-frame deletion mutants for those candidate proteins of most interest and the subsequent use of the *lecA*-*lux* reporter in these strains to gain a more accurate understanding of the function of these regulators.

Although the results of the western blots are encouraging, they give a holistic idea of LecA production at a single point in time; that is, the LecA production that has occurred at a single point in time as a result of the myriad upstream signalling events in several different networks. The effects of a specific protein on *lecA* regulation can be masked by the effects of other proteins, and this is evident when comparing the results of western blots carried out in the presence of *pqsE* overexpression or PqsE depletion with those carried out under conditions of normal *pqsE* expression. Clearly, PqsE has such a pronounced effect on *lecA* expression that it is able to overcome the effects of mutations in other regulators, therefore masking the functions of those proteins in the wider regulatory network. This convergence of multiple regulatory pathways to produce a single outcome poses a particular problem when studying a microorganism as complex as *P. aeruginosa*.

4.4. Conclusions.

The work presented in this Chapter aimed to look further into the regulatory network controlling the expression of the *lecA* gene, which encodes an important virulence factor involved in the maturation of *P. aeruginosa* biofilms. In promoter pulldown experiments using the *lecA* promoter as bait, a large number of candidate regulatory proteins were detected bound to the promoter. To investigate these candidate regulators, deletion mutants were constructed and western blots carried out to determine their LecA production phenotypes. These experiments validated the results of the promoter pulldowns, confirming that a large number of the candidate proteins do indeed have a regulatory effect on *lecA* expression. PqsE is absolutely required for LecA production, and the work described here supports this. The positive effect of PqsE on the *lecA* promoter is potent enough to overcome nearly all mutations in candidate regulators; however, mutation of two proteins, AlgP and GbuR, abolished LecA production even in the presence of basal levels of PqsE. These two proteins are therefore prime candidates for further investigation, since they may transduce PqsE effect to the *lecA* promoter. In all, the results of this study have opened new avenues of investigation with respect to *P. aeruginosa* regulatory control of virulence by proposing new upstream pathways that link *lecA* expression to the expression of biofilm components.

Chapter Five.

The PqsE-Mediated Repression of the *pqsA* Promoter.

5.1. Overview.

The *Pseudomonas aeruginosa* 2-alkyl-4-quinolone (AQ) system is intricately interlinked with the *rhl* and *las* quorum sensing systems and centres around the two predominant AQ signalling molecules, HHQ and PQS[149,150]. The synthesis of HHQ and PQS is coordinated from the *pqsA* promoter by PqsR and RhlR[145,376].

5.1.1. The Regulation of the *pqsA* Promoter.

Ultimately, the *pqsA* promoter is positively regulated indirectly by the *las* quorum sensing system, which also activates *pqsH* and *pqsR* expression. PqsH activity then increases the conversion of HHQ into PQS, which activates PqsR and in turn allows it to induce the transcription of the *pqsABCDE* operon via the *pqsA* promoter[149,150]. PqsR, a LysR-type transcriptional regulator (LTTR), directly binds the *pqsA* promoter via a LTTR consensus binding sequence centred 121 bp upstream from the transcriptional start site, as shown in Figure 5.1[376]. A transcriptional start site has been mapped to the −71 position relative to the ATG start codon of the *pqsA* gene[145,376,377]. However, Doetsch *et al.*[378] mapped a second transcriptional start site to the −339 position relative to the ATG start codon. In the same study, an additional transcriptional start site was mapped 31 bp upstream of the *pqsB* open reading frame, within the *pqsA* gene.

The *pqsA* promoter region also contains two *lux* box-like sequences. Although Wade *et al.*[145] found that neither LasR nor RhlR bind the *pqsA* promoter, Xiao *et al.*[377] have identified the distal *lux* box relative to the *pqsA* gene as the binding site through which RhlR represses *pqsA* promoter activity. This is located 311 bp upstream of the *pqsA* start codon.

However, LasR binding to the *pqsA* promoter has not been demonstrated, and the second *lux* box is not required for *pqsA* regulation[377].



Figure 5.1. The *pqsA* Promoter. The region directly upstream of the *pqsA* open reading frame (red) contains several regulatory elements. Two transcriptional start sites (TSS, purple) have been identified at the -71 and -339 positions relative the beginning of the *pqsA* open reading frame. An LTTR-specific motif (green) is present 121 bp upstream of the *pqsA* start codon, allowing PqsR interaction with the *pqsA* promoter. Two lux boxes have also been identified, (orange) although only the distal *lux* box 1 is believed to be involved in the regulation of *pqsA* by RhlR. Arrows indicate primer annealing sites used to amplify the promoter region for promoter pulldowns, both in pilot experiments and this study [145,377,378].

5.1.2. PqsE-Mediated Regulation of *pqsA* Promoter Activity.

In addition, Rampioni *et al*.[152] found that PqsE is able to negatively regulate the transcription of the *pqsABCDE* operon. The uninduced PAO1−N *pqsEind* conditional mutant, which does not produce PqsE, is still able to produce HHQ and PQS confirming that PqsE is not involved in the biosynthesis of these signal molecules. However, overexpression of *pqsE* abolishes the production of PQS and HHQ[149,152]. That PqsE is also required for the response of cells to PQS[150] and has been shown to regulate the production of pyocyanin, rhamnolipids and elastase in a RhlR-dependent manner[151], as well as being involved in AQ-independent *lecA* expression and swarming motility[152] suggests an important regulatory role for the protein in *P. aeruginosa* virulence. However, the mechanism through which PqsE regulates the expression of target genes remains to be elucidated.

Since PqsE is unlikely to be a DNA-binding protein, the effect of PqsE at the *pqsA* promoter region is likely to be indirect, the result of a chain of events possibly involving additional mediators that transduce the effect of PqsE to the target promoter[152]. To investigate this, promoter pulldown experiments were previously carried out in this laboratory using PAO1-N *pqsEind* cytoplasmic cell extracts harvested from overnight cultures in combination with the *pqsA* promoter region illustrated in Figure 5.1 (G. Rampioni, personal communication). The experiment revealed six candidate proteins that bound the promoter under different conditions. AsrA, an aminoglycoside-induced stress response protease[379] and PhpA, an aminopeptidase that is able to bind DNA and alter *algD* expression[380] were identified only in promoter pulldown samples obtained using wild type and uninduced *pqsEind* cell extracts. ClpC, a predicted chaperone protein, NirQ, a transcriptional regulator involved in the denitrification response[381], and PA4843, a predicted two component response regulator were identified only in those pulldown experiments using uninduced *pqsEind* cell extracts. Only one protein, PhaF, was found to bind the *pqsA* promoter when *pqsE* overexpression was induced. PhaF

has been discussed in Chapter 4, where it was consistently identified bound to the *lecA* promoter under all conditions tested and across all timepoints, and consequently classified as a non-specific DNA-binding protein due to its role in binding the chromosome during polyhydroxyalkanoate granule segregation in cell division[343,344]. In the *pqsA* promoter pulldown, however, PhaF appeared to bind the *pqsA* promoter at a high level specifically under a single condition and so it was included as a candidate protein for further investigation. A number of additional proteins were identified in this initial pulldown, but not studied further; for example, LasR, RhlR and PqsR were identified bound to the *pqsA* promoter when *pqsE* was not expressed, but not when *pqsE* was overexpressed, which is interesting given that *pqsA* promoter activity is repressed when *pqsE* is overexpressed[145].

In-frame deletion mutations were constructed in this laboratory within the genes encoding the six candidate proteins AsrA, PepA, ClpC, NirQ, PhaF and PA4843, both in the wild type PAO1-N and the PAO1-N *pqsEind* backgrounds, and *pqsA-lux* reporter studies were carried out to determine the effects of the mutations on the activity of the *pqsA* promoter. Unfortunately, this experiment concluded that none of the candidate proteins were involved in the transduction of the PqsE effect to the *pqsA* promoter. In the mutants created in the PAO1-N background, the deletions had little, if any, effect on the activity of *pqsA-lux*. In the mutants created within the *pqsEind* background, the deletion of the candidate proteins reduced the *pqsA* promoter activity only in the absence of PqsE. (G. Rampioni, personal communication) These results suggested that, although the candidate proteins act as positive regulators of the *pqsA* promoter, their effect is masked in the PAO1−N background by the negative effect of *pqsE*. Furthermore, overexpression of *pqsE* in the candidate mutant strains still abolished *pqsA* promoter activity, indicating that none of the candidate proteins were involved in the PqsE-mediated *pqsA* repression. The ability of PqsE to mask events occurring at the *pqsA* promoter reflects the findings discussed in Chapter 4, where PqsE was

able to mask the effects of mutations in potential regulators of *lecA* expression.

At the *pqsA* promoter, this masking effect may occur due to the ability of PqsE to autoregulate its own expression. In the wild type *P. aeruginosa* strain, the expression of the *pqsABCDE* operon leads to the production of PqsE, which then represses the transcription of the whole operon via the *pqsA* promoter, including the *pqsE* gene itself. This maintains the homeostasis of AQ production. When a potential positive regulator of the *pqsA* promoter is mutated, *pqsABCDE* transcription is reduced as a result. This leads to a concomitant reduction in PqsE-mediated repression of *pqsA* promoter activity and hence, in the wild type background, *pqsA*-*lux* expression remains similar between the wild type and mutant strains. However, in the absence of PqsE, the effect of these mutations in candidate positive regulators of the *pqsABCDE* operon becomes apparent due to the complete removal of PqsE-mediated repression.

Interestingly, PqsR, the transcriptional regulator of the *pqsABCDE* operon, was not detected bound to the *pqsA* promoter when *pqsE* was overexpressed in the promoter pulldown experiments. When considered alongside experiments previously carried out in this laboratory, a tentative hypothesis for the PqsE-mediated repression of the *pqsA* promoter began to emerge (G. Rampioni, personal communication). In a Δ*pqsA*Δ*pqsH*Δ*pqsLpqsEind* strain that is unable to synthesise AQs but expresses *pqsR*, addition of exogenous HHQ and PQS is able to induce the expression of *pqsA*-*lux*. However, when *pqsE* expression is induced, this AQ-mediated induction does not occur, suggesting that PqsE may be able to impair the PqsR-dependent activation of the *pqsA* promoter by HHQ and PQS.

A recent study used rule-based computer modelling to suggest a mechanism for the PqsE-dependent regulation of virulence factors such as pyocyanin. The model proposed that PqsE acts as a receptor for an autoinducer such as HHQ or PQS, and that formation of the complex would lead to upregulation of pyocyanin production[382]. However, a separate

study by Yu *et al.*[153] had previously demonstrated that PqsE does not form complexes with either PQS or AHLs. The model assumed that *pqsR, pqsBCD* and *pqsE* mutant strains would produce pyocyanin at maximum level, and therefore PqsE-mediated regulation must occur via interaction of PQS or HHQ with PqsE, dependent on PqsR[382]. However, this model is not supported by experimental evidence. PqsE-mediated upregulation of pyocyanin production occurs independently of PQS and PqsR, and *pqsE* mutants do not produce pyocyanin[151,152]. Thus, PqsE control of the *pqsA* promoter is likely to be via an unconventional mechanism.

5.1.3 Aims of this Chapter.

In this Chapter, the aim of the work presented was to investigate the events at the *pqsA* promoter in order to elucidate the role of PqsE in the repression of *pqsABCDE* transcription. Promoter pulldown experiments were carried out at specific timepoints using the *pqsA* promoter as bait. A number of candidate regulatory proteins that bound the promoter regions were subsequently identified. PAO1-W transposon mutants were obtained for these candidate proteins, and work carried out to modify an existing bioreporter to allow characterisation of their ability to regulate the *pqsA* promoter in both wild type *P. aeruginosa* and *pqsEind* backgrounds. This work is currently ongoing to develop a new *pqsA-lux* bioreporter that will allow validation of the promoter pulldown results presented here.

5.2. Results.

5.2.1. The Activity of the *pqsA* Promoter.

Previously, the *pqsA* promoter pulldowns had been carried out using cell extracts obtained from PAO1-N and PAO1-N *pqsEind* 16 hour overnight cultures (G. Rampioni, personal communication). However, according to growth and bioluminescence experiments using the *pqsA-lux* bioreporter, the peak of the *pqsA* promoter activity occurs after approximately 7.5 hours of growth (data not shown). This was determined using cultures grown statically in a microplate reader. Since the level of aeration may have an effect on the activity of the *pqsA* promoter[383], the expression experiment was repeated using PAO1-N and PAO1-N *pqsEind* cultures grown in culture flasks with agitation at 200 rpm to ensure effective aeration of the cultures. The strains were grown in the presence or absence of 1mM IPTG. Hourly readings were taken over a 12 hour period. The results, shown in Figure 5.2, showed that all the strains grew similarly. The activity of the *pqsA-lux* promoter fusion peaked in the PAO1-N strain following approximately 5 hours of growth, corresponding to an OD_{600nm} of 0.5; however, in the uninduced PAO1-N *pqsEind* strain, *pqsA* promoter activity peaked at a slightly higher level compared with the PAO1-N strain, and this level was also maintained for a longer period of time in the uninduced PAO1-N *pqsEind* strain. As expected, *pqsA-lux* expression in the induced PAO1-N *pqsEind* strains was negligible.

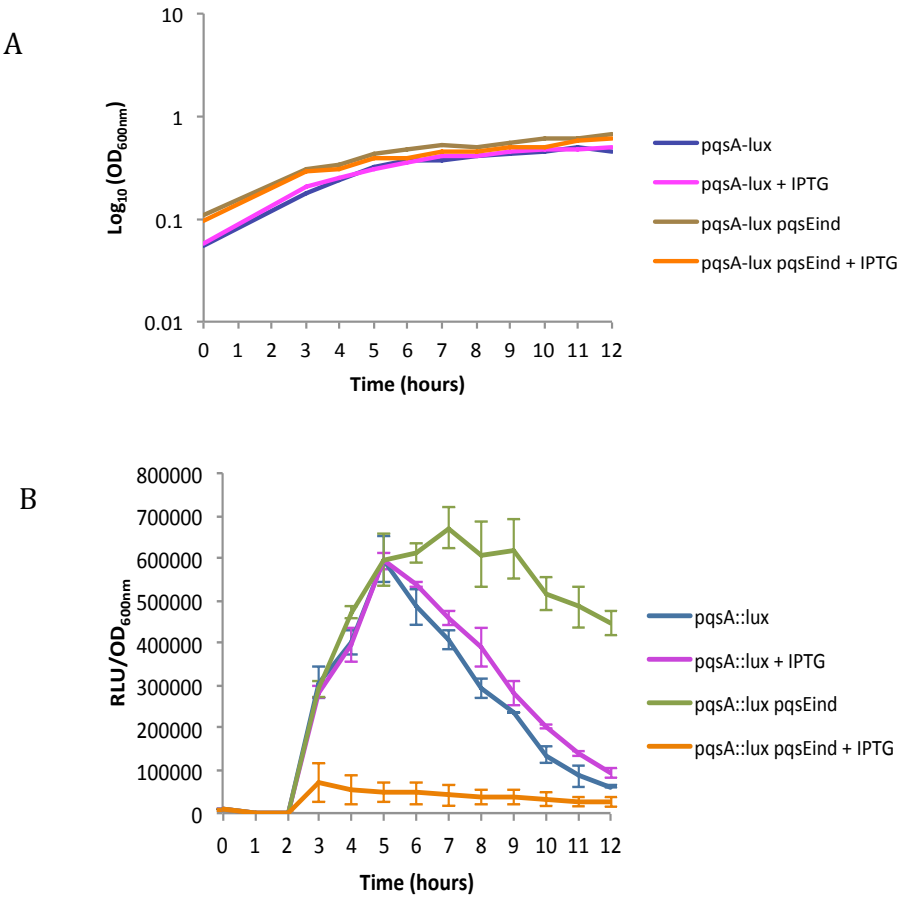


Figure 5.2. The Expression of *pqsA-lux*. PAO1-N (*pqsA::lux*)and PAO1-N *pqsEind* (*pqsA::lux pqsEind*) were grown in culture flasks at 37°C with shaking for 12 hours, in the presence (+IPTG) or absence (-IPTG) of 1 mM IPTG. Following inoculation of the culture media, bacteria were allowed to grow for 3 hours before hourly readings commenced. Both OD_{600nm} and the relative light units (RLU) were measured. Log_{10} growth curves (A) showed that all strains grew similarly in both the presence and absence of IPTG. The expression of the *pqsA-lux* promoter fusion was measured as proportional to RLU output during growth. RLU values were normalised to obtain the RLU per OD_{600nm} (RLU/OD), which is proportional to the amount of light produced per cell. The *pqsA-lux* expression curves (B) indicated that the peak of *pqsA* expression occur after ~5 hours of growth for the PAO1-N cultures. Expression of *pqsA* in the induced PAO1-N *pqsEind* strain peaked slightly later at approximately 7.5 hours. Error bars show 2x standard error calculated across three experimental replicates.

5.2.2. The Protein Profile at the *pqsA* Promoter.

The *pqsA* promoter pulldown experiments were therefore carried out using PAO1-N and PAO1-N *pqsEind* cell extracts harvested at OD_{600nm} 0.5, using the *pqsA* promoter region as bait. The promoter region was amplified from PAO1-N genomic DNA using the biotinylated primers B–PqsA_prof and B-PqsA-pror as shown in Figure 5.1. The PAO1-N *pqsEind* strain was grown both in the presence and absence of 1 mM IPTG for induction of *pqsE* expression. Protein samples obtained from the *pqsA* promoter pulldown experiments were resolved by SDS-PAGE, as shown in Figure 5.3. A very large number of bands were observed of varying densities in all of the *pqsA* promoter pulldown protein samples, many of which migrated very close together in SDS-PAGE. Although it was not possible to carry out protein identification analysis for all of the observed bands by LCMS/MS, representative bands of interest were excised for analysis. The bands chosen were those that appeared to change in density dependent upon *pqsE* expression. Particularly of interest was a cluster of three bands evident between approximately 46 kDa and 58 kDa. These were some of the most distinct protein bands observed, and these also showed the most notable differences in response to changes in *pqsE* expression. The LCMS/MS results are summarised in Table 5.1.

Figure 5.3. The Effect of PqsE on the Protein Profile at the *pqsA* Promoter. Promoter pulldowns were carried out using the *pqsA* promoter region in combination with cell extracts harvested from wild type PAO1 Nottingham (PAO1-N) and a PAO1-N with an IPTG-inducible *pqsE* conditional mutation (PAO1-N *pqsEind*) to determine the effect of normal *pqsE* expression (PAO1-N), *pqsE* overexpression (+IPTG), and absence of PqsE (-IPTG) on the protein profile at the *pqsA* promoter. *pqsE* overexpression was induced by the addition of 1mM IPTG to PAO1-N *pqsEind* cultures. Cell extracts were harvested at OD_{600nm} 0.5. Control experiments were carried out using either *pqsA* promoter DNA only or cell extract only. Bands excised for LCMS/MS analysis are indicated on the gel and colour-coded for clarity. The experiment was repeated three times to ensure reproducibility of the band migration pattern, and a representative gel is shown. Orange - PAO1-N bands; blue – PAO1-L *pqsEind* -IPTG; green – PAO1-L *pqsEind* +IPTG. The colour-coded numbers also correspond to the LCMS/MS results shown in Table 5.1.

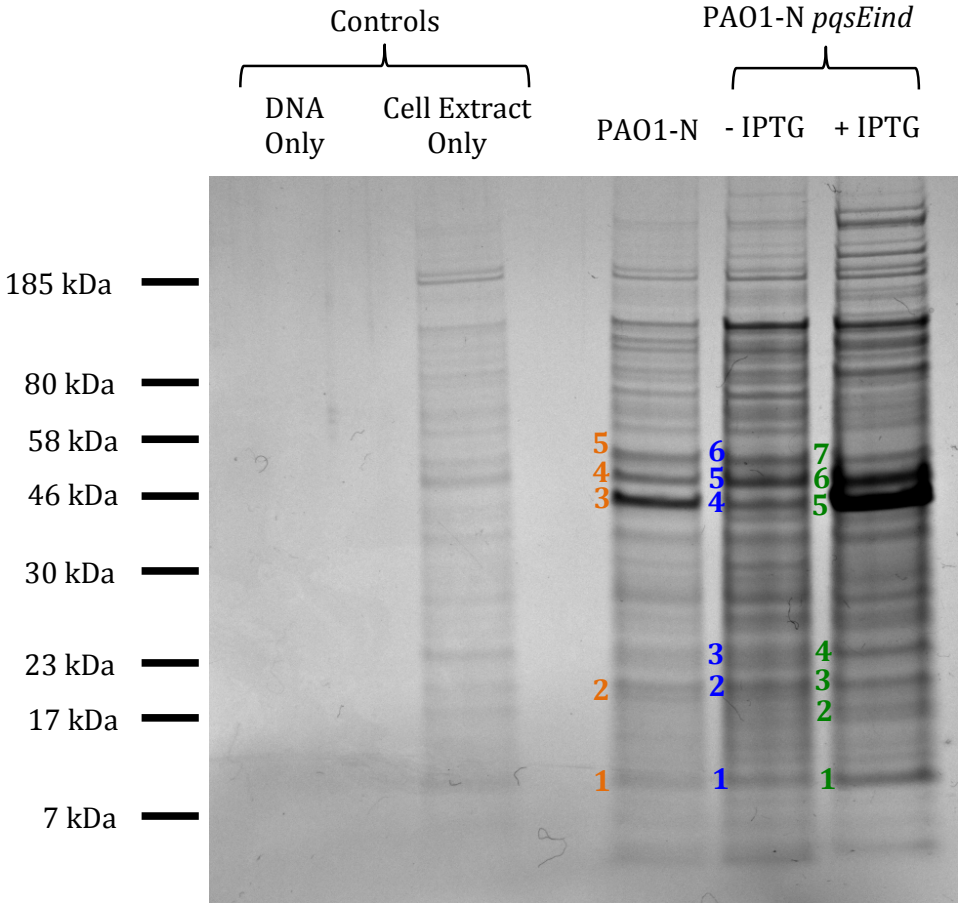


Table 5.1. LCMS/MS Identification of the Proteins Bound to the *pqsA* Promoter in the Presence of Different Levels of *pqsE* Expression. Protein bands excised from the SDS-PAGE gel shown in Figure 5.3 were analysed by LCMS/MS, identifying a mixture of proteins within each band. The band numbers and colours correspond to those shown in Figure 5.3; Orange - PAO1-N (A); blue – PAO1-N *pqsEind* –IPTG (B); green – PAO1-N *pqsEind* +IPTG (C). Proteins were classified according to PseudoCap function; for clarity, only uncharacterised proteins and proteins of predicted or confirmed regulatory function are shown here. Individual proteins were assigned a relative abundance value according to their unweighted LCMS/MS spectrum counts. Values in brackets represent the highest protein abundance value within the band to give an idea of scale. LCMS-MS analysis was carried out only once per band. The LCMS/MS results Tables are continued on subsequent pages.

A

PAO1-N					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	15 kDa	No Proteins of Interest			
2	23 kDa	Vfr	10	(10)	Transcriptional Regulator
		AguR	9		Transcriptional Regulator
		RoxR	8		Transcriptional Regulator
		PA3801	6		Uncharacterised Protein
3	46 kDa	PA2705	7	(28)	Uncharacterised Protein
4	50 kDa	ClpX	19	(27)	ATP-dependent Protease Subunit
		AlgP	15		Transcriptional Regulator
		AlgB	6		Transcriptional Regulator
		PilR	6		Transcriptional Regulator
		SadB	5		Transcriptional Regulator
5	57 kDa	FleQ	33	(33)	Transcriptional Regulator
		PA3455	11		Predicted Polyphosphate Kinase

B

PAO1-N *pqsEind* - IPTG					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	15 kDa	No Proteins of Interest			
2	23 kDa	Vfr	9	(11)	Transcriptional Regulator
		RoxR	8		Transcriptional Regulator
		ErdR	7		Transcriptional Regulator
		PA1894	7		Uncharacterised Protein
		Dnr	5		Transcriptional Regulator
3	27 kDa	RhlR	21	(21)	Transcriptional Regulator
		BfmR	6		Transcriptional Regulator
		PA5208	6		Predicted Transcriptional Regulator
		ParR	6		Transcriptional Regulator
		AmgR	6		Transcriptional Regulator
		PhoB	6		Transcriptional Regulator
		Anr	5		Transcriptional Regulator
4	46 kDa	No Proteins of Interest			
5	50 kDa	ClpX	23	(33)	ATP-dependent Protease Subunit
		AlgB	18		Transcriptional Regulator
		AlgP	17		Transcriptional Regulator
		DctD	13		Transcriptional Regulator
		PilR	8		Transcriptional Regulator
		SadB	6		Transcriptional Regulator
6	57 kDa	FleQ	15	(28)	Transcriptional Regulator
		PA3455	13		Predicted Polyphosphate Kinase
		FleR	9		Transcriptional Regulator
		CbrB	6		Transcriptional Regulator

C

PAO1-N *pqsEind* + IPTG					
Band	**Band Size**	**Protein**	**Relative Abundance**		**Function**
1	15 kDa	No Proteins of Interest			
2	20 kDa	PA0171	9	(16)	Uncharacterised Protein
		PA4352	8		Predicted Stress Protein
		DadR	6		Transcriptional Regulator
3	23 kDa	ClpP2	14	(14)	ATP-dependent Protease Subunit
		ErdR	8		Transcriptional Regulator
		PA2731	7		Uncharacterised Protein
		Vfr	7		Transcriptional Regulator
		PA2066	6		Uncharacterised Protein
		RoxR	6		Transcriptional Regulator
4	27 kDa	RhlR	21	(21)	Transcriptional Regulator
		Anr	5		Transcriptional Regulator
		BfmR	5		Transcriptional Regulator
5	46 kDa	PA2705	5	(55)	Uncharacterised Protein
6	50 kDa	ClpX	33	(33)	ATP-dependent Protease Subunit
		AlgP	15		Transcriptional Regulator
		DctD	8		Transcriptional Regulator
		PA5209	8		Uncharacterised Protein
		PilR	7		Transcriptional Regulator
		AlgB	6		Transcriptional Regulator
		SadB	5		Transcriptional Regulator
7	57 kDa	FleQ	20	(29)	Transcriptional Regulator
		CbrB	10		Transcriptional Regulator
		PA3455	6		Predicted Polyphosphate Kinase
		PA4132	6		Predicted Transcriptional Regulator

169 proteins were identified within 18 bands, a number of which were characterised or predicted transcriptional regulators and uncharacterised proteins (Table 5.1). Components of the Clp ATP-dependent proteases were also frequently identified. The Clp proteases function as chaperone proteins, but can also play a role in regulation; ClpX and ClpP have previously been implicated in the regulation of alginate overexpression and mucoidy, whilst ClpA was identified as a regulator of LecA[118,384]. Other proteases such as MucD and HflK also appeared to bind the *pqsA* promoter, and PhaF was also present in all pulldown samples (data not shown).

Vfr, ClpX, SadB, AlgB, AlgP, PilR and FleQ were found bound to the *pqsA* promoter in all backgrounds tested, along with the uncharacterised protein PA3455. AguR, a predicted regulator of carbon and nitrogen metabolism, and PA3801 were identified only in the wild type background. A number of transcriptional regulators were identified only in the absence of PqsE within the uninduced *pqsEind* background, including FleR, PhoB, AmgR, ParR and Dnr.

Furthermore, some regulators bound the *pqsA* promoter only in the *pqsEind* background regardless of whether *pqsE* expression had been induced or not. Amongst these were ErdR, RhlR, DctD, BfmR and CbrB. The presence of RhlR, but not LasR, is in agreement with previous studies; however, it is interesting that RhlR was not identified in the wild type background. In contrast with previous studies[145,377] and pilot experiments (G. Rampioni, personal communication), PqsR was not observed in these pulldown experiments.

PA2705 bound the *pqsA* promoter only in the presence of PqsE. However, overexpression of *pqsE* facilitated the binding of several additional proteins to the promoter. These were the largely uncharacterised proteins PA4132, PA4352 and PA2731, along with the transcriptional regulator DadR.

5.2.3. Verification of PAO1-W Transposon Mutants.

To further investigate the results of the *pqsA* promoter pulldowns, a total of 18 proteins were chosen as candidate regulators of the *pqsA* promoter. These included Vfr and FleQ, for which PAO1-L in-frame deletion mutants were constructed in Chapter 4. PAO1-W transposon mutants were obtained carrying Tn5 insertion elements within the genes encoding the candidate proteins, and these were confirmed by PCR as previously described in Chapter 4, Section 4.2.7. The results of the PCR screening experiments are summarised in Table 5.2. All 16 PAO1-W transposon mutants screened positive for the presence of a transposon-specific PCR product in the correct position within the appropriate genes. 15 PAO1-W transposon mutants also screened positive for the absence of the wild type candidate gene, whilst the *fleR* mutant produced a wild type *fleR* PCR product that was also present in the PAO1-W control; however, since the PAO1-L Δ*fleR* strain screened positive for the presence of the correct transposon it will be assumed that this strain is correct.

The IPTG-inducible *pqsEind* conditional mutation was also introduced into each strain and verified as described in Chapter 4, Section 4.2.8 (results not shown).

Table 5.2. Verification of PAO1-W Transposon Mutants. For this study, 15 PAO1-W transposon mutants were obtained. To confirm that the Tn5 insertions were present within those genes encoding the candidate regulators of the *pqsA* promoter, the transposon mutants were screened by PCR. Using primer pairs in which one primer was transposon-specific and the other was gene-specific, all 16 transposon mutants screened positive for the presence of the transposon in the correct position and orientation within the appropriate open reading frame (transposon present). The transposon mutants were also screened using primers flanking the gene of interest. Mutants were confirmed by the absence of PCR product (absence of gene). Δ*fleR* displayed similar PCR products to their respective wild type controls, and so were not confirmed by this PCR. ✓- confirmed; ✗ - not confirmed.

PAO1-W Transposon Mutant	**PCR Confirmation**	
	Transposon Present	Wild Type Gene Absent
Δ*dctD*	✓	✓
Δ*algB*	✓	✓
Δ*pilR*	✓	✓
Δ*algP*	✓	✓
Δ*bfmR*	✓	✓
Δ*amgR*	✓	✓
Δ*cbrB*	✓	✓
Δ*erdR*	✓	✓
Δ*sadB*	✓	✓
Δ*fleR*	✓	✗
Δ*phoB*	✓	✓
Δ*parR*	✓	✓
ΔPA5208	✓	✓
ΔPA4132	✓	✓
ΔPA4352	✓	✓

5.2.4. Construction of a *pqsA-lux* Bioreporter.

5.2.4.1. Replacement of the Antibiotic Resistance Marker in miniCTX::*pqsA-lux*.

In order to confirm the role of the candidate proteins at the *pqsA* promoter and determine their involvement in PqsE-mediated repression of the *pqsABCDE* operon, the miniCTX::*pqsA-lux* bioreporter[143] was used.

The miniCTX::*pqsA-lux* bioreporter is selectable by tetracycline and therefore poses a problem in terms of its use in the PAO1-W transposon mutants, which also carry a tetracycline resistance marker within the Tn5 insertion element. In order to overcome this problem, the *tet* gene within miniCTX::*pqsA-lux* was disrupted via insertion of a β-lactamase resistance gene, as shown in Figure 5.4(A).

In silico analysis of the miniCTX::*pqsA-lux* vector revealed a unique EagI site within the *tet* resistance gene. Thus, the *bla* gene was amplified from pBluescript along with its predicted promoter region using the primers Bla_f and Bla_r, which contained EagI-specific recognition sequences at the 5' ends (Figure 5.4(B)). The resulting 1,095 bp PCR product was digested using EagI, ligated into similarly digested miniCTX::*pqsA-lux* (Figure 5.4(C)), and confirmed by PCR using the primers Bla_f and Bla_r (Figure 5.4(D)). DNA sequencing of the plasmid using the primers BlaSeq_f and BlaSeq_r flanking either end of the *tet* gene verified the sequence of the *bla* insertion and its correct position and orientation within the *tet* gene (results not shown). This new *pqsA-lux* bioreporter was designated pRB901.

Transformation of *E. coli* S17-1 λ*pir* with pRB901 yielded carbenicillin-resistant colonies that were sensitive to tetracycline, confirming the replacement of the tetracycline resistance marker with a functional carbenicillin resistance marker. The *pqsA-lux* construct was also functional, since the S17-1 pRB901 colonies produced light (data not shown); however, repeated attempts to both transform and conjugate this plasmid into PAO1-L and PAO1-W were unsuccessful for reasons that are

not clear. DNA sequencing confirmed that the integrase gene within pRB901 was intact (data not shown).

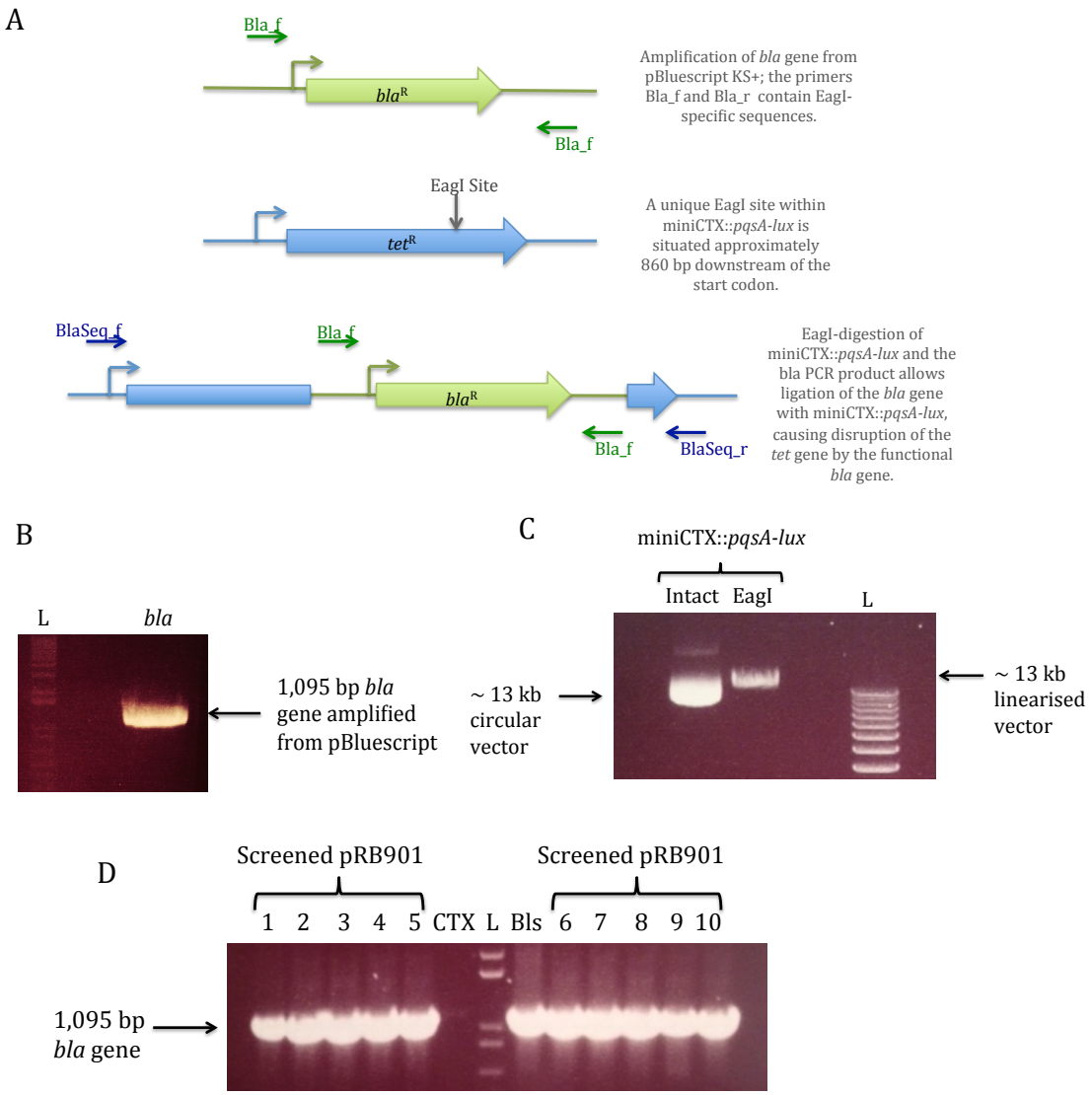


Figure 5.4. Construction of pRB901. (A).The tetracycline resistance marker in miniCTX::*pqsA-lux* was disrupted by insertion of a functional β-lactamase resistance marker; arrows show the annealing sites of the primers used. (B) The *bla* gene was amplified from pBluescript KS+, yielding a 1,095 bp PCR product. (C) Digestion of miniCTX::*pqsA-lux* with EagI linearised the vector, indicated by the presence of a single band (EagI) compared with the undigested control (intact). (D) Ligation of the *bla* gene into miniCTX::*pqsA-lux* was successful, since PCR screening of the plasmids pRB901 (1-10) using primers Bla_f and Bla_r indicated a 1,095 bp band. PBls was used as a positive control (Bls); miniCTX::*pqsA-lux* was used as a negative control (CTX). L denotes DNA ladder.

5.2.4.2. Transfer of the *pqsA-lux* Reporter Fusion to a Different Vector.

An attempt was then made to transfer the *pqsA-lux* reporter fusion into an alternative vector carrying a more appropriate resistance marker. The miniCTX::*pqsA-lux* vector was double digested using XbaI and EagI to gain better separation of fragments, yielding three fragments approximately 3,510 bp, 1,164 bp and 8, 388 bp (Figure 5.5).

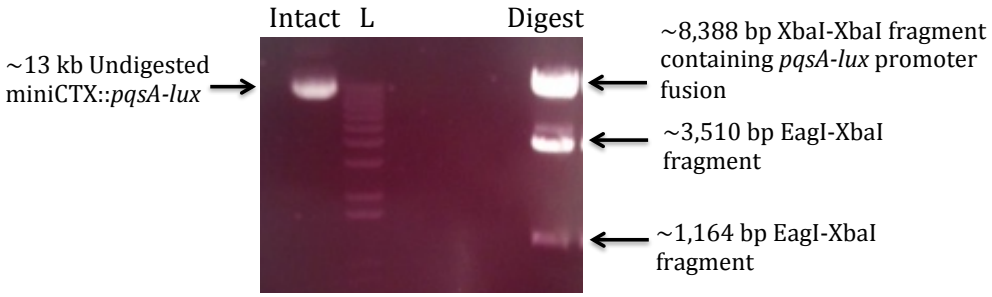


Figure 5.5. Removal of the *pqsA-lux* Reporter Construct from miniCTX::*pqsA-lux*. The *pqsA-lux* promoter fusion can be digested from the miniCTX::*pqsA –lux* bioreporter via the XbaI-specific sites flanking either end of the *pqsA-lux* construct. Use of XbaI alone produces two fragments similar in size; therefore, to obtain better separation of the fragments, a double digest was carried out using XbaI, and also EagI. EagI digestion occurred within the tetracycline resistance marker, in the opposite fragment to that containing the promoter fusion. Three fragments were therefore produced, including the ~8,388 bp fragment containing the intact *pqsA-lux* promoter fusion. For size comparison, undigested miniCTX::*pqsA-lux* was also resolved on the agarose gel (intact). L denotes DNA ladder.

The 8, 388 bp XbaI-XbaI fragment containing the *pqsA-lux* reporter fusion was ligated into XbaI-digested pBluescript and sequenced using the generic T7 forward primer to confirm that the construct was intact (data not shown). The pBls::*pqsA-lux* plasmid, designated pRB902, was also screened for the presence of the *pqsA* promoter using the primers PpqsActx_f and PpqsActx_r.

PCR screening of pRB902 suggested that the *pqsA-lux* fusion contained two *pqsA* promoter fragments, represented by two bands of 532 bp and 1,064 bp as shown in Figure 5.6(A). This was confirmed by DNA sequencing. A PstI site was observed between the two promoter fragments, and another was found to be present at the far end of the *lux* operon within the pBluescript backbone. Therefore, an attempt to excise the *pqsA-lux* fusion from pRB902 and at the same time remove the distal promoter sequence relative to the *lux* reporter genes using the two PstI sites initially appeared to be successful, since PCR screening of the purified PstI-PstI *pqsA-lux* fragment showed a single PCR product of approximately 500 bp (Figure 5. 6(B)).

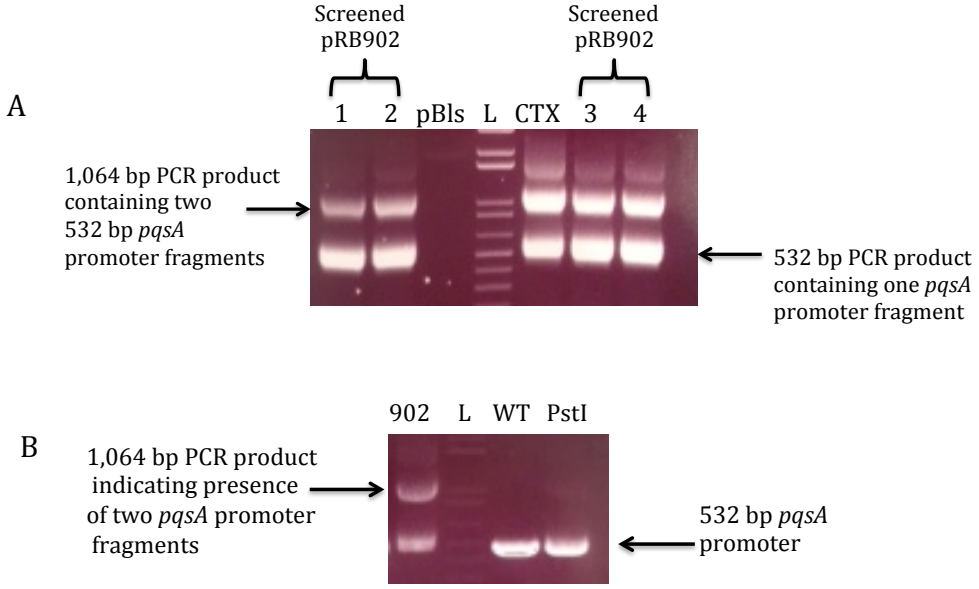


Figure 5.6. PCR of the *pqsA-lux* Promoter Fusion. PCR screening of pRB902 (pBls::*pqsA-lux*) using the primers PpqsActx_f and PpqsActx_r to verify the presence of the *pqsA* promoter revealed that two promoter fragments were present within the construct (A). These were also present in the original miniCTX::*pqsA-lux* bioreporter (CTX), but not in the pBluescript (pBls) negative control. PstI digestion of pRB902 released an ~8 kb fragment which was used as template in PCR experiments again using primers PpqsActx_f and PpqsActx_r. Compared with the double PCR product obtained for pRB902 (902), the PstI-PstI *pqsA-lux* fragment (Pst) showed a single 532 bp PCR product showed only one PCR product ~530 bp in size, confirming the presence of a singe intact *pqsA* promoter. Wild type PAO1-N genomic DNA (WT) was screened as a positive control, also showing a single band.

The PstI-PstI *pqsA-lux* fragment was cloned into PstI-digested pBK–miniTn7ΩGm to create the pRB1000 *pqsA-lux* bioreporter containing a gentamicin resistance marker. However, this cloning strategy was also ultimately unsuccessful. Following ligation, PstI digestion of pRB1000 allowed the observation of an insert of approximately 8 kb, likely to contain the PstI-PstI *pqsA-lux* fragment (Figure 5.7(A)), and this fragment was not observed upon PstI digestion of the original pBK–miniTn7ΩGm vector (Figure 5.7(B)). Although both *E. coli* and *P. aeruginosa* colonies carrying the pRB1000 construct produced light, consistent with the presence of the *lux* genes, they did so at the same level in both wild type PAO1-N and PAO1-N Δ*pqsA* strains (results not shown), and the *pqsA* promoter could not be amplified from pRB1000 (Figure 5.7(C)).

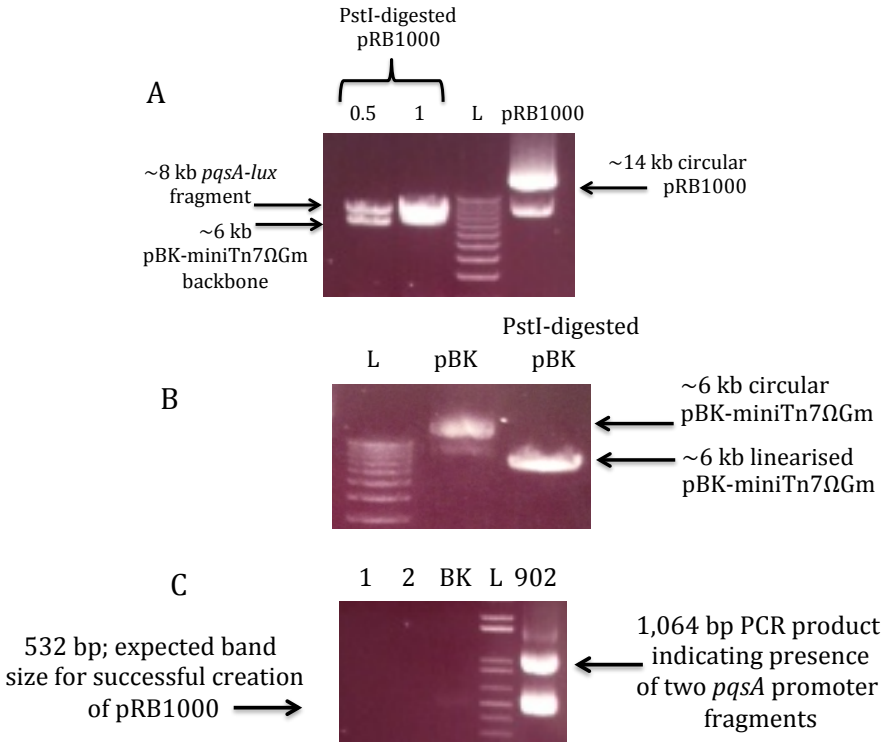


Figure 5.7. The PstI-PstI *pqsA-lux* Construct in pRB1000. The PstI-PstI *pqsA-lux* promoter fusion fragment appeared to be present in pRB1000 when 0.5 µl (but not 1 µl) of digested DNA was resolved by DNA gel electrophoresis, indicated by an 8 kb insert that was observed following PstI digestion (A). The 8 kb insert was not present in the original pBK-miniTn7ΩGm vector (pBK) (B). However, PCR amplification of the *pqsA* promoter region from pRB1000 using primers PpqsActx_f and PpqsActx_r was unsuccessful on 2 occasions (1-2), suggesting that only part of the *pqsA-lux* promoter fusion is present within pRB1000 (C). Intact vectors were used as controls; pRB1000, pBK- miniTn7ΩGm (pBK) and pRB902 (902).

The forward and reverse primers originally used to amplify the *pqsA* promoter were thought to contain HindIII and BamHI restriction sites respectively[143]. However, upon investigation of the pRB902 XbaI-XbaI *pqsA-lux* sequence, it transpired that a PstI site was present in place of the BamHI site. This means that the original information obtained regarding miniCTX::*pqsA-lux* bioreporter was incorrect, and explains the presence of the PstI site in the centre of the *pqsA-lux* promoter construct. The DNA sequencing readout was limited to approximately 1 kb, therefore the second PstI site was not observed; however, since it is present in the primers used to amplify the promoter, the assumption is that a second PstI site is present just after the *pqsA* ATG start codon in the proximal fragment relative to the *lux* genes, as shown in Figure 5.5. Therefore, complete PstI-digestion of the *pqsA-lux* reporter fusion would result in removal of both promoter fragments from the *lux* reporter genes. It is possible that the observation of single promoters in the DNA fragments obtained from PstI digestion (Figure 5.6(B)) represented incompletely digested *pqsA-lux* fusions, which could be at very low concentrations in ligation mixtures and therefore it is unlikely that these would be integrated into the final vector. DNA sequencing of pRB902 also revealed the presence of a 16 bp sequence of unknown origin between the two *pqsA* promoters.

At the time of writing, work is ongoing to complete the construction of a new bioreporter suitable for determination of *pqsA-lux* activity within the candidate *P. aeruginosa* mutant strains. Reconstruction of a *pqsA-lux* bioreporter within the pBK-miniTn7ΩGm may even allow the cloning of the same promoter region used in the pulldown experiments in this study, which is shorter than that used in miniCTX::*pqsA-lux*. This might be more appropriate within the context of validating the findings of the promoter pulldowns.

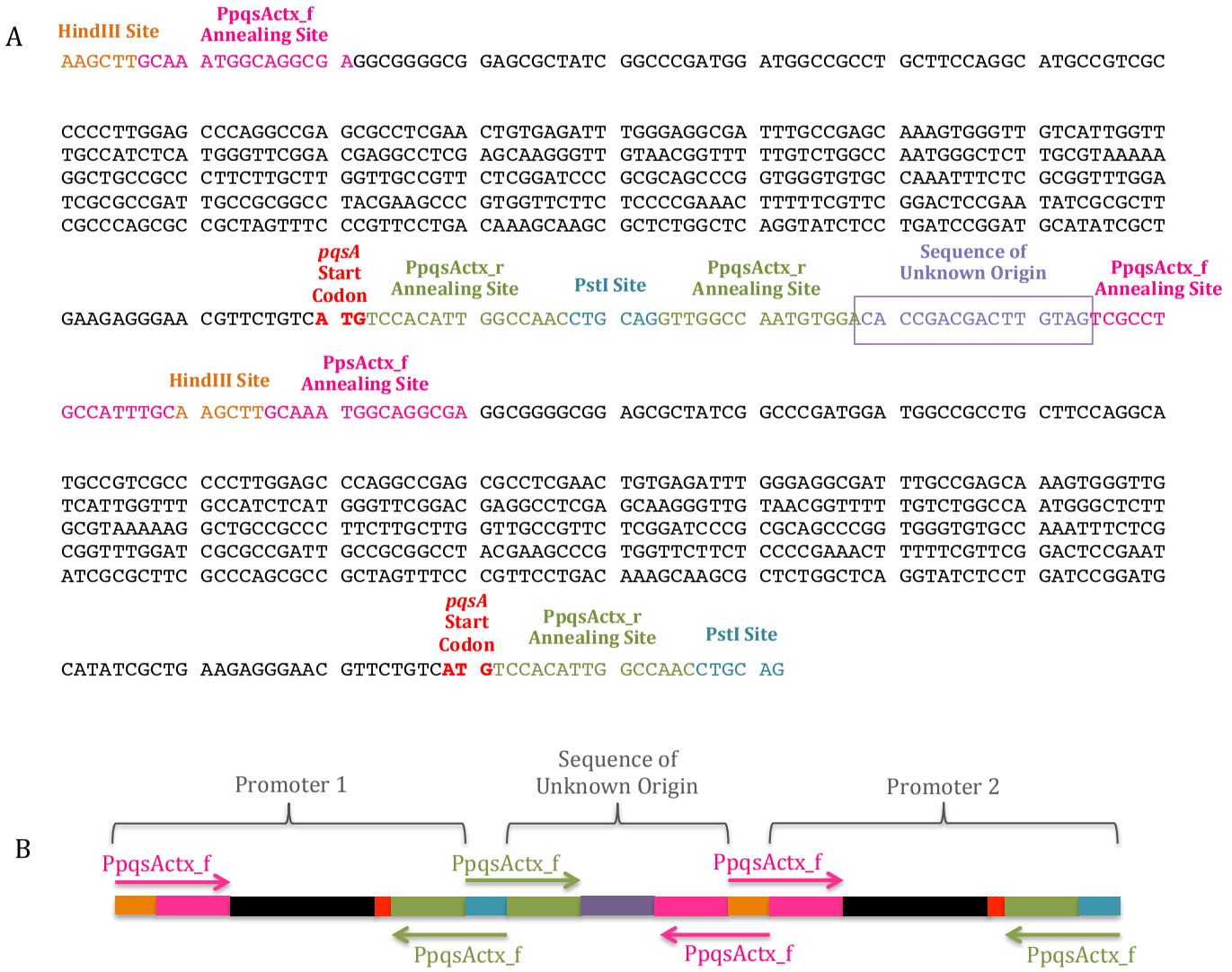


Figure 5.8. The *pqsA-lux* Reporter Fusion in miniCTX::*pqsA-lux*. DNA gel electrophoresis and DNA sequencing revealed that the *pqsA-lux* promoter fusion contains two copies of the *pqsA* promoter region. These are separated by a ~50 bp DNA sequence containing primer-derived sequences and a sequence of unknown origin. Sequencing also showed that the construct had been made using the PstI restriction site, rather than BamHI, which is why the promoter region was not present in PstI-Pst *pqsA-lux* fragments, since PstI digestion would remove the promoter regions from the *lux* genes. (A) shows the likely DNA sequence and primer annealing sites of the *pqsA* promoter region in miniCTX::*pqsA-lux*. This is simplified in the schematic in (B). In both diagrams: Black – *pqsA* promoter; red – *pqsA* start codon; orange – HindIII site; blue – PstI site; Pink – forward primer annealing site; green – reverse primer annealing site. Purple highlights the sequence of unknown origin. Arrows show the primer extension direction.

5.3. Discussion.

The work carried out in this Chapter aimed to elucidate the regulation of *pqsA* promoter activity, and potentially identify novel mediators involved in the PqsE-dependent repression of the *pqsA* promoter. The promoter pulldown technique was used as a starting point to identify proteins bound to the *pqsA* promoter at the peak of promoter activity in the wild type PAO1-N background, as determined by *pqsA-lux* reporter studies. The results of the wild type promoter pulldown were then compared with promoter pulldowns carried out using PAO1-N *pqsEind* cell extracts, either lacking expression of *pqsE*, or overexpressing *pqsE*. The findings revealed that a number of transcriptional regulators were able to bind the *pqsA* promoter regardless of the *pqsE* expression levels, such as Vfr, SadB and FleQ. The role of Vfr in the regulation of the *pqsA* promoter may be difficult to determine, since it is able to regulate the expression of myriad target genes; therefore, the potential direct Vfr-mediated regulation of the *pqsA* promoter could occur in concert with indirect Vfr-mediated regulation via, for example, RhlR[162,377].

Both SadB and FleQ have previously been well characterised as regulators of virulence. SadB, for example, has been shown to be required for *P. aeruginosa* surface attachment and the formation of biofilms[373], potentially by transducing the effect of c-di-GMP signalling to target promoters, such as that of the *pel* operon[385]. In Chapter 4, SadB was proposed to be involved in a regulatory pathway controlling *lecA* expression in which FleQ was also involved. However, although FleQ was found to bind the *lecA* promoter directly in *lecA* promoter pulldown experiments, SadB was not. It will therefore be interesting to see how the mutation of these regulatory proteins affects the activity of the *pqsA* promoter.

In the *pqsA* promoter pulldown experiments, FleR bound the *pqsA* promoter only in the absence of PqsE. FleR positively regulates the expression of flagellar biosynthetic genes, and is itself under the positive control of FleQ[386,387]. If FleQ does regulate the *pqsA* gene as well as the

lecA gene, flagellar genes and *pel* operon, it would suggest that FleQ is a global regulator of virulence that may be heavily involved in biofilm formation[115,353].

Several other proteins were identified in the *pqsA* promoter pulldowns that were also observed in the *lecA* promoter pulldowns. Most notably, AlgP bound the *pqsA* promoter under all conditions. In Chapter 4, the effect of *algP* mutation on *lecA* expression could not be masked by the homeostatic PqsE levels within the wild type PAO1-W background. In the light of this finding, it will therefore be interesting to determine whether the deletion of the *algP* gene also abrogates either the activity or the PqsE-mediated repression of the *pqsA* promoter. If AlgP does regulate *pqsE* expression, it may be that the AlgP mediated regulation of *lecA* expression occurs indirectly via PqsE.

The regulator of biofilm formation, BfmR, bound the *pqsA* promoter in pulldown experiments using PAO1-N *pqsEind* cell extracts. However, MifR was not observed in these experiments. This seems to suggest specificity of *pqsA* promoter binding that is limited only to BfmR, although, given the interdependence of the BfiSR, MifSR and BfmSR upon each other, it is likely that BfmR is subject to upstream regulatory pathways[355].

Other proteins identified in the *pqsA* promoter pulldowns that were also identified as regulators of *lecA* expression include PA5208, PA4132, PilR and CbrB. AlgB, AmgR and DctD also bound the *pqsA* promoter in this study, but were found in Chapter 4 to have little or no effect on the expression of *lecA*.

The number of candidate *pqsA* regulators that have been proposed to have a role in surface attachment and biofilm formation is striking, and perhaps reflects the role of PQS in biofilm development; previously, Diggle *et al.*[134] showed that addition of exogenous PQS to *P. aeruginosa* cultures was able to promote the formation of biofilms.

Several new transcriptional regulators and uncharacterised proteins bound the *pqsA* promoter dependent upon PqsE. For example, ParR and PhoB, amongst others, bound the promoter only in the absence of PqsE. This is interesting in light of the fact that *pqsA* promoter activity

increases in the absence of PqsE, and might suggest these proteins positively regulate *pqsA* promoter activity under certain conditions. ParR is part of the two component ParSR system and regulates the resistance of *P. aeruginosa* to aminoglycoside and polymyxin antibiotics[388]. ParR is a particularly important regulator given that polymyxin antibiotics are currently considered the 'last resort' treatment for pseudomonal infections[389]. However, transcriptomic analysis did not identify *pqsA* as a ParR-regulated gene[388].

PhoB, on the other hand, has been shown to positively regulate both the *rhlR* and *pqsR* genes under conditions of phosphate limitation. A predicted PhoB consensus sequence was identified in the *pqsR* promoter, and the production of PQS was increased under phosphate limited conditions dependent upon PhoB, presumably via induction of *pqsR* expression[390]. Considering that PhoB bound the *pqsA* promoter only in the absence of PqsE under conditions in which *pqsA* is overexpressed and PQS production is increased, it will be intriguing to investigate the role of PhoB in the relationship between PqsE and the *pqsA* promoter. It is worth noting, however, that Jensen *et al.*[390] did not identify a PhoB consensus sequence within the *pqsA* promoter sequence.

Some proteins bound the *pqsA* promoter only when *pqsE* was overexpressed. These were mainly uncharacterised proteins, but included PA4352. According to Boes *et al*.[391], PA4352 is a universal stress protein that is essential for the *P. aeruginosa* response to anaerobic stress. Interestingly, this protein is itself activated by Anr, which was also identified in the *pqsA* promoter pulldown experiments but not chosen as a candidate protein. The PA4352 gene is under the control of two promoters, one of which is active only during stationary phase growth in both anaerobic and aerobic conditions. Investigations revealed that this second promoter was not under the control of RpoS, Anr, RhlR or LasR, hinting at the further complexity of *P. aeruginosa* gene regulation networks[391]. Finally, ErdR, a two component response regulator, bound the *pqsA* promoter only when PAO1-N *pqsEind* cell extracts were used in pulldown experiments. This *luxR*-type protein is involved in the regulation

of aerobic ethanol metabolism. The cognate sensor protein of ErdR is cytoplasmic, and is believed to respond to intracellular signals[392].

The proteins identified that display differential binding to the *pqsA* promoter region dependent on *pqsE* expression are prime candidates for further investigation, since these may play a role in transducing PqsE effect to the promoter. It must be considered that the presence of so many proteases such as ClpX, ClpP, HflK and MucD might also suggest post-translational regulatory mechanisms, increasing the complexity of events at the *pqsA* promoter.

The lack of the 27 kDa protein PqsR at the *pqsA* promoter in the pulldown experiments is unexpected, particularly given the previous findings of Wade *et al.*[145] and the pilot experiments described in Section 5.1.2. In the pilot experiments, PqsR appeared to be displaced from the *pqsA* promoter when *pqsE* was overexpressed. The differences in observations may reflect the differences in experimental techniques; cell extracts harvested from overnight cultures compared with cell extracts harvested from earlier time points are likely to be very different with respect to protein content. Indeed, the SDS-PAGE analysis of promoter-bound proteins showed far fewer protein bands when cell extracts were harvested later. The sheer number of protein bands detected in the promoter pulldowns during this study meant that it was not possible to sequence all of them, both from the point of view of cost and difficulty in the excision of distinct bands in areas of dense band distribution. The 18 bands sequenced were a representative selection displaying obvious changes in density across samples; however, PqsR may be present in the adjacent, fainter bands. It must be considered that further sequencing may have identified additional proteins of interest. It is remarkable that such a large number of transcriptional regulators were identified in such a relatively small selection of protein bands; this may imply a highly complex protein binding profile at the *pqsA* promoter, of which this study has revealed only a small part. On the other hand, such large numbers of proteins could also represent a large amount of non-specific binding to promoter DNA, and therefore a limitation of the assay.

In order to determine whether the candidate proteins do indeed play a role in the regulation of the *pqsA* activity, the intention was to carry out characterisation of PAO1-W and PAO1-L strains in which the genes of interest had been mutated using a chromosomally inserted *pqsA-lux* reporter fusion. Unfortunately, this was complicated by the intrinsic tetracycline resistance of the PAO1-W transposon mutants, which meant that it was not possible to use the miniCTX::*pqsA-lux* bioreporter.

As a result, two strategies were utilised in making a new bioreporter. The first strategy was to disrupt the tetracycline resistance marker in the miniCTX::*pqsA-lux* vector via the insertion of a β-lactamase resistance marker. Although this vector was functional in *E. coli*, it could not be transformed or conjugated into *P. aeruginosa*. The second strategy, which involved transfer of the *pqsA-lux* reporter fusion from miniCTX::*pqsA-lux* into a different vector encoding a different antibiotic resistance marker, unexpectedly revealed that the miniCTX::*pqsA-lux* contained two copies of the *pqsA* promoter region, possibly due to a tandem ligation during the original construction. Whilst the effect of this may only be to proportionally increase the amount of light produced by *pqsA-lux*, it was deemed appropriate to reconstruct the bioreporter such that only one promoter fragment would be present. However, it became evident that the restriction sites present in the *pqsA-lux* promoter fusion would not allow cloning of an intact promoter region into the miniTn7 shuttle vector chosen.

Therefore, work is continuing to complete a new *pqsA-lux* bioreporter. Subsequently, strains will be characterised for their ability to induce the activity of the *pqsA* promoter in both wild type and *pqsEind* backgrounds. This will hopefully shed new light on the manner through which regulation of the *pqsA* promoter occurs, particularly with respect to PqsE involvement.

5.4. Conclusions.

As in Chapter 4, the work presented in this Chapter aimed to use the promoter pulldown technique to investigate the regulation of a gene, in this case, *pqsA*. Promoter pulldowns revealed a large number of regulatory proteins bound to the *pqsA* promoter, some of which were chosen as candidates for further investigation with a view to exploring whether any of them transduced the negative effect of PqsE to the *pqsA* promoter. Although mutants for a number of interesting proteins were obtained, including AlgP and FleQ, ultimately, further investigations were not possible due to problems with the construction of a bioreporter that could be used to analyse *pqsA* expression in the mutant strains. This work is ongoing, and will hopefully in the future contribute new information regarding the regulation of the PQS system, a key *P. aeruginosa* virulence determinant.

Chapter Six.
Conclusions.

Pseudomonas aeruginosa infections are a leading cause of morbidity and mortality, particularly in the clinical environment and amongst individuals with underlying health conditions[1]. The inherent antibiotic resistance and genetic flexibility of *P. aeruginosa* has facilitated the acquisition of even higher levels of antibiotic resistance, such that currently available anti-pseudomonal drugs are becoming increasingly ineffective[11].

The development of new therapeutics against *P. aeruginosa* infections requires a fundamental understanding of the events that occur during pathogenesis. To this end, three distinct aspects of *P. aeruginosa* pathogenesis were chosen for investigation; the interaction of *P. aeruginosa* with the host, the regulation of a specific virulence factor involved in pathogenesis, and the regulation of a quorum sensing system that itself regulates virulence.

Some mechanisms of pathogenesis are conserved across bacterial species[236]. Bearing in mind some of the parallels between the pathogenesis of *P. aeruginosa* and other opportunist pathogens such as *N. meningitidis*[249,252], the ability of the *P. aeruginosa* outer membrane proteins PilQ and OprI were investigated as potential ligands for the binding of the host 37 kDa laminin receptor precursor and galectin-3, which is able to form complexes with the 37 kDa laminin receptor precursor during the maturation process to form the mature 67 kDa laminin receptor [292].

The findings of the whole cell pulldown technique, in which *P. aeruginosa* cells were mixed with free recombinant 37 kDa laminin receptor precursor, showed that *P. aeruginosa* strains bind the 37 kDa laminin receptor precursor at low levels, and that this binding may be mediated at least in part by PilQ and OprI. The process of coming to this conclusion highlighted the importance of tailoring experimental design to

the particular species under investigation. ELISA experiments had previously been successfully used to investigate the binding of *N. meningitidis*, *H. influenzae* and *S. pneumoniae* to the 37 kDa laminin receptor precursor[232]. However, the use of this technique to study *P. aeruginosa* binding was less effective due to the highly adhesive properties of the strain, which possibly led to non-specific adhesion of the strain to the plastic microtitre plate. Similarly, the involvement of PilQ and OprI in the binding of *P. aeruginosa* to galectin-3 was not proven due to the ability of the bacterial cells to interact with galectin-3 via bacterial lipooligosaccharide[266]. Since galectin-3 may be present within the mature 67 kDa laminin receptor[292], it is possible that this could be a mechanism through which *P. aeruginosa* interacts with the mature isoform *in vivo*.

The work carried out in Chapter 3 used the recombinant 37 kDa laminin receptor precursor, which does not undergo any post-translational modification when expressed in *E. coli*. However, on the surface of eukaryotic cells, the laminin receptor exists in both the precursor isoform and the mature isoforms, which have undergone modification such as acylation[277]. *In vitro* experiments using the recombinant precursor isoform allow isolation of the protein-protein interactions so that it can, in theory, be studied without the interference of other cell surface mediators; however, it may not reflect the events *in vivo*. Further work could be carried out to determine whether these modifications occurring in laminin receptor precursor maturation perhaps potentiate *P. aeruginosa* binding to the 37/67 kDa laminin receptor, perhaps using cultured cell monolayers or via the purification of the mature laminin receptor isoform from eukaryotic cells.

P. aeruginosa PilQ may also be subject to regulation in response to environmental signals[306], which may impact upon the results of experiments such as those carried out in Chapter 3. However, there is conflicting evidence as to whether quorum sensing regulates the proteins involved in twitching motility, such as PilQ[393,394].

This thesis also investigated the regulation of LecA, an archetypal quorum sensing-controlled virulence factor. Using the *lecA* promoter

region as bait for DNA-binding proteins, a number of regulatory proteins were observed binding the *lecA* promoter at several time points in growth. Previous studies had already proposed RhlR, MvaT, RpoS and RsmA as regulators of LecA production[118,163,177]. In work presented here, RhlR and MvaT bound the *lecA* promoter region, whilst RpoS did not, either suggesting that it was simply not detected in the assay, or that it may exert its effect indirectly. However, this conflicts with the findings of Winzer *et al.*[163], where expression could be induced from the *lecA* promoter within an *E. coli* background in a RpoS-dependent manner.

From the results of the *lecA* promoter pulldown experiments, 22 candidate regulators of the *lecA* gene were identified. Validation of these candidate proteins confirmed that 18 were able to positively or negatively regulate the expression of the *lecA* gene. This indicated the efficacy of the promoter pulldown technique in the identification of novel regulators in *P. aeruginosa* and extended the current knowledge regarding the controls to which *lecA* expression is subject.

Overexpression of *pqsE* in an IPTG-inducible *pqsEind* conditional mutant leads to the concomitant overexpression of the *lecA* gene[152]. Accordingly, overexpression of *pqsE* also increased the number of transcriptional regulators that bound the *lecA* promoter in pulldown experiments. It was also observed that homeostatic levels of PqsE in the wild type PAO1-W background were able to mask the effects of mutations in candidate regulators of *lecA* expression, such that their effects were only evident when *pqsE* was not expressed, therefore confirming the importance of PqsE in the regulation of LecA production.

Nevertheless, basal PqsE levels were not able to mask the effects of *algP* mutation or *gbuR* mutation, both of which abolished LecA production in the PAO1-W background. LecA was also barely detectable in the *glpR* mutant. Interestingly, *gbuR* was proposed to be a RpoS-regulated gene[165]; however, neither AlgP, GlpR nor GbuR have previously been associated with LecA or PqsE.

PqsE is an important regulator of virulence gene expression via an unknown mechanism. It has also been identified as a negative regulator of

the *pqsA* promoter, and thus PQS production[151,152]. In order to gain more insight into how the *pqsA* promoter is regulated, and to observe whether PqsE effect is transduced via altered protein binding to the *pqsA* promoter, promoter pulldowns were again utilised.

The striking number of protein bands observed upon SDS-PAGE resolution of the protein samples obtained from the *pqsA* promoter pulldowns suggested a very large number of proteins bound to the promoter region at the peak of *pqsA* expression. This implied that the promoter is under the control of myriad regulatory elements beyond PqsR and RhlR[145,377]. LCMS/MS protein identification of a very small proportion of the protein bands found an unexpected number of transcriptional regulators, 17 of which were chosen as candidate regulators of *pqsA* expression for further investigation.

Interestingly, the proteins that bound the *pqsA* promoter showed considerable overlap with those identified at the *lecA* promoter in Chapter 4. This could be due to lack of protein binding specificity; however given the involvement of the PQS system, via PQS and PqsE, in the regulation of biofilm formation[143], and the production of virulence factors such as LecA, the findings of Chapters 4 and 5 taken together beg the question as to whether the shared candidate proteins may regulate *lecA* expression directly, via the regulation of the *pqsA* promoter, or both. One protein that was observed both in the *lecA* and *pqsA* promoter pulldowns is AlgP. Mutation of the *algP* gene abolished LecA production in the wild type PAO1-W background. In the PAO1-W Δ*algP pqsEind* strain however, where *pqsE* expression occurs independently of the *pqsA* promoter, *pqsE* overexpression is able to rescue the production of LecA from the effect of the *algP* mutation. The AlgP protein was also observed to bind the *pqsA* promoter in the pulldown experiments. If AlgP transpires to be a positive regulator of the *pqsA* promoter, this may explain why *lecA* expression is abolished completely in the wild type PAO1-W strain.

Although it is possible to speculate, it is difficult to come to any firm conclusions on the basis of the work described in Chapters 4 and 5. The LecA western blots ultimately show that the *lecA* candidate regulatory

proteins do indeed regulate LecA expression, and allow the proposal of a novel regulatory pathway to which *lecA* expression may be subject, involving SadB, AmpR, AlgU and AmrZ converging upon FleQ, a novel negative regulator of LecA. Nevertheless, the western blot results do not confirm that the effect of the candidate regulators upon the *lecA* promoter is direct, and they also take into account the post-transcriptional and post-translational regulation of LecA production. Thus, protein-DNA gel shift assays could be used to confirm the direct, specific interactions of the candidate proteins with the *lecA* or *pqsA* promoter regions, whilst *lecA-lux* promoter fusion experiments would confirm whether the effect of a regulatory protein occurs at the transcriptional level, as well as allow the full *lecA* expression curve to be observed. Unfortunately, the use of the existing miniCTX::*lecA-lux* bioreporter (L. Baker, unpublished), which confers tetracycline resistance to transconjugants, was problematic in this study due to the intrinsic tetracycline resistance of the PAO1-W transposon mutant strains.

In an attempt to confirm the role of the *pqsA* candidate regulatory proteins using the *pqsA-lux* promoter fusion, work was carried out to modify the *miniCTX::pqsA-lux* bioreporter[143] to make it suitable for use in the PAO1-W transposon mutants. At the time of writing, however, this work has thus far been unsuccessful. It is hoped that the reconstruction of the *pqsA-lux* bioreporter will be completed in due course to allow characterisation of the PAO1-W transposon mutants with respect to *pqsA* expression. Nevertheless, the work described in Chapter 5 hints at the extent of the complexity of events at the *pqsA* promoter, and proposes a number of novel regulators for consideration.

One theme running throughout this thesis is the ability of one *P. aeruginosa* mediator to mask the effect of another, for example the masking of *P. aeruginosa* interactions with the 37 kDa laminin receptor precursor by the non-specific adhesion of the strain to abiotic surfaces, or the masking of the effect of regulatory proteins on *lecA* expression by the effect of PqsE. These observations serve to demonstrate the complexity of

P. aeruginosa, in the sense that one outcome can be achieved through multiple mechanisms.

In conclusion, the work presented here has shown that, although *P. aeruginosa* shares some traits with *N. meningitidis*, it does not bind the 37 kDa laminin receptor precursor at comparable levels, suggesting that *P. aeruginosa* may not use the 37/67 kDa laminin receptor during adhesion to host tissues *in vivo*. Furthermore, the regulation of a specific *P. aeruginosa* virulence factor, LecA, has been demonstrated to be subject to at least 18 novel regulators, adding further complexity to what is already known. A modified hypothetical regulatory network governing *lecA* regulation has been proposed, and the importance of PqsE in the expression of LecA has been confirmed. Finally, 16 candidate regulators of the *pqsA* promoter, have been described. Characterisation experiments to confirm the involvement of these proteins in *pqsA* promoter activity are ongoing, with a view to identify novel regulators of the PQS system. This thesis therefore suggests that the regulatory systems within *P. aeruginosa* are of a complexity such that their full extent are not yet known, and that specific genes can be subject to highly intricate control mechanisms.

Chapter Seven.

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