LECTIN-MEDIATED BIOFILM MATURATION, QUORUM SENSING AND *Pseudomonas aeruginosa* INFECTIONS IN CYSTIC FIBROSIS

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Abstract

Chronic infections with *Pseudomonas aeruginosa* are primarily responsible for the decline in lung function and ultimate mortality in cystic fibrosis patients. The overall aim of this project was to elucidate some of the molecular mechanisms governing the pathogenesis of *P. aeruginosa* in the cystic fibrosis lung. This was with particular reference to (a) quorum sensing, a cell-to-cell communication system controlling the production of virulence determinants in a population density dependent manner using diffusible signal molecules and (b) the pseudomonas lectins, LecA and LecB, which are known to contribute to biofilm formation. Serial sputum samples were collected from adult and paediatric patients with cystic fibrosis and a cohort of clinical *P. aeruginosa* isolates was assembled. Using bioreporters, these isolates were shown to synthesise a range of quorum sensing signal molecules. Furthermore, the direct detection of these *P. aeruginosa* products from infected sputum samples in conjunction with patient clinical data implied an association between sputum quorum sensing signal molecule level and cystic fibrosis disease status, response to intravenous antibiotics and the presence of non-culturable *P. aeruginosa*.

Quorum sensing also makes an important contribution to *P. aeruginosa* biofilm maturation, antibiotic tolerance and resistance to host defences. There is evidence that the quorum sensing regulated lectins LecA and LecB contribute to biofilm development and this was investigated using different biofilm assays, including the flowchamber biofilm system. This work demonstrated that LecA contributed to biofilm maturation in both laboratory and clinical strains and hydrophobic galactosides were shown to be able to inhibit biofilm development. The putative biofilm target ligand for LecA was tentatively identified as the Psl exopolysaccharide. Mutants deficient in either *lecA* or *lecB* produced defective biofilms, which could be inhibited and/or dispersed by galactosides or furanosides respectively, including novel synthetic furanoside dendrimers. The latter proved inhibitory to both laboratory and clinical *P. aeruginosa* isolates and constitute a potential novel therapeutic.

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Abbreviations

% v/v	Percentage volume per volume
% w/v	Percentage weight per volume
ABC	ATP binding cassette
ABTS	2,2h-Azino-di-[3-ethylbenzthiazoline-sulfonate (6)]
AHL	*N*-acyl-L-homoserine lactone
AQ	2-alkyl-4-quinolone
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage fluid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BP	Band pass (confocal microscope emission filter)
BSA	Bovine serum albumin
Cb	Carbenicillin
CBM	Center of Biomedical Microbiology
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CLSM	Confocal laser scanning microscopy
CT	Computerised tomography
DCM	Dichloromethane
DIC	Differential interference contrast
DIG-NHS	Digoxigenin-3-0-succinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester
dH_2O	Deionised water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Dexynucleoside triposphate
DTT	Dithiothreitol
DTU	Danish Technical University
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunoSorbent assay
EPS	Extracellular polymeric substance
EtOH	Ethanol
FEV_1	Forced expiratory volume in one second
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
g	Gram
g	Centrifugal force
GFP	Green fluorescent protein
GLM	General linear model
Gm	Gentamicin
GP	General practitioner
h	Hour
H_a	Alternative hypothesis
H_0	Null hypothesis
H_2O	Water
HHQ	2-heptyl-4-quinolone
HPLC	High pressure liquid chromatography
ID	Inner diameter
i.e.	id est (Latin for “that is to say’)
IPTG	Isopropyl-β-D-thiogalactoside
IMS	Industrial methylated spirit
IV	Intravenous
kDa	Kilodalton
kb	Kilobase pair
L	Litre
LB	Luria Bertani medium
LC-MS	Liquid chromatography – Mass spectroscopy
LP	Long pass (confocal microscope emission filter)
LPS	Lipopolysaccharide
MS	Mass spectroscopy
Mbp	Mega base pairs
MeOH	Methanol
min	Minute

ml	Millilitre
mM	Millimolar concentration
MOA	*Marasmium oreades agglutinin*
MOPS	4-morpholinepropanesulfonic acid
MRMTT	Multiple Reaction Monitoring Triggered Traps
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
nm	Nanometre
NPF	*p*-Nitrophenyl-α-L-fucose
NYB	Nutrient yeast broth
Ω	Ohm
OD	Outer diameter
OD_{600}	Optical density at 600 nanometres
OP	Oropharyngeal cultures
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDL	Poly-D-lysine
PI	Propidium iodide
PIA	Pseudomonas isolation agar
PON	Paraoxonase
PQS	Pseudomonas quinolone signal (2-heptyl-3-hydroxy-4-quinolone)
RAPD	Random amplified polymorphic DNA analysis
R&D	Research and Development
RLU	Relative light unit
RP-HPLC	Reverse phase liquid chromatography
r	Pearson's correlation coefficient
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SI	Sputum induction
TAE	Tris-acetate-EDTA buffer

Tc	Tetracycline
TEMED	N, N, N', N'-tetramethylethylenediamine
3D	Three-dimensional
TLC	Thin layer chromotography
T_m	Melting temperature
Tris	Tris hydeoxymethyl aminomethane
µl	Microlitre
UV	Ultraviolet
V	Volt

Chapter 1: Introduction

1.1 General Introduction

Microorganisms are often viewed as simple creatures, however the study of microbial development has shown that they are capable of complex differentiation and behaviours (O'Toole *et al.*, 2000a). This is well illustrated by the tiny fraction which have the capacity to cause disease and continue to threaten human health and welfare. A major challenge is the emergence of bacterial strains exhibiting resistance to multiple traditional antibiotic classes.

An appreciation of the fact that bacteria need to adopt and regulate particular mechanisms in order to grow and survive *in vivo* is the basis for the study of bacterial pathogenicity. The aim of understanding this capacity to cause disease is to identify new targets for intervention and provide alternative strategies for disease control. In recent times this has been facilitated by advances in molecular techniques, which enable the manipulation and investigation of these microorganisms in dynamic and diverse ways.

One intensely studied example is *Pseudomonas aeruginosa*. This ubiquitous environmental organism demonstrates considerable nutritional and metabolic versatility and adaptability. This in part reflects the fact that is has a very large genome (6.3 mega base pairs [Mbp]; 1.7 Mbp larger than *Escherichia coli*) containing a high proportion of regulatory genes, giving it a genetic complexity approaching that of a simple eukaryotic organism (Stover *et al.*, 2000).

P. aeruginosa is the most important human pathogen of its genus and draws on an impressive array of mechanisms to infect almost any external site or organ (Williams *et al.*, 2000). Most community infections are mild and superficial, but in hospital patients, infections are more frequent, more severe and more varied. In fact, it is one of the most common Gram-negative bacteria found in hospital-acquired infections, representing a major cause of pneumonia, urinary tract, surgical wound, burn wound and bloodstream infections (Van Delden and Iglewski, 1998).

Chronic pulmonary infections with *P. aeruginosa* are the major cause of morbidity and mortality in cystic fibrosis (CF) patients (Lyczak *et al.*, 2002). This disorder is the most common autosomal recessive condition amongst Caucasians, with a frequency of about 1 in 2,500 livebirths (Ratjen and Doring, 2003), affecting approximately 60,000 individuals worldwide (Gibson *et al.*, 2003). Despite the discovery of the mutated gene encoding a defective chloride channel in epithelial cells, no curative treatment is currently available. With symptomatic treatment alone, life expectancy has improved considerably over the last few decades and the predicted median survival for babies born in the 21st century is now more than 50 years (Dodge *et al.*, 2007).

The lung environment of these patients appears to provide a unique niche that promotes chronic microbial colonisation, with *P. aeruginosa* being the most common isolate recovered (Cystic Fibrosis Foundation, 2006). Indeed, once established, its treatment is hugely problematic and eradication almost impossible. The reasons for this are themselves the subject of intense study and widely overlap with exciting areas of work that are unravelling aspects of bacterial communication and community living.

It is apparent that the remarkable ability of *P. aeruginosa* to cause disease is not only the consequence of individual attributes. Its success can also be explained by its behaviour as a population, where individual members can communicate (termed quorum sensing [QS]) and hence live in communities (known as biofilms). Understanding the mechanisms of biofilm formation provides insight into how populations of *P. aeruginosa* persist in the environment of the CF lung and may identify much needed targets for intervention. A promising new focus is the molecular basis for the contribution of the sugar-binding lectins LecA and LecB to biofilm development.

Hence this introduction aims to present what is already known in this field by first briefly examining the general pathogenicity of *P. aeruginosa* and then concentrating on the specific pathogenicity of this organism in CF. In doing so, the aim is to establish the context within which research into QS and lectin-mediated biofilm maturation is based.

1.2 General pathogenicity of *P. aeruginosa*

1.2.1 Characteristics of *P. aeruginosa*

P. aeruginosa is a Gram-negative rod belonging to the family Pseudomonadaceae. It is non-sporing and non-capsulate and usually motile by means of one or two polar flagella. It is a strict aerobe except in the presence of nitrate and grows readily on a wide variety of culture media over a wide temperature range. Typical colonies and their surrounding medium are greenish-blue due to the production of a soluble blue phenazine pigment, pyocyanin and the yellow-green fluorescent pigment pyoverdin, which acts as a major siderophore. Energy is derived from carbohydrates by oxidative rather than fermentative metabolism. In culture it emits a sweet grape-like odour that is easily recognised and all strains give a rapid positive oxidase reaction and this is a useful preliminary test (Govan *et al.*, 2003).

1.2.2 Epidemiology of *P. aeruginosa*

P. aeruginosa is a cosmopolitan organism, growing and surviving in almost any environment, though it does have a preference for moist surroundings. As such, human colonisation occurs at sites such as the axilla, ear and perineum and moisture is also a key factor in hospital reservoirs of *P. aeruginosa* which include respiratory equipment, sinks, medicines and disinfectants. Whilst carriage of the organism by healthy individuals in the community is relatively low, hospitalisation can lead to greatly increased colonisation rates and this frequently precedes overt infection (Pollack *et al.*, 2000). Patient-to-patient transmission may occur directly, via the hands of medical staff or from contaminated equipment (Govan *et al.*, 2003).

1.2.3 Pathogenesis: a successful opportunist

Unlike the so-called ‘primary pathogens’, *P. aeruginosa* does not possess the specific genetic traits to cause disease in healthy people with intact immune systems. It can only cause disease in those individuals whose natural defences against bacterial infection are impaired in some way and is thus referred to as an ‘opportunistic pathogen’.

Often such immunocompromised individuals are treated with antibiotics in an attempt to create a means of defence against infecting organisms. Such treatment inevitably and

inadvertently disrupts the host's indigenous microflora, the very first biological barrier against subsequent colonisation. In fact, *P. aeruginosa* exploits the situation even further for as a common environmental organism, it happens to be innately resistant to many of the traditional antibiotics developed against frequent human pathogens (Martinez and Baquero, 2002). Escalation of treatment with further exposure to different classes of antibiotics has led to the development of problematic multidrug-resistant strains.

1.2.4 Virulence determinants of *P. aeruginosa*

As a pathogenic organism, *P. aeruginosa* not only invades and grows within a host (i.e. causes infection) but it also does harm to the host (i.e. causes disease). To achieve this, it must gain entry into the host, multiply in the extra- or intracellular body compartment, resist host defences, damage host tissues to cause the symptoms and signs of disease and finally disseminate through the host and/or to another host. To perform this multistage adaptive process *in vivo*, *P. aeruginosa* synthesises a wide range of virulence determinants (Williams, 2002), which can be cell-associated or extracellular.

Cell-associated factors such as type IV pili, nonpilus adhesins, flagella, alginate and lipopolysaccharide (LPS) are thought to mediate adherence of the organism to altered epithelium of mucosal surfaces and allow initial colonisation of host tissues. Subsequently, *P. aeruginosa* produces several extracellular factors, which can cause extensive tissue damage, bloodstream invasion and dissemination. These include proteases (LasB elastase, LasA elastase, alkaline protease), haemolysins (phospholipase C, rhamnolipid), exotoxin A and exoenzyme S, pyocyanin and lectins (Pollack *et al.*, 2000) (Van Delden, 2004). The extracellular release of these substances is achieved by dedicated molecular machineries termed secretion pathways and *P. aeruginosa* utilises types I, II and III (Filloux *et al.*, 1990) (Guzzo *et al.*, 1991) (Yahr *et al.*, 1997).

1.2.5 The regulation of virulence

Gene expression, necessary for the production of virulence determinants, is expensive in terms of energy utilisation and therefore it makes sense that this process is subject to sophisticated regulatory mechanisms. These enable bacteria to sense and respond appropriately (i.e. adapt) to the chemical and physical cues in their surroundings. One such system allows bacteria to perceive the density of the surrounding bacterial

population and to coordinately respond to this information by regulating various genes. This regulatory phenomenon has been termed ‘quorum sensing’ (QS) because it reflects the requirement for a quorate population of bacterial cells prior to the activation of target genes (Fuqua *et al.*, 1994).

1.2.6 Quorum sensing

In simple terms, QS can be thought of as a regulatory circuit consisting of three components: a small signal molecule called an autoinducer, the gene coding for the autoinducer synthase protein and the gene for a response regulator protein. At low population densities, individual bacterial cells make the autoinducer at a basal rate, which then diffuses or is pumped out of the cell. As the population grows, the concentration of the autoinducer increases. At a threshold concentration, it binds to and activates a response regulator which in turn directly or indirectly activates or represses target gene expression (Diggle *et al.*, 2007a). The gene which encodes for the autoinducer synthase is itself activated by the autoinducer-activated response regulator and hence a positive-feedback loop is established. In this way, the resulting increase in gene expression can reach 1,000-fold.

In 1970 the *lux* system was the first QS system to be described following investigation into cell density-dependent bioluminescence in the Gram-negative marine bacterium *Vibrio fischeri*. It was found that the supernatants of high density cultures of *V. fischeri* contained a substance, which at a critical concentration, would induce light emission in cultures at low cell density (Nealson *et al.*, 1970). This was the autoinducer *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981), a member of the *N*-acyl-L-homoserine lactone family (AHL), synthesised by LuxI. At a critical concentration, it binds to LuxR and together this complex binds to a regulatory region known as the *lux* box. Hence there is increased transcription of the downstream genes *luxCDABE,* which make the luciferase and fatty acid reductase complex required for light production (Engebrecht and Silverman, 1984) (Figure 1-1). In this way, cell-cell communication enables individual cells to detect when the circumstances are right to partake in the energy-expensive production of light, which is only effective at the population level.

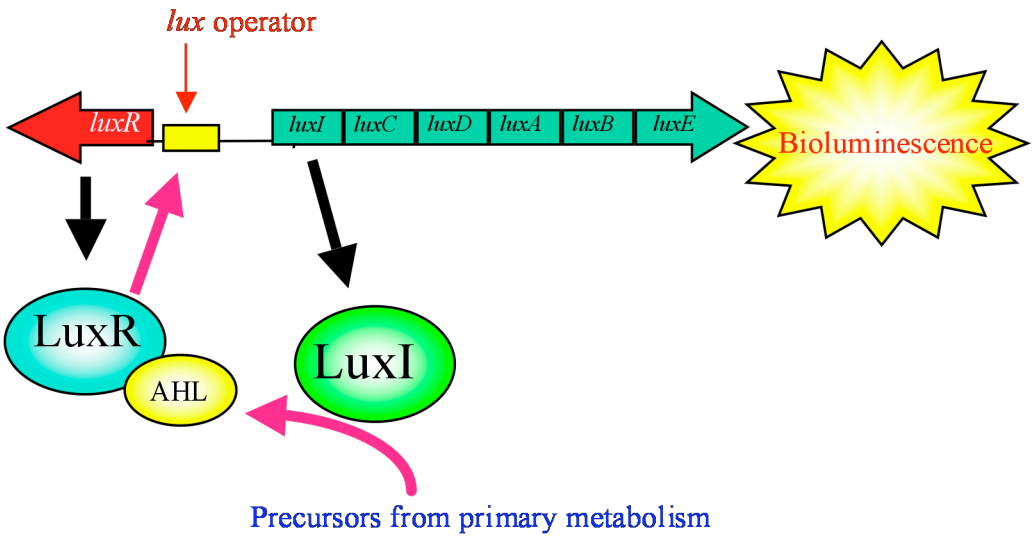


Figure 1-1 Bioluminescence mechanism in *V. fischeri.*

In the early 1990s, the discovery that 3-oxo-C6-HSL regulated production of the β-lactam antibiotic carbapenem by the terrestrial plant pathogenic bacterium *Erwinia cartovora* suggested that QS systems may be more widespread throughout the bacterial kingdom. To test this hypothesis, plasmid-based AHL-biosensors were engineered to detect the accumulation of these signal molecules in spent culture supernatants (Bainton *et al.*, 1992). This approach revealed that QS occurs in a range of Gram-negative organisms, which possess LuxRI homologues and that 3-oxo-C6-HSL belongs to a family of AHL autoinducer molecules. The application of sophisticated chemical purification techniques such as high pressure liquid chromatography (HPLC) revealed that AHLs consist of a homoserine lactone moiety derived from amino acids, linked to a *N*-acyl side-chain of between 4 and 14 carbon atoms, with or without either an oxo- or a hydroxy- moiety at the C3 position (Chhabra *et al.*, 2004).

The AHL lactone ring is readily hydrolysed under alkaline conditions to form the corresponding *N*-acylhomoserine compound ('ring open' form), which is inactive as a QS signal molecule (Yates *et al*., 2002). Furthermore, the 3-oxo-AHLs can also undergo an alkali-driven rearrangement reaction to form the corresponding tetramic acids,

compounds with iron chelating and antibacterial activities (Kaufmann *et al*., 2005). Examples of the these structures produced by *P. aeruginosa* are presented further on in Figure 1-3.

Gram-positive bacteria are also known to quorum sense although they do not produce AHLs as signal molecules. Instead, they utilise post-translationally modified peptides which are recognised by receptor kinases and this sets off a signalling cascade that activates the target relevant genes (Sturme *et al.*, 2002).

1.2.7 Quorum sensing in *P. aeruginosa*

The first cell-to-cell signalling system described in *P. aeruginosa* was found to regulate expression of the *lasB* gene (coding for elastase) and was therefore named the *las* system (Passador *et al.*, 1993). This consisted of the AHL autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), produced by the LasI synthase, and the LasR transcriptional activator protein (Gambello and Iglewski, 1991) (Pearson *et al.*, 1994).

A second system is also present, named *rhl* because of its ability to control the production of rhamnolipid. In this case, the signal molecule is *N*-butanoyl-L-homoserine lactone (C4-HSL), made by RhlI, which binds to the transcriptional activator RhlR (Latifi *et al.*, 1995).

These two QS circuits have been found to regulate the genes for a number of virulence determinants in *P. aeruginosa* to varying degrees. These include *toxA* (exotoxin A), *lasB* (LasB elastase), *lecA* (LecA lectin), *rhlAB* (rhamnolipids), *aprA* (alkaline protease), *hcnAB* (hydrogen cyanide), *katA* (catalase), *sodA* and *sodB* (superoxide dismutases) and *xcpR* and *xcpP* (type II secretion systems) (Brint and Ohman, 1995) (Chapon-Herve *et al.*, 1997) (Gambello *et al.*, 1993) (Glessner *et al.*, 1999) (Hassett *et al.*, 1999) (Latifi *et al.*, 1995) (Pessi and Haas, 2001) (Winzer *et al.*, 2000). In addition, synthesis of type IV pili and siderophores as well as biofilm maturation have also been shown, at least in part, to be regulated by QS (Glessner *et al.*, 1999) (Stintzi *et al.*, 1998) (Davies *et al.*, 1998). Overall, it has been estimated that between 3 and over 10 % of the genome of *P. aeruginosa* may be controlled to some degree by QS (Whiteley *et al.*, 1999) (Schuster *et al.*, 2003) (Wagner *et al.*, 2003).

Whilst there is considerable overlap in the genes regulated by these two systems, it is also evident that they do not function independently of each other. In fact, the LasR-3-oxo-C12-HSL complex activates the expression of *rhlR* and *rhlI* placing the *las* system above the *rhl* system in a signalling hierarchy (Latifi *et al.*, 1996). This represents the core of the *P. aeruginosa* QS system and ongoing work has demonstrated the presence of many additional layers of complexity.

For example, in contrast to light-production in *V. fischeri* and carbapenem production by *E. carotovora,* the addition of exogenous AHLs did not advance the expression of several QS-dependent genes such as *lecA*, *lasB* or *rhlR* (Diggle *et al.*, 2002). Therefore population size, translated into an increase in AHLs, is not the only determinant controlling multiple genes. Rather, it appears that the expression of certain genes is coordinated with growth phase and growth environment by additional regulators. These include the positive regulators GacA (Reimmann *et al.*, 1997) and Vfr (Albus *et al.*, 1997) and those that act as repressors, such as RsaL (de Kievit *et al.*, 1999), RsmA (Pessi *et al.*, 2001), MvaT (Diggle *et al.*, 2002) and the third homologue of LuxR, QscR (Chugani *et al.*, 2001). Recently, a fourth LuxR homologue, VqsR, has been identified as a major virulence regulator in the QS hierarchy of *P. aeruginosa* (Juhas *et al.*, 2004). The alternative sigma factor RpoS also plays a role, which may allow the regulation of virulence determinants with survival in stationary phase (Winzer *et al.*, 2000) (Schuster *et al.*, 2004).

In 1999, Pesci *et al.* demonstrated that a non-AHL signal produced by *P. aeruginosa* was able to activate *lasB* expression and whose own synthesis and bioactivity was mediated via the *las* and *rhl* systems respectively. The molecule responsible for this non-AHL-mediated QS signalling pathway was purified and chemically identified as 2-heptyl-3-hydroxy-4-quinolone and termed the 'Pseudomonas quinolone signal' (PQS). In fact, PQS belongs to the family of 2-alkyl-4-quinolones (AQs), which were first identified in the 1940s and have been previously studied for their antimicrobial properties. Other major molecules belonging to this family produced by *P. aeruginosa* include 2-heptyl-4-quinolone (HHQ), 2-nonyl-4-quinolone (NHQ) and 2-heptyl-4-quinolone *N*-oxide (HHQNO).

A five-gene operon termed *pqsABCDE* is responsible for the synthesis of AQs in *P. aeruginosa* (Gallagher *et al.*, 2002). PQS binds to and activates the LysR-type transcriptional regulator PqsR (also known as MvfR), which in turn induces the

expression of the *pqsABCDE* operon. The *pqsA-D* genes are involved in the biosynthesis of the PQS precursor HHQ from anthranilate. A further gene termed *pqsH*, encoding for a predicted FAD-dependent mono-oxygenase, is required for the conversion of HHQ into PQS. The expression of *pqsH* is, at least in part, controlled by LasR, thus linking the AHL and AQ signalling pathways (Deziel *et al.*, 2004). Whilst the exact function of PqsE is unknown, it has recently been reported that it acts as a regulator that is independent of PqsR and PQS, but dependent on the *rhl* quorum sensing system (Farrow *et al.*, 2008) (Fletcher, Pustelny, Diggle, Cámara & Williams, unpublished observations).

Whilst other bacterial species have been shown to make AQs (Diggle *et al.*, 2006a), to date, *P. aeruginosa* is the only organism known to make PQS. Indeed, the PQS signalling system plays an important role in pathogenesis by regulating the production of diverse virulence factors, including elastase, pyocyanin and LecA lectin as well as affecting biofilm formation (Diggle *et al.*, 2003; Gallagher *et al.*, 2002) (Cao *et al.*, 2001).

See Figure 1-2 for an overview of *P. aeruginosa* QS signalling systems and Figure 1-3 for the structures of the major QS signal molecules and their derivatives.

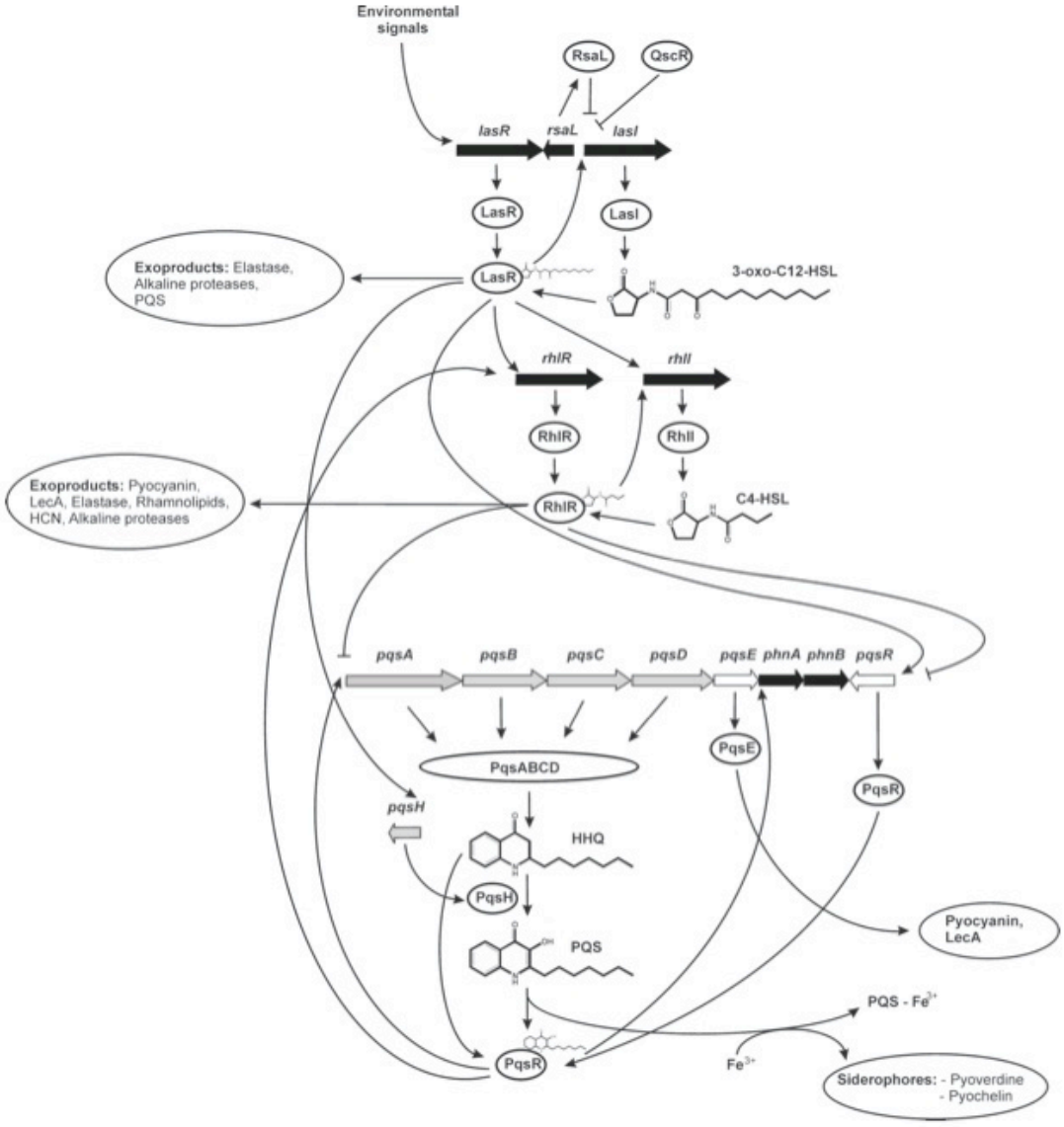


Figure 1-2 An overview of QS in *P. aeruginosa*.
The QS cascade is induced at high population cell densities when within the cell, the LasR response protein binds to a critical concentration of 3-oxo-C12-HSL signal that has been produced by neighbouring cells and taken up from the surrounding environment. This results in activation of the *las* QS system and the production of a number of QS-regulated phenotypes. Activation of the *las* system is also important in the induction of the *rhl* QS system that is required for the production of *rhl*- controlled phenotypes.
AHL and AQ-dependent QS are linked since LasR/3-oxo-C12-HSL is required for full expression of *pqsH* and positively regulates *pqsR*. Both *pqsA* and *pqsR* are repressed by the action of the RhlR/C4-HSL system. ↓ represent positive regulation. ⊥ represent negative regulation.

(Dubern and Diggle, 2008)

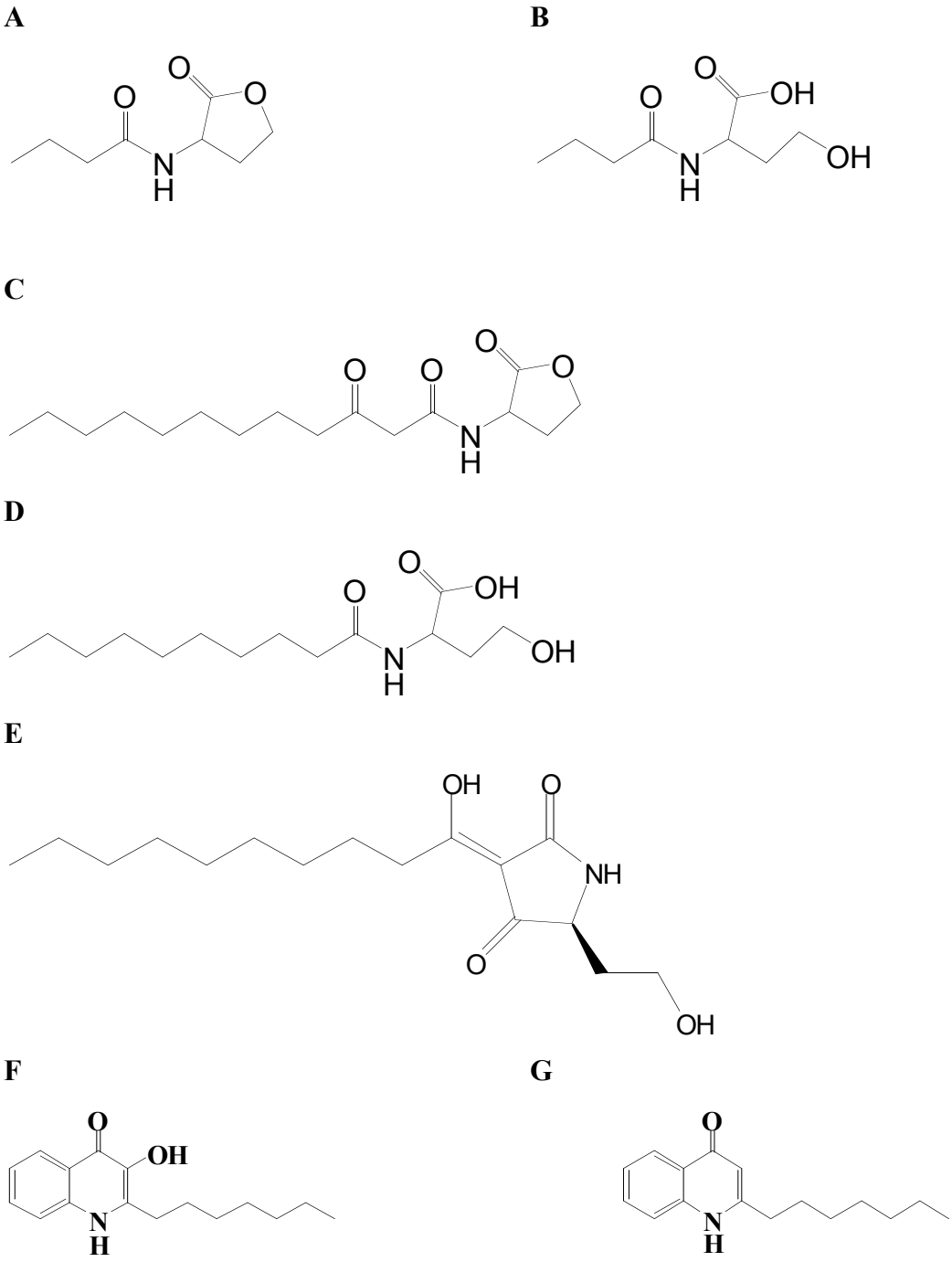


Figure 1-3 Structures of the major QS signal molecules and their derivatives.
(A) C4-HSL (B) ring open C4-HSL (C) 3-oxo-C12-HSL (D) ring open 3-oxo-C12-HSL (E) 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione [tetramic acid] (F) PQS (G) HHQ

1.2.8 The implications of quorum sensing in *P. aeruginosa*

It is interesting that many pathogenic AHL producers tend to be opportunists, which can exist in multiple environments. In contrast, pathogens which uniquely colonise and cause infections in humans, such as *Haemophilus influenzae*, do not appear to employ QS circuits (Swift *et al.*, 1999). So by regulating diverse physiological processes including virulence, QS enables adaptation to a niche and hence a survival advantage for these microbial opportunists.

It is the timing of this regulation that is particularly relevant to the establishment of infection. QS ensures that *P. aeruginosa* virulence determinant production occurs maximally when the invading organism has reached a critical population density sufficient to instigate a unified attack and therefore overwhelm the host before it has time to mount an effective defence.

In vivo evidence for the role of QS in the regulation of virulence comes from testing strains of *P. aeruginosa* that contain deletions in one or more QS genes in various models of infection. These include a neonatal mouse model of acute pneumonia and a burned mouse model of infection (Tang *et al.*, 1996) (Pearson *et al.*, 2000) (Rumbaugh *et al.*, 1999a). In these studies, the mutant strains were found to induce less tissue destruction and reduced cases of pneumonia than wild-type *P. aeruginosa*. Interestingly, the QS mutants were not completely avirulent. This emphasises the multifactorial nature of virulence and fact that other factors play a role in regulating pathogenesis.

Increasingly, it is recognised that the QS signal molecules themselves can interact with eukaryotic cells and hence directly affect the host, acting as virulence determinants in their own right. For example, there are several reports that 3-oxo-C12-HSL is a potent immune modulator which impacts on cytokine production and immune cell activity *in vitro* and *in vivo* (Pritchard, 2006). In addition, this molecule has been shown to exert a pharmacological effect on the cardiovascular system, inhibiting vasoconstrictor tone of both pulmonary and coronary blood vessels from the pig (Lawrence *et al.*, 1999) and triggering bradycardic effects in rats (Gardiner *et al.*, 2001). Hence, this AHL QS signal molecule itself may enhance *P. aeruginosa* survival in the host by increasing nutrient supply via the bloodstream and downregulating host defences. Furthermore, there is evidence that PQS is a more potent immune modulator than 3-oxo-C12-HSL and

together, they are capable of acting synergistically to inhibit T-cell proliferation (Hooi *et al.*, 2004).

Clearly, as global regulatory systems, the QS circuits represent novel and attractive therapeutic targets for the attenuation of virulence. Possible strategies include depletion of the signal molecule, either by its destruction or the inhibition of its synthesis, or alternatively to block transmission of the signal by antagonising the formation of the AHL-LuxR complex (Williams, 2002). Such approaches would overcome conventional antibiotic resistance mechanisms and reduce the selective pressure for the emergence of resistant strains. However, as there is no bactericidal action, effective host defences would be needed for bacterial clearance and this may limit such an approach in the immunocompromised, a target patient population for opportunistic pathogens. As such, QS inhibitors may have a prophylactic role or be used in synergy with more conventional classes of antibiotic (Williams, 2002).

Overall QS has changed our view of bacteria as simple unicellular organisms existing in isolation, when in fact they are capable of complex and sophisticated patterns of multicellular behaviour (Williams *et al.*, 2000). With communication comes community living and this pattern of existence is crucial to the understanding of the pathogenesis of *P. aeruginosa* in the CF lung.

1.3 The pathogenicity of *P. aeruginosa* in CF

1.3.1 Parasite-host interaction

In the clinical sense, the virulence of an organism is a manifestation of a complex parasite-host relationship in which the capacity of the organism to cause disease is considered in relation to the resistance of the host (Ala'Aldeen *et al.*, 2003). Nearly all clinical cases of *P. aeruginosa* infection are associated with compromise of the host. In patients with general immunosuppression such as those with AIDs or neutropenia following chemotherapy, *P. aeruginosa* is one of a number of potential bacterial, viral and fungal pathogens. Such scenarios, therefore, do not yield information which is specific to the pathogenesis of *P. aeruginosa* (Lyczak *et al.*, 2000). However, in CF, the genetic defect leads to a particular phenotype that makes these patients especially susceptible to lung infections with *P. aeruginosa*. In this environment, *P. aeruginosa* selects certain virulence determinants from its repertoire to behave in a specific way. It

is interesting to review what is currently known about this host environment and the molecular and cellular mechanisms of the parasite's behaviour.

1.3.2 CF: the host phenotype

CF is an autosomally recessive inherited disease affecting cells of the exocrine glands. It is characterised by mucus hypersecretion, chronic bacterial infection and airway inflammation, as well as exocrine pancreatic insufficiency and being underweight. The disorder predominantly occurs in Caucasian populations of European ancestry, who have a carrier frequency of 1 in 25. Overall, the condition has an incidence of about 1 in 2500 livebirths (Ratjen and Doring, 2003)

1.3.3 Pathology: the genetic defect

The understanding of the genetic basis of CF was initiated and advanced by the work of Dorothy Andersen. In 1938 she published a detailed study of 49 patients (Andersen, 1938) that for the first time allowed CF to be thought of as a single disease with diverse effects rather than a loose collection of related disease states of the alimentary and respiratory tracts. In 1946 she published a pedigree type analysis of CF in 20 affected families (Andersen and Hodges, 1946). The disease was found to occur with a frequency approximating 25% in the affected families, the value expected for an autosomal recessive disorder inherited in a classical Mendelian fashion.

An additional clue to aetiology was provided by di Sant'Agnese and colleagues in 1953 who found that the sweat of CF patients contained abnormally high concentrations of sodium, chloride and potassium (Di Sant'Agnese *et al.*, 1953). This ultimately led to the work that demonstrated that CF patients have abnormalities in chloride conductance in and out of cells (Quinton and Bijman, 1983).

The cause of this defect in chloride conductance and of its mode of inheritance was determined in 1989, a milestone in CF research, when the gene encoding for the defect that manifests as CF was identified and cloned (Kerem *et al.*, 1989) (Riordan *et al.*, 1989). Chromosomal walking experiments confirmed the localisation of the gene to the long arm of chromosome 7 (Rommens *et al.*, 1989). The gene itself is large, spans 250 kilobases (kb) and is composed of 27 exons (Zielenski *et al.*, 1991). It is transcribed into

a 6.5-kb messenger RNA that encodes the 1,480 amino acid protein termed the cystic fibrosis transmembrane regulator (CFTR).

The CFTR protein, located in the apical membranes of epithelial cells, is a member of the ATP binding cassette (ABC) family of transporters. It contains two nucleotide-binding domains that bind and hydrolyse ATP, two dual sets of membrane-spanning segments that form the channel, and a central regulatory (R) domain. The R domain is unique to CFTR and is highly charged with numerous phosphorylation sites for protein kinases A or C (Figure 1-4).

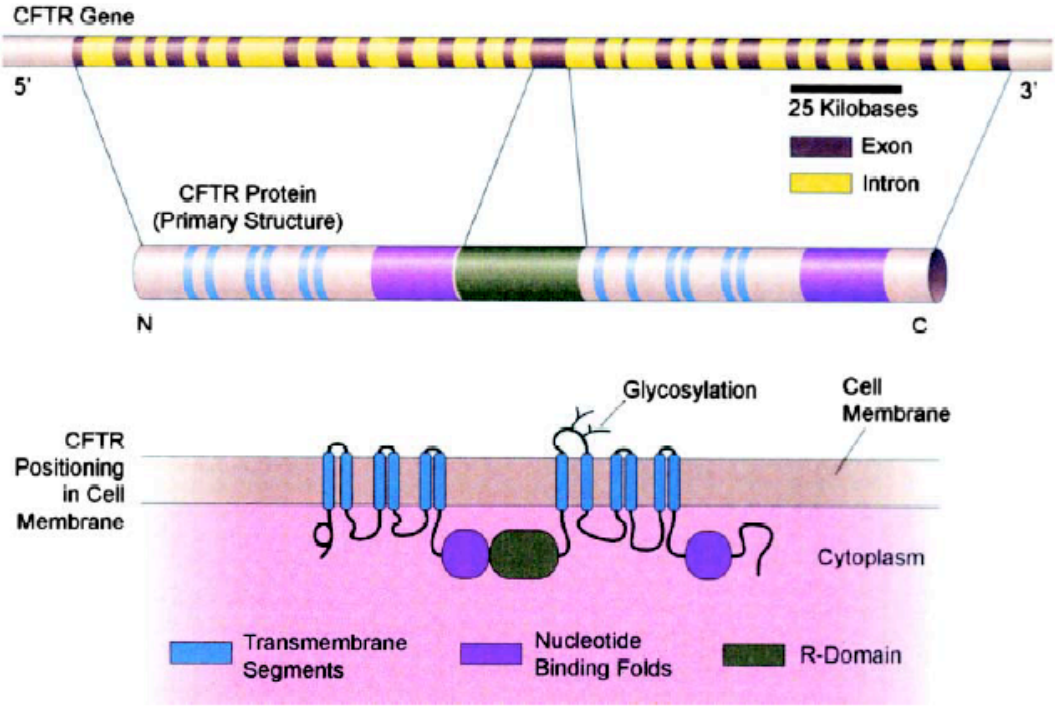


Figure 1-4 The cystic fibrosis transmembrane conductance regulator (CFTR) gene and its encoded polypeptide
(Gibson *et al.*, 2003)

The CFTR protein has a dual role as a cAMP-sensitive chloride channel of low conductance with no preference for the direction of chloride transport and as a conductance regulator due to its ability to regulate other protein channels and transporters. In the CF sweat gland, chloride absorption is hindered by defective CFTR function. As a result, sweat that reaches the skin surface has higher than normal chloride concentration (>60 mEq) and this provides the basis for the diagnostic sweat test. In the airway epithelium, the CFTR defect results in loss or reduction of chloride secretion

into the airways. The physiological effects of this defect on the salt concentration and volume of the airway surface fluid is an area of debate and the competing theories are discussed further on in the context of the pathophysiology of the CF lung disease.

Over 1500 naturally occurring mutations in the CFTR gene have been described so far, (Cystic Fibrosis Foundation Mutation database, http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html, accessed 09.06.08). The vast majority involve three or fewer nucleotides and result in predominantly amino acid substitutions, frameshifts, splice site or nonsense mutations. CFTR mutations can be grouped into six classes that reflect the biosynthetic pathway and function of the CFTR (Vankeerberghen *et al.*, 2002):

1 CFTR is not synthesised
2 defective processing
3 defective regulation
4 defective conductance
5 partly defective production or processing
6 defective regulation of other channels

Classes 1-3 are the most common, with the most frequent mutation worldwide belonging to class 2, caused by deletion of phenylalanine at position 508 of CFTR (phe508del, until recently known as ΔF508) (Ratjen and Doring, 2003). Interestingly, the incidence of this mutation varies between ethnic groups, for example 82% of patients with CF have phe508del in Denmark in contrast to only 32% in Turkey (Morral *et al.*, 1994). This has led to the theory that selective pressure has been operating, perhaps due to a heterozygote advantage such as protection against fatal infections. Although several theories have been proposed suggesting a selective advantage for phe508del heterozygotes such as resistance to secretory diarrhoea from cholera (Gabriel *et al.*, 1994) or protection against bronchial asthma (Schroeder *et al.*, 1995) no confirmatory data is available.

Attempts to link mutations in CFTR to severity of lung disease have not been successful (The Cystic Fibrosis Genotype-Phenotype, 1993) and it is suggested that environmental factors, genes other than CFTR, or both, modify development, progression and disease severity (Ratjen and Doring, 2003).

1.3.4 Diagnosis

Although the genetic basis is well understood, the diagnosis of CF is usually made clinically and not genetically. The disease leads to pathological changes in organs that express CFTR, including secretory cells, sinuses, lungs, pancreas, liver and reproductive tract. These result in clinical signs and symptoms which can be used to make a preliminary diagnosis. Additionally, a positive family history or a positive finding at newborn screening can be informative. The diagnosis can then be confirmed by tests of abnormal ion concentration (sweat chloride concentration or nasal epithelium potential difference) and genotyping of the most common mutations. Clinical tests which do not directly assess the CFTR defect can also aid diagnosis, for example tests of pancreatic insufficiency or sinus radiographs looking for the total opacification of the paranasal sinuses commonly seen in this disorder (Ratjen and Doring, 2003). See Figure 1-5 for the clinical signs of CF.

Chronic airway disease

Chronic cough

Airway colonisation with pathogens

Persistent abnormalities on chest radiograph

Airway obstruction

Clubbing

Pansinusitis

Nasal polyps

Gastrointestinal disease

Meconium ileus, distal obstruction syndrome, rectal prolapse

Pancreatic insufficiency, pancreatitis

Biliary cirrhosis

Failure to thrive, oedema with hypoproteinaemia,

deficiency of fat-soluble vitamins

Pseudo-Bartter's syndrome (salt wasting with metabolic alkalosis)

Infertility due to obstructive azoospermia

Figure 1-5 Clinical signs of CF
(Ratjen and Doring, 2003)

1.3.5 Screening

Neonatal screening programmes have been introduced in many countries with 50 % of all patients in the USA diagnosed by the age of 6 months (Cystic Fibrosis, 2006). Modern screening programmes are based on a two-step approach: first, test for immune-reactive trypsin in dried blood spots; and second confirm result by DNA analysis in positive cases. Only a few studies have evaluated screening and whether early diagnosis will affect long-term outcome continues to cause controversy (Merelle *et al.*, 2001).

1.3.6 Monitoring

Routine clinical investigations provide the means for assessing pulmonary status and are used to monitor disease progression and response to treatment. These include laboratory investigations (white cell count and C reactive protein) and pulmonary imaging (chest X-rays and high resolution CT scanning). Lung function testing with spirometry or plethysmography is the principal measure of pulmonary status in individuals with CF older than 5 years of age. Serial measurements document stability or progression of airway obstruction and air trapping. These measures are also useful in documenting acute changes associated with pulmonary exacerbations and response to therapy.

1.3.7 The parasites

While the gene defect results in a myriad of clinical problems for the patient, pulmonary disease, in the form of chronic pulmonary infection, accounts for most morbidity and mortality (Lyczak *et al.*, 2002). In fact, CF can be described as "an inherited susceptibility to bacterial respiratory infections" (Tummler and Kiewitz, 1999) caused by surprisingly few bacterial pathogens. Overall, *P. aeruginosa* is the most common isolate, followed by *Staphylococcus aureus* and *H. influenzae* (Cystic Fibrosis Foundation, 2006). See Figure 1-6.

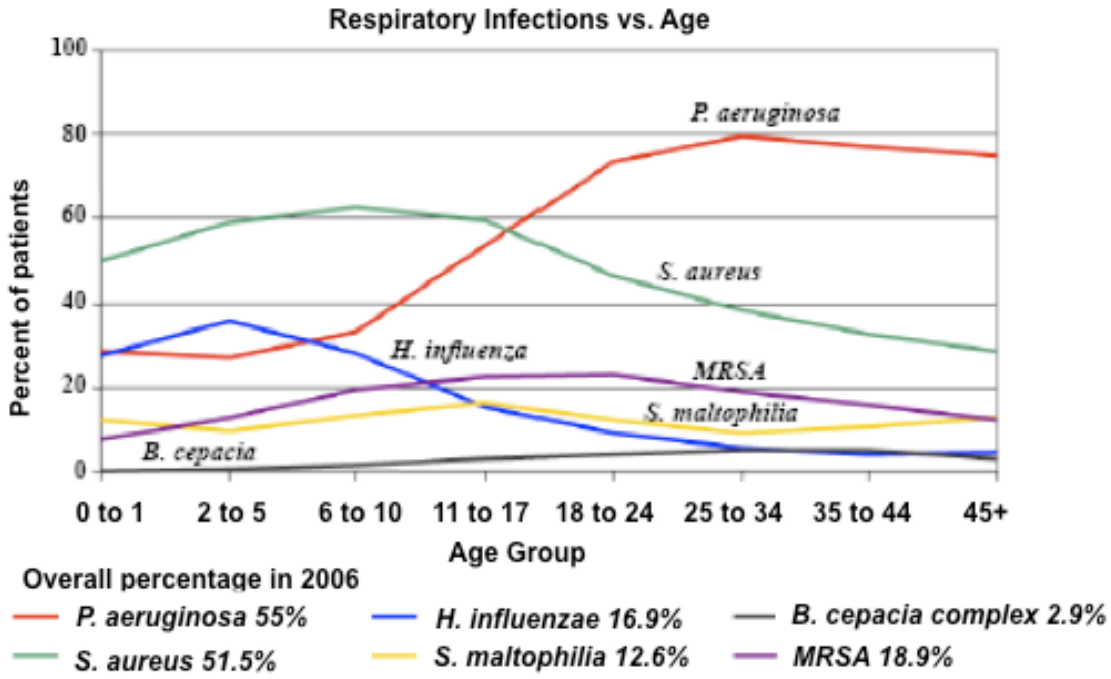


Figure 1-6 Age-specific prevalence of airway infections in patients with CF.
(Cystic Fibrosis Foundation, 2006)

S. aureus was the first pulmonary pathogen recognised in patients with CF and in the present day, it is often the first organism cultured from the respiratory tract of young children with CF. Historically, the development of antistaphylococcal penicillins has been associated with significant improvements in patient longevity. Clearly the presence of *S. aureus* in the lower respiratory tract is representative of a pathological situation and there is a consensus amongst clinicians about a beneficial effect from treatment of *S. aureus* associated with clearance of the organism from the sputum. However, the degree of pathology associated with its presence in the lungs has never been adequately assessed in CF patients and there is no data indicating that its treatment leads to improved lung function or other clinical benefit (Lyczak *et al.*, 2002). There are reports in the literature which question the benefit of prophylactic antistaphylococcal therapy (Ratjen, 2001) (Stutman *et al.*, 2002) and there is evidence that the presence of *S. aureus* and the absence of *P. aeruginosa* predicts long-term survival in CF patients after the age of 18 years (Huang *et al.*, 1987) (Hudson *et al.*, 1993).

While essentially all patients prior to the 1950s died by the age of 10 years, by the 1990s approximately one-third were surviving to adulthood (Fitzsimmons, 1993). This increased mean survival has had a dramatic impact on the nature of CF as an infectious

disease since the longer survival of patients has created opportunities for infection with organisms other than *S. aureus*.

H. influenzae is the third most commonly recovered bacterium from the respiratory tracts of CF patients, typically children. The infecting organism is nontypeable and is therefore not prevented by childhood immunisation against *H. influenzae* type b. Data related to the pathogenic potential of nontypeable *H. influenzae* are virtually nonexistent yet many clinicians regard the possibility of this organism colonising the lung as significant enough to warrant therapy.

Today *P. aeruginosa* is the most prevalent pulmonary pathogen in CF patients, irrespective of geographical location. Yet this has not always been the situation. Prior to 1946 the reported prevalence of CF pseudomonal infections was low (Di Sant'Agnese and Andersen, 1946), however a variety of sources indicate that during the 1960s, *P. aeruginosa* became the most prevalent organism in the airways of these patients (Pier, 1985) and this coincided temporally with the introduction of regional centres which specialised in CF care. Whilst these centres have promoted standardised principles of multidisciplinary therapy, which has made an important contribution to increasing the mean survival of patients (Ramsey, 1996), there is evidence to support the theory that they are potential sites for the increased risk of spread of *P. aeruginosa* (Pedersen *et al.*, 1986).

CF patients can acquire *P. aeruginosa* in their respiratory tracts at any time with most studies indicating that 70-80 % are infected by their teens (Lyczak *et al.*, 2002). In fact, there is evidence that most infection probably occurs within the first 3 years of life, which is much earlier than previously believed. The diversity of clones seen suggests that most clinical isolates originate from the environment (Burns *et al.*, 2001), however, there is mounting evidence that some widespread clonal lineages are apparently contracted through cross infection from other CF patients (Jones *et al.*, 2001). Risk factors for initial *P. aeruginosa* airway infection in patients with CF diagnosed by newborn screening included female sex, homozygous phe508del genotype and *S. aureus* isolation (Maselli *et al.*, 2003). Individuals fortunate enough to avoid colonisation show survival rates twice that of colonised individuals (Hutchison and Govan, 1999).

Other organisms that are identified later in the course of CF airways disease include *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, methicillin-resistant *S. aureus*, fungi including *Aspergillus* and nontuberculous mycobacteria. *B. cepacia* was first described as a significant pathogen among this patient population in 1984 (Isles *et al.*, 1984) and is now known to include at least nine genomovars or genomic species, collectively referred to as *B. cepacia* complex. Whether strategies to reduce the prevalence of *P. aeruginosa* infections could lead to the situation where *B. cepacia* complex or one of the other emerging pathogens dominates in the CF airways is unknown, but a worrying possibility.

1.3.8 Diagnostic microbiology

It is accepted that the upper respiratory tract is colonised by normal flora while the lower respiratory tract is maintained sterile by various defences of the host. Therefore any organism recovered from the lower airway is considered a pathogen. A significant issue in the diagnostic microbiology of CF is sampling of the airways. Expectorated sputum is currently the preferred source of airway secretions in CF subjects and is composed of lower respiratory tract secretions along with nasopharyngeal and oropharyngeal secretions, cellular debris and microorganisms. It is accepted to be an accurate indicator of lower airway microbiology (Thomassen *et al.*, 1984) (Henig *et al.*, 2001), with insignificant levels of oropharyngeal contamination (Gilljam *et al.*, 1986). However subjects with mild disease, young subjects or those not colonised with *P. aeruginosa* may not produce much if any sputum.

Alternative sources of specimens are oropharyngeal cultures (OP) or throat swabs, which are often used in young children, broncheolar lavage fluid (BAL) and sputum induction (SI). Studies comparing OP and BAL cultures indicate that OP cultures have high specificity (i.e. a negative culture is useful in ruling out lower airway infection) but poor sensitivity (i.e. a positive culture is not reliable to make the diagnosis of *P. aeruginosa* in the lower airway) (Rosenfeld *et al.*, 1999). Whilst culture of BAL fluid is considered a more sensitive measure of infection in nonexpectorating patients, the procedure is more invasive and requires sedation, thus increasing risk and cost. One important caveat is that BAL fluid samples are obtained from only a small portion of the lung, leaving the possibility that pathogens might be present in parts of the lung not sampled by lavage.

Hypertonic saline induction of sputum has been reported to be a good surrogate for lower airway sampling for both microbiology and inflammatory markers, in both adult and older paediatric patients with CF. In a comparison of culture results from expectorated sputum, BAL fluid and induced sputum, similar detection rates for bacteria and fungi were identified with all three sample sources (Henig *et al.*, 2001). Currently, such a method may have an important role as a research tool and is not a routine feature of clinical practice.

1.3.9 The environmental niche

The causal associations between mutant forms of the CFTR and susceptibility to lung infection remain controversial. Several competing theories exist which attempt to define the host factors that create the unique environmental niche within which *P. aeruginosa* dominates.

The cell-receptor hypothesis suggests that CF cell organelles are more acidic (Poschet *et al.*, 2001) or alkaline (Imundo *et al.*, 1995) than organelles from normal cells and that altered pH leads to reduced sialysation of glycoconjugates on CF epithelial cell membranes. Increasing numbers of asialoGM1 molecules (a receptor for many bacterial respiratory pathogens) may result in increased binding of *P. aeruginosa* (Poschet *et al.*, 2001) and *S. aureus* (Imundo *et al.*, 1995). CFTR itself has been characterised as a receptor for *P. aeruginosa* with the outer core region of the LPS acting as the ligand. When CFTR displays normal function, the cell can internalise and kill the pathogen. By contrast, phe508del CFTR cannot bind this pathogen, leaving bacteria free to multiply in the airway lumen of the lungs of these patients (Pier *et al.*, 1996).

Alternative hypotheses debate the contribution of the defective CFTR to the nature of the biphasic mucus layer in CF. This consists of a low-viscosity liquid perciliary layer within which cilia beat, causing the mucus to flow unidirectionally toward the oesophagus. Above this is a more viscous layer consisting of high molecular weight mucins. These are glycoproteins whose properties are altered by water content, ion concentrations and pH. It is within this upper layer that microorganisms and other particles become trapped, often binding to the carbohydrate side chains of mucins.

Hence the salt-defensin or compositional hypothesis is based on the assumption that in the CF airway there is a raised mucus salt concentration (Smith *et al.*, 1996;

Widdicombe, 2001). Since the activity of airway antimicrobial peptides or defensins is inactivated by a salt concentration greater than 50 mmol/L, bacteria can multiply on the respiratory epithelial cell surfaces of these patients, leading to infection.

The tonicity of airway fluid is still the matter of much debate. Thus the competing isotonic fluid depletion hypothesis proposes isotonic salt concentrations as a result of abnormal sodium absorption from the airway lumen, coupled with a failure of CFTR to secrete chloride (Matsui *et al.*, 1998) leading to a water/volume depleted periciliary liquid. Water loss increases overall mucus viscosity and impairs mucociliary clearance and cough clearance. Bacteria invading the CF lung are trapped in this viscous mucus on top of respiratory epithelial cells. At this stage, the anoxic mucus hypothesis proposes that the bacteria encounter microaerophilic or anaerobic growth conditions (because of abnormal oxygen consumption of the CF cell). These growth conditions trigger a switch of *S. aureus* and *P. aeruginosa* from non-mucoid to mucoid cell-types, the main phenotype in the CF lung (Worlitzsch *et al.*, 2002).

No single hypothesis alone adequately explains why only a select group of organisms occur, why they show a particular sequence of colonisation and particular phenotypes such as mucoidy occur. What is established, however, is that there is no evidence for a systemic immunodeficiency in CF to explain this chronic endobronchial infection. There is no increased frequency or severity of infections outside the respiratory tract and patients with CF have normal responses to standard immunisations (Chmiel *et al.*, 2002). In this sense, airway infections with *P. aeruginosa* are unique and further clarification of the host factors, which enhance the propensity for this opportunist to initially colonise, is awaited.

1.3.10 Adaptation to a niche

A defining feature of *P. aeruginosa* lung infections in CF is chronicity. The use of genomic fingerprinting has shown that most individuals become colonised with a single clone of *P. aeruginosa* that persists throughout the patient's lifetime (Romling *et al.*, 1994). Further, when sputum from a single patient is plated out, a broad spectrum of clonal morphology is seen, reflecting the remarkable adaptation and diversification of a single clone to various niches in the lung (Tummler and Kiewitz, 1999).

In addition to spatial adaptation, characteristic temporal changes are seen. Early and sometimes intermittent colonisation of the lung occurs with organisms which resemble environmental isolates in their phenotype. They are motile, free swimming (planktonic) with a nonmucoid phenotype and smooth lipopolysaccharide (LPS). With time, certain phenotypic characteristics appear to be selected in the CF airways and chronic colonisation by a transformed nonmotile, sessile, mucoid phenotype with a rough LPS contained within a complex biofilm is seen (Hutchison and Govan, 1999) (Tummler and Kiewitz, 1999). Given that chronic infection with *P. aeruginosa* is the main proven perpetrator of lung function decline and ultimate mortality in CF patients, understanding these mechanisms which enable *P. aeruginosa* to persist and resist treatment are crucial.

1.3.11 The Biofilm Mode of Growth

Light and electron microscopy of sputum and post-mortem samples have revealed that rather than existing as isolated organisms, *P. aeruginosa* forms microcolonies or biofilms in the CF lung (Lam *et al.*, 1980) (Worlitzsch *et al.*, 2002). Additional physiological evidence shows a change in QS signal profiles comparing planktonic and biofilm *P. aeruginosa* organisms (Singh *et al.*, 2000).

A biofilm is a structured community of bacterial cells (single or mixture of species) enclosed in a self-produced polymeric matrix. Biofilms are widely seen in natural environments and are said to occur whenever bacteria come into contact with a surface. Interestingly, in the CF lung, the pseudomonads have been seen to colonise the bronchiolar lumen and it is unclear whether the respiratory epithelium or mucus itself represents the anchoring surface (Worlitzsch *et al.*, 2002) (Hasset *et al.*, 2002).

Biofilms are highly hydrated structures, consisting predominantly of water and only 10-20 % bacteria. They have a clear structure of polysaccharide penetrated by minute anatomising water channels as a primitive circulation (Costerton *et al.*, 1994) and gradients of oxygen, nutrients, waste and signalling factors exist. The environmental heterogeneity creates a heterogeneous population of bacterial cells (Mah and O'Toole, 2001), although they typically show reduced growth compared with planktonic bacteria (Brown *et al.*, 1988). In fact the complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms (Costerton *et al.*, 1995).

Whilst recognised for about 100 years, detailed molecular study of biofilms only began three decades ago and *P. aeruginosa* is among the best studied biofilm formers (O'Toole, 2003). This has been achieved *in vitro* by examining static growth on inert surfaces or in specially constructed flow-chambers, which allow a more representative simulation of *in vivo* conditions. Animal models of biofilm bacteria can be established in rats and mice by intratracheal inoculation of bacteria in agar beads and alginate beads (Hoiby *et al.*, 2001). The recent advances in confocal laser scanning microscopy (CLSM) allow examination of living fully hydrated biofilms in three-dimensions. *In situ* gene expression can also be evaluated microscopically by the use of green fluorescent protein (GFP) fusion reporter constructs.

1.3.12 Biofilm formation and regulation

It is widely accepted that the planktonic-biofilm transition is a complex and highly regulated bacterial developmental process. Current models suggest that biofilm formation involves particular stages or bacterial phenotypes that are well conserved among a wide range of organisms. These include a reversible then irreversible attachment of organisms, maturation of the biofilm and then dispersion. Bacteria within each stage of development are generally believed to be physiologically distinct from cells in other stages of development and in mature biofilms all stages of development may be present to some degree at the same time (Sauer *et al.*, 2002).

Microbial factors that enable biofilm formation are beginning to be characterised and include those that facilitate motility and adherence as well as those contributing to the biofilm architecture. The initiation and regulation of these factors is thought to involve environmental conditions and cues such as nutrient and oxygen availability, hydrodynamics and the nature of the surface adhered to (Hall-Stoodley and Stoodley, 2002) (Geesey, 2001). These then trigger multiple complex genetic regulatory pathways, which may show redundancy and overlap. Like other developmental pathways, a number of different regulators are involved. For example, Crc is a global carbon metabolism regulator which has also been shown to regulate the genes involved in early biofilm formation (O'Toole *et al.*, 2000b). A *crc* mutant had attenuated biofilm formation suggesting that Crc may be part of a signal transduction pathway that relays signals (such as carbon availability) and thereby regulates the transition from planktonic to biofilm growth.

The possibility of a role for QS in biofilm formation was first hypothesised by Williams and Stewart (Williams *et al.*, 1994). Indeed, QS seems ideally suited for bacteria in a diffusion-limited environment such as a biofilm. The detection of *lasR* transcripts (Storey *et al.*, 1998) and AHLs in CF sputum (Middleton *et al.*, 2002) and the production of PQS by *P. aeruginosa* isolates from CF airways (Guina *et al.*, 2003) is compelling evidence that it does indeed plays a role *in vivo* in the pathogenesis of this organism in the CF lung.

Much interest and rapid progress in biofilm research followed the publication of a paper by Davies and colleagues (Davies *et al.*, 1998) in which evidence for a correlation between biofilm formation and QS in *P. aeruginosa* was presented. They hypothesised that because QS requires a sufficient density of bacteria, signals would not be expected to participate in the initial stages of biofilm formation, attachment and proliferation. However they may be involved in differentiation. In support of this, they demonstrated that *lasI* mutants incapable of synthesising 3-oxo-C12-HSL formed biofilms with abnormal structure (thin and undifferentiated) that were more sensitive to the detergent biocide sodium dodecyl sulphate (SDS).

Along side this, O'Toole and Kolter (O'Toole and Kolter, 1998) conducted a simple genetic screen in which random transposon mutants were grown in microtitre plates and those that did not form biofilms were investigated further. They found that flagellar motility was required for primary adhesion and that type IV pili were essential for cellular aggregation. In a following paper, Glessner and coworkers (Glessner *et al.*, 1999) demonstrated that both the *las* and *rhl* QS systems are required for type-IV pilus-dependent twitching motility. C4-HSL in particular appeared to influence both the export and surface assembly of surface type IV pili, while 3-oxo-C12-HSL played a role in maintaining cell-cell spacing and associations required for effective twitching motility.

Subsequent research, however, has challenged these original findings. In a study investigating the influence of alginate production on biofilm structure in the laboratory strain PAO1, the wildtype control biofilm was flat and appeared more like the QS *lasI* mutant biofilm reported above. This was attributed to differences in media composition (Hentzer *et al.*, 2001). In fact, Beatson *et al.* (Beatson *et al.*, 2002) have suggested that the results obtained by Davies *et al.* (1998) and Glessner *et al.* (1999) were most likely due to the occurrence of spontaneous secondary mutations in key regulatory genes in

the strains shared by these two groups. Additionally, Heydorn and colleagues have provided evidence that twitching motility is not required for microcolony formation and that cell-cell signalling via *lasI-lasR* QS is not required for the development of mature biofilms (Heydorn *et al.*, 2002).

This debate serves to illustrate the challenges of studying the role of QS in biofilm formation and of biofilm formation itself. Whilst compelling evidence from research in other species leaves no doubt that QS is essential for the development of biofilms (Lynch *et al.*, 2002), as a global regulator of operons encoding different functionalities, mutations in the QS control system will have diverse effects with potentially complex consequences for the expression of many genes. Other regulators, experimental conditions, such as the nutritional environment (Shrout *et al*., 2006) and the methods of analysis will also have an impact. Ongoing research aims to develop the methodologies and technologies, which can accommodate these complexities inherent in the investigation of biofilms (Heydorn *et al.*, 2000b) (O'Toole, 2003).

1.3.13 The extracellular polymeric substance (EPS) matrix

A key feature of biofilms is that they enable *P. aeruginosa* to persist in the CF lung and this has been attributed to the nature of the biofilms developing in this unique environment. The hallmark of a mature biofilm is the production of an extracellular matrix or EPS, which determines its architecture and may also contribute to its strength and material properties (Hall-Stoodley and Stoodley, 2002). The chemistry of the EPS is complex and includes polysaccharides, nucleic acids and proteins, although their nature and relative contributions are ill-defined.

Historically, research has focused on the role of alginate, a polymer of the uronic acids mannuronic and guluronic acid and alginate overproduction leads to the mucoid phenotype of *P. aeruginosa*. This appears to be a unique adaptation to the CF lung environment as it is not seen in infections elsewhere in the body and mucoid isolates typically revert to the nonmucoid phenotype when grown on laboratory medium (Nivens *et al.*, 2001). The mechanism for the overproduction of alginate is complex and requires several regulatory proteins that act in a hierarchical regulatory cascade (Wozniak and Ohman, 1994). Possible triggers for this conversion include hypoxia (Worlitzsch *et al.*, 2002), hydrogen peroxide (Mathee *et al.*, 1999) and contact with a surface (Davies *et al.*, 1993). Alginate is thought to promote persistence of

P. aeruginosa by offering a degree of protection in the harsh lung environment in which bacteria are continually subjected to oxidative stress and attack by the immune system (Simpson *et al.*, 1988).

In keeping with its contribution to the chronicity of lung disease, there is evidence that alginate production enhances biofilm structure and increases biofilm resistance to the antibiotic tobramycin (Nivens *et al.*, 2001) (Hentzer *et al.*, 2001). Yet the relationship between biofilm formation and the conversion to mucoidy remains unclear. Notably, alginate is not actually required for biofilm formation (Nivens *et al.*, 2001). Both mucoid and nonmucoid strains readily form biofilms and this demonstrates that alginate cannot be the universal structural matrix. Whilst it is accepted that its expression and role may be important under certain environmental conditions, the suggestion that other exopolysaccharides might be important must be entertained.

Another candidate molecule is LPS: a major component of the cell wall of Gram-negative bacteria that consists of a hydrophobic lipid A region, a central core oligosaccharide region and a repeating polysaccharide portion termed the O antigen. LPS molecules are important for the structure of individual bacterial cells and also mediate interactions with the neighbouring environment. Indeed, changes in the LPS are characteristically seen in the CF lung and are thought to contribute to biofilm formation. For example, a transition from a 'smooth' to a 'rough' phenotype by the loss of the O antigen is a typical feature of chronic infection and studies have shown that rough mutants form a more stable biofilm (Flemming *et al.*, 1998).

Several groups have undertaken studies to identify alternative polysaccharide candidates for the biofilm matrix and two loci have been discovered. The *pel* genes produce a glucose-rich matrix polysaccharide that was found to be essential for biofilm formation at the air-liquid interface of standing cultures (pellicles) and for biofilm structure in laboratory strains PA14 and PAK (Friedman and Kolter, 2004a) (Vasseur *et al.*, 2005). This Pel polysaccharide is biochemically and genetically distinct from the second polysaccharide identified: Psl (polysaccharide synthesis locus). The *psl* operon was found to be essential for biofilm formation in strains PAO1 and ZK2870 (Friedman and Kolter, 2004b; Jackson *et al.*, 2004) and has been shown to be mannose and galactose-rich (Ma *et al.*, 2007).

Whilst polysaccharides are the most-studied component of the EPS, there is a paucity of data as to their exact nature. Ultimately this comes down to the difficulties in studying polysaccharides, which is in part due to the difficulty in the separation and extraction of EPS from bacterial cells as well as the complexities of analysing polysaccharides, which can be highly branched with a wide variety of linkages and side groups (Hall-Stoodley and Stoodley, 2002). Given that it is widely accepted that carbohydrates represent important binding ligands in biofilms, an alternative and promising avenue of investigation concerns their receptors.

1.3.14 Lectins

"Lectins are ubiquitous proteins that bind carbohydrates specifically and reversibly and may therefore aggregate cells and glycosylated macromolecules" (Garber *et al.*, 1992) such as the EPS of bacterial biofilms.

P. aeruginosa synthesises two lectins termed LecA and LecB (formerly PA-IL and PA-IIL respectively). LecA was discovered because of the ability of *P. aeruginosa* cell-free extracts to haemagglutinate papain-treated human erythrocytes (Gilboa-Garber, 1972) and it was the first bacterial lectin to be purified by the use of affinity chromatography (Gilboa-Garber *et al.*, 1972). Subsequently, LecB was discovered in extracts of the same bacterium isolate when cultured in a different medium (Gilboa-Garber, 1982). The combined sugar-binding spectra of these two lectins cover a wide range of human antigens (ABH, P and I systems) that are common to all human cells and tissues and this may well be related to the reported ability of *P. aeruginosa* to infect most of them (Imberty *et al.*, 2004).

The *lecA* gene consists of 366 bp and codes for a 51 kDa tetrameric protein composed of four 12.75 kDa subunits, each made up of 121 amino acids (Gilboa-Garber *et al.*, 1972). Calcium together with a unique network of hydrogen bonds generates a binding site endowed with a selective specificity for D-galactose and its derivatives. Equilibrium dialysis and haemagglutination inhibition tests by Garber and colleagues (Garber *et al.*, 1992) have shown that LecA binds hydrophobic derivatives of thiogalactose and galactose better than D-galactose itself.

The 47 kDa lectin LecB is also a tetramer, with each subunit consisting of 114 amino acids. Its gene, *lecB* (345 bp) is located about 867.5 kb away from *lecA* on the

P. aeruginosa chromosome. There are two calcium ions at the binding site, which shows affinity for L-fucose and other monosaccharides such as mannose (Gilboa-Garber *et al.*, 2000). See Figure 1-7 for the crystal structures of the lectin/cognate-carbohydrate complexes and Table 1-1 for a comparison of the properties of the two lectins.

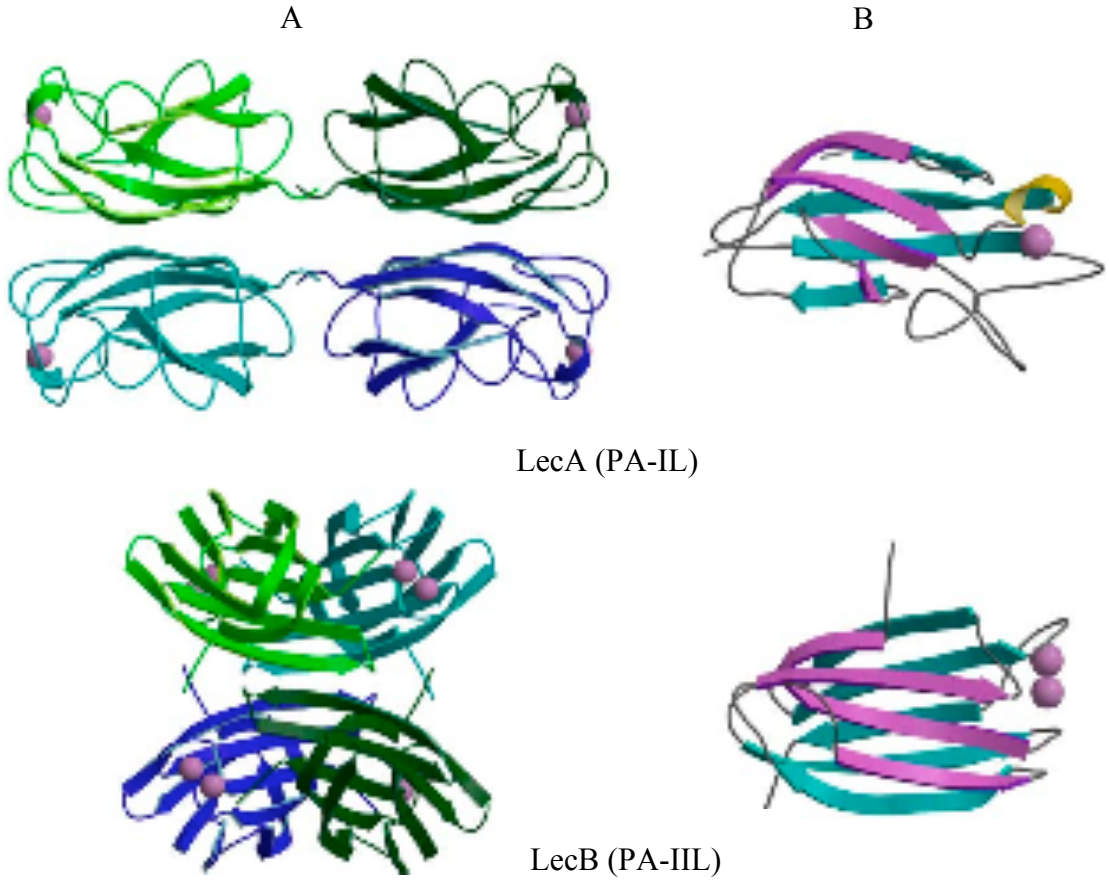


Figure 1-7 Crystal structure of the LecA/galactose and LecB/fucose complexes.
(A) Tetramer with stick representation of monosaccharide and space-filling representation of calcium ions. (B) Monomer with the β-sheets represented by two colours.
(Imberty *et al.*, 2004)

Table 1-1 Comparison of LecA (PA-IL) and LecB (PA-IIL) properties
(Imberty *et al.*, 2004)

Property	PA-IL	PA-IIL
Gene	*lecA* (366 bp)	*lecB* (345 bp)
Swiss-Prot/TrEMBL accession number	Q05097	Q9HYN5
Number of amino acids (without initiation Met)	121	114
Molecular mass (Da)	12,753	11,732
PI	4.94	3.88
Oligomeric state	Tetrameric	Tetrameric
Cations observed in crystal structures	1 Ca^{2+} [a]	2 Ca^{2+} [a]
Monosaccharide in the binding site (pdb code)	D-Galactose (1OKO)	L-Fucose (1G7L, 1OXC) D-Mannose (1OVS, 1OUR) D-Mannose (1OVS, 1OUR)

[a] Biochemical studies indicated the additional presence of Mg^{2+} in PA-IL, and Mg^{2+} and Zn^{2+} in PA-IIL.

Sudakevitz and Gilboa-Garber (Sudakevitz and Gilboa-Garber, 1987) have demonstrated that LecA and LecB are present in the extracts of most *P. aeruginosa* strains, including hospital isolates. Whilst most of their activity is stored intracellularly, small but significant fractions of these lectins are present on the cytoplasmic membrane, on the outer membrane and in the periplasmic space (Glick and Garber, 1983). It is said that the lectins are released from bacteria following their lysis and this may occur secondary to the disruption of cells by host defences (Imberty *et al.*, 2004). An alternative theory is that the lectins are secreted by the bacterial cell, possibly via the type II pathway (Stacey, 2003), however the *lecA* gene does not appear to carry a signal sequence.

For many Gram-negative bacteria, lectin-carbohydrate interactions are involved in microbial pathogenicity, especially by enhancing bacterial adherence to epithelial cells (Beuth *et al.*, 1990). For example, Laughlin *et al*. (Laughlin *et al.*, 2000) demonstrated that LecA not only aids adhesion of *P. aeruginosa* to intestinal epithelial cells in lethal gut-derived sepsis in the mouse, but also induces a permeability defect, allowing cytotoxic exoproducts, including exotoxin A, to cross the epithelial barrier. There is evidence that LecA promotes adherence to the respiratory epithelium (Plotkowski *et al.*, 1989) and significantly, Bajolet-Laudinat *et al.* (Bajolet-Laudinat *et al.*, 1994) have

demonstrated a dose-dependent cytotoxic effect of LecA on respiratory epithelial cells *in vitro*. In addition, a search of sequence databases for proteins displaying similarities to these lectins yielded no positive hit for LecA (Imberty *et al.*, 2004), suggesting that any pathogenic role it may play in the lung is unique to *P. aeruginosa.*

In fact, recent microarray work by Whiteley and colleagues has shown that *lecA* is over 1000-fold induced during anaerobic growth and as such, is the most highly regulated anaerobic gene that their group has seen (Whiteley 2004, personal correspondence). This has great significance given that there is evidence that CF mucus is essentially anaerobic and that *P. aeruginosa* seems to thrive as biofilms under these conditions (Borriello *et al.*, 2004) (Hasset *et al.*, 2002).

The regulation of lectins in *P. aeruginosa* is complex and involves the *las* and *rhl* QS systems. Most work has been undertaken on *lecA* expression, which has been shown to be dependent on RhlR/C4-HSL. Furthermore, LecA production is growth-phase dependent and requires RpoS (Diggle *et al.*, 2002) (Winzer *et al.*, 2000). Additional regulatory elements exist such as the post-transcriptional regulator RsmA (Pessi *et al.*, 2001). PQS is also needed and its presence can overcome the cell-density but not growth-phase dependency of *lecA* regulation (Diggle *et al.*, 2003).

The role of MvaT, a novel global regulator of virulence gene expression in *P. aeruginosa* is also being unravelled. A mutation in *mvaT* results in enhanced *lecA* expression and the addition of exogenous AHLs to this mutant advances *lecA* expression, suggesting that MvaT is involved in growth phase-dependent regulation (Diggle *et al.*, 2002). Interestingly, the phenotype of this MvaT mutant demonstrates an increased ability to form biofilms and the question of whether this is the result of altered lectin production was raised (Stacey, 2003).

To investigate this hypothesis, Diggle *et al*. (Diggle *et al.*, 2006b) demonstrated that *lecA* is expressed in *P. aeruginosa* PAO1 wildtype biofilms and provided evidence that in contrast to this parent strain, a *lecA* mutant was unable to form mature biofilms. A mutant defective in MvaT, which overproduces LecA, was observed to form significantly thicker biofilms, which covered a correspondingly greater surface area. The addition of exogenous galactosides with a strong affinity for LecA abolished biofilm formation on stainless steel and dispersed pre-formed biofilms.

Further clues detailing this proposed role for LecA in biofilm formation arise from the realisation that *lecA* is not the only gene upregulated in the enhanced biofilm-forming *mvaT* mutant. A recently identified gene cluster, termed *cupA*, is also negatively controlled by MvaT (Vallet *et al.*, 2004). This is believed to be important in the assembly of putative fimbrial structures and may therefore play an important role in the initial colonisation of surfaces (Vallet *et al.*, 2001). There is data to suggest that whilst *cupA* genes play an important role in the early stages of biofilm formation, the *lecA*-encoded LecA contributes to cell-cell interaction and hence biofilm maturation at a later stage (Vallet *et al.*, 2004) (Diggle *et al.*, 2006b). The hypothesis that it contributes to the structure of biofilms by means of its ability to bind carbohydrates is lent support by the observation that purified LecA preparations always contain traces of bound LPS (Gilboa-Garber, 1997).

Work has also been undertaken to investigate whether LecB contributes to *P. aeruginosa* biofilms. Diggle *et al*. found that the addition of a furanoside with a strong affinity for LecB, at a concentration likely to be effective, did not have an effect on biofilm formation (Diggle *et al.*, 2006b). However data published by Tielker and colleagues has provided evidence that LecB is located in the outer membrane and that a LecB-deficient mutant was impaired in biofilm formation (Tielker *et al.*, 2005). The report that this defect in biofilm formation is due to lack of pilus biosythesis in the *lecB* mutant strain raises the important point that some of the ascribed functions of LecA and LecB may be secondary effects on other systems rather than direct effects of the lectins themselves (Sonawane *et al.*, 2006).

1.3.15 The significance of the biofilm mode of growth

Whilst the details of biofilm formation have yet to be clarified, the huge significance of biofilms cannot be disputed and is the fuel which powers research in this field. Biofilms are ubiquitous in the environment and in man. Whilst precise figures are lacking, it has been estimated that biofilms are associated with 65 % of nosocomial infections (Licking, 1999) and account for over 80 % of microbial infections in the body (http://grants.nih.gov/grants/guide/pa-files/PA-03-047.html). In response, there is growing concern that the adoption of biofilm concepts in medical microbiology lags far behind the acceptance of these notions of bacterial growth in adjacent areas of research, such as microbial ecology and industrial microbiology (Costerton, 2001).

Whilst much research has focused on single species *P. aeruginosa* biofilms, mixed biofilms are more representative of those which occur in nature. In the CF lung, *P. aeruginosa* and the emerging human pathogen *B. cepacia* may coexist in biofilms and this environment may provide the means for interaction between these two organisms. Both species are know to employ QS and investigations by Riedel *et al.* (Riedel *et al.*, 2001) provide evidence for unidirectional cross-talk, with *B. cepacia* capable of perceiving AHL QS signals of *P. aeruginosa* but the latter not responding to the AHL signals of the former. Interestingly, although *S. aureus* uses a different peptide-based QS system, the exogenous addition of AHLs produced by *P. aeruginosa* to cultures of *S. aureus* affected virulence-determinant production by this organism, suggesting that QS may allow *P. aeruginosa* to displace *S. aureus* in the CF lung (Qazi *et al*., 2006). In contrast, it has been reported that HQNO from *P. aeruginosa* can lead to the development of antibiotic resistant and difficult to detect small colony variants of *S. aureus* (Hoffman *et al*., 2006). This could allow *P. aeruginosa* to benefit from useful *S. aureus* metabolites, such as iron (Mashburn *et al*., 2005).

A major role of biofilms is their contribution to the persistence of *P. aeruginosa* in the CF lung. This strategy has been characterised as tenacious survival rather than aggressive virulence and seems to be the result of biofilm resistance to treatment and protection from the host immune response.

The intrinsic ability of *P. aeruginosa* to develop resistance to many commonly used antibiotics is not well understood but has been speculated to be due to the structure of the cell wall, the extensive linkage of outer membrane proteins to the LPS or the extremely hydrophilic nature of the outer membrane, which may exclude many hydrophobic antibiotics (Lyczak *et al.*, 2002). However, these individual mechanisms of antibiotic resistance do not seem to be responsible for the protection of bacteria in a biofilm and other multicellular strategies have been proposed. These include failure of the antibiotic to penetrate the full depth of the biofilm, an altered chemical microenvironment within the biofilm affecting antibiotic action and speculation that some biofilm bacteria may adopt a protective, spore-like state (Stewart and Costerton, 2001).

Hence, antibiotic therapy (developed against planktonic cells and selected because of results of planktonic assays) typically reverses the symptoms caused by planktonic bacteria released from the biofilm but fails to kill the biofilm itself. For this reason

biofilm infections, such as *P. aeruginosa* infections in the CF lung, typically show recurring symptoms or exacerbations necessitating repeated cycles of symptomatic but not curative antibiotic therapy (Costerton *et al.*, 1999).

The immune response is an important player in the host-parasite interaction, yet its ineffectiveness against biofilms is another major reason why *P. aeruginosa* persists in the CF lung. Sessile *P. aeruginosa* communities release antigens whilst growing as biofilms and very high concentrations of antibodies to *P. aeruginosa* are seen in the circulating blood and in the lungs. These react with their specific antigens in the outer reaches of the matrices but neither their bactericidal nor opsonising capabilities are realised and in fact may lead to a worse clinical outcome because they may cause immune complex damage to surrounding tissues (Costerton *et al.*, 1999). Overall, this overstimulated yet frustrated immune response fails to clear the biofilms and together, infection and inflammation lead to tissue destruction, loss of function and ultimately, the demise of the patient.

1.4 Perspectives

Overall, *P. aeruginosa* infection in CF is a complex series of events involving host abnormalities secondary to CFTR dysfunction, exacerbated by consequent cycles of bacterial adaptations and host responses. Ultimately, the point of trying to unravel this interaction is in order to identify novel targets for intervention.

Current cornerstones of treatment are nutritional regimes, physical airway clearance exercises and antibiotic chemotherapy. These are delivered by multidisciplinary clinical teams who adhere to strict infection control measures. Given the intensity of research in the field, treatment strategies are flexible in order to incorporate new advances such as the use of recombinant human DNase I as a mucolytic and the use of macrolides as antiinflammatory agents. There are great expectations for the development of CF gene transfer therapy, antiinflammatory therapy and effective immunotherapy as presently, lung transplantation is the final therapeutic option for patients with end-stage lung disease.

There is no doubt that chronic infection with *P. aeruginosa* is the major cause of ill-health and death in this patient population. Whilst the use of antibiotics leads to improvement in lung function (the best predictor of survival in these patients), timing

and regime are largely empirical and the rise of resistant strains poses new challenges. However, thinking about bacterial populations as connected organisms capable of concerted multicellular activities may provide researchers with the opportunity to target multicellular behaviour and the formation of multicellular structures such as biofilms.

In fact, methods for disrupting QS, including destruction of AHLs or interference with their binding, are being developed. For example, Hentzer *et al.* (Hentzer *et al.*, 2002) have demonstrated that suppression of thicker more complex biofilm structures was possible using a synthetic furanone to inhibit QS. Alongside this, new diagnostic systems, which have the capacity to interpret and indicate the presence of QS will be needed (Williams, 2002). Indeed if a rise in AHLs and PQS in CF sputum correlates with clinical deterioration, they may represent useful diagnostic biomarkers which provide additional information about pathogenic behaviour. The ability to detect the lectin LecA may indicate that biofilm development is occurring and given its cytotoxicity, may also provide a non-invasive marker of airway inflammation and damage.

It is hoped that the recent characterisation of the crystal structures of the two lectins may open up a route for the design of carbohydrate-based compounds that would act as efficient inhibitors of bacterial adhesion and biofilm formation (Cioci *et al.*, 2003) (Loris *et al.*, 2003). A preliminary report, based on a one patient case, has described an anti-adhesion based therapy of respiratory *P. aeruginosa* infection using a fucose and galactose containing solution (von Bismarck *et al.*, 2001). Yet to make this work applicable to the disruption of *P. aeruginosa* infections in the CF lung, investigation of LecA production by isolates from CF sputum and further details of the specific contribution of these lectins to biofilms are needed.

1.5 Project aims

Despite major advances in CF research, premature mortality occurs in this important and vulnerable patient group in the prime of their lives. Given that *P. aeruginosa* is the major challenge, unravelling the mechanisms of its unique adaptation and pathogenicity is urgently needed in order to develop new treatment strategies to improve health and survival. This project was set in the context of exploring the QS related mechanisms governing the pathogenesis of *P. aeruginosa* in the CF lung, including the role of the pseudomonas lectins in biofilm formation. The specific aims of the project were:

1) To isolate and characterise a cohort of clinical *P. aeruginosa* strains with respect to their QS phenotype and establish the conservation and expression of *lecA* amongst these isolates.

2) To assess the ability to detect QS signal molecules in sputum from CF patients and determine whether there was any association between the level of these molecules and the clinical status of the patients.

3) To establish a flowchamber biofilm system and the associated COMSTAT and IMARIS technology required to undertake detailed *in vitro* biofilm study.

3) To investigate the contribution of LecA to biofilm maturation in both laboratory and clinical strains and identify its biofilm target ligand. Thereafter to explore the ability of anti-lectin galactosides and furanosides, including novel synthetic furanoside dendrimers, to disrupt biofilms with a view to identifying novel therapeutics to tackle *P. aeruginosa* biofilms.

Chapter 2: Materials and Methods

2.1 Patient Recruitment

Applications were submitted to Nottingham Research Ethics Committee 1 and Nottingham City Hospital Research and Development (R&D) department for the clinical research project entitled "Lectin-mediated biofilm maturation, quorum sensing and *Pseudomonas aeruginosa* infections in cystic fibrosis". Full approval for the study protocol and associated documentation was granted by Ethics and R&D in April 2004, with no amendments required. Two subsequent research monitoring visits were performed by the Nottingham City Hospital Research Governance Manager and her recommendations were implemented. See Appendix 1 for the approved study protocol.

The study was a prospective, longitudinal cohort study among a population of CF patients in Nottinghamshire. Patients were recruited from the Adult and Paediatric CF outpatients' clinics at Nottingham City Hospital, UK from May 2004 until August 2005. The diagnosis of CF had been established previously in all of these subjects by genetic testing and/or sweat test. To be included in the study, subjects had to be sputum producers. The presence of *B. cepacia* was an exclusion criterion. There was no cut off level for the forced expiratory volume in 1 second (FEV_1).

Suitable patients were identified by the CF nurse specialists and given patient information leaflets to read. Informed voluntary consent was obtained by myself or one of the CF doctors. One signed copy of the consent form was given to the patient, one was filed in the notes and one kept with the study documentation. If the patient agreed, their general practitioner (GP) was informed of their involvement in the study. See Appendix 2 for examples of patient information leaflets, consent forms and GP letter.

2.2 Sputum collection

Voluntarily expectorated sputum samples were collected by the lung function technicians or the CF nurse specialists following spirometry. Samples were obtained at each routine clinic visit at approximately two monthly intervals throughout the study

period. Specimen pots were labelled with the patient's Nottingham City hospital number to maintain confidentiality. These were transported routinely on hospital transport to the Microbiology specimen reception at the Queen's Medical Centre the same afternoon. Specimens were stored at 4 ºC overnight and then collected and transported in an airtight plastic box to the Centre for Biomolecular Sciences for further analysis.

Each sample was allocated a unique study code consisting of the letter A (for adult patients) or P (for paediatric patients), an individual patient number and the date it was produced. For example, sample A001-291204 was provided by adult patient number 1 on 29 December 2004.

Analysis of sputum samples was undertaken in a Class 1 microbiological safety cabinet within a Category 3 safety facility in order to prevent exposure to airborne droplets or particles generated in handling infected sputum samples.

2.3 Clinical data collection

Clinical data collection proformas were prepared (Appendix 3) and used to analyse patient clinical records. On the date a particular sputum sample was collected for analysis, the corresponding clinical data was recovered by Dr Paramita Cifelli, Specialist Registrar in Paediatrics, Nottingham City Hospital. This included predicted and actual spirometry values, pulse oximetry, white cell count, C reactive protein, sputum microbiology, the presence of clinical symptoms of a pulmonary exacerbation (Rosenfeld *et al.*, 2001) and antibiotic use.

2.4 Bacterial strains

2.4.1 Laboratory bacterial strains

All laboratory bacterial strains used in this study are listed in Table 2-1.

Table 2-1 Laboratory bacterial strain characteristics

Strain	Characteristics	Reference/Source
***Escherichia coli*:**		
DH5α	F^-, φ80d*lacZΔM15*, Δ(*lacZYA-argF*)$_{U169}$, *deoR*, *recA1*, *endA1*, *phoA*, *hsdR17*(r_K^-, m_K^+), *supE44*, *thi-1*, *gyrA96*, *relA1*	GibcoBRL, Life Technologies
JM109	*recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, thi Δ(lac-proAB), F' [traD36, $proAB^+$, lacIq, lacZΔM15]	(Yanisch-Perron *et al.*, 1985)
S17-1 λ*pir*	*thi, pro, hsdR, hsdM*$^+$, *recA*, RP4-2-Tc::Mu-Km::Tn7, λ*pir*	(Simon *et al.*, 1983)
***Pseudomonas aeruginosa*:**		
PAO1	Wild-type, Nottingham strain	Holloway collection
PAO1 *lecA*::*lux*	*lecA*::*luxCDABE* genomic reporter fusion in PA01	(Winzer *et al.*, 2000)
PAO1Δ*lecA*	*lecA* chromosomal deletion mutant derived from PAO1	(Stacey, 2003)
PAO1Δ*lecB*	*lecB* chromosomal deletion mutant derived from PAO1	This study
PAO1 pUCP18::gfpmut3.1	PAO1 containing pUCP18::gfpmut3.1	(Stacey, 2003)
PAO1Δ*lecA* pUCP18::gfpmut3.1	*lecA* chromosomal deletion mutant derived from PAO1 containing pUCP18::gfpmut3.1	This study
PAO1 *lecA'*::*lux*Δ*pqsA*	*pqsA* chromosomal deletion mutant derived from PA01 *lecA::lux*	(Diggle *et al.*, 2006a)

2.4.2 Clinical bacterial isolates

P. aeruginosa clinical isolates were recovered by streaking a 1 µl loopful of neat sputum on Pseudomonas Isolation Agar (PIA). Plates were incubated at 37 ºC for 24 to 48 h. Each resulting colony type was Gram-stained and tested for oxidase activity.

Those that consisted of rapidly oxidase positive Gram-negative rods were restreaked onto fresh PIA and incubated for a further 24 to 48 h.

The resulting colonies were photographed using a Nikon Coolpx995 digital camera under light and dark-phase conditions. A record of colony morphology, pigment production and mucoidy was made.

2.5 Plasmids

All plasmids used in this study are listed in Table 2-2.

Table 2-2 Plasmids used

Name	Description	Reference
pUCP18::gfpmut3.1	pUCP18 containing the *gfpmut3.1* gene, ligated in to MCS with *Hind*III and *Bam*HI	(Stacey, 2003)
pSB1142	AHL reporter plasmid containing the *P. aeruginosa lasR* gene and *lasI* promoter fused to *luxCDABE* operon from *Photorhabdus luminescens*, pACYC184 derived (Tc^R)	This laboratory Unpublished
pSB536	AHL reporter plasmid containing the *Aeromonas hydrophila ahyR* gene and *ahyI* promoter fused to *luxCDABE* operon from *Photorhabdus luminescens*, pAHP13 derived (Amp^R)	(Winson *et al.*, 1998)
pBLS	pBluescript KS cloning vector; ColE1 replicon (Amp^R)	Stratagene
pBLS::*lecB*	A 4.4 kb SacI, KpnI PAO1 chromosomal DNA fragment containing *lecB* in pBLS (Amp^R)	This study
pBLSΔ*lecB*	pBLS containing *lecB* flanking regions and deleted *lecB* gene (Amp^R)	This study
pEX18Gm	$oriT^+$ $sacB^+$, gene replacement vector with MCS from pUC18 (Gm^R)	(Hoang *et al.*, 1998)
pEX18GmΔ*lecB*	pEX18Gm containing *lecB* flanking regions and deleted *lecB* gene (Gm^R)	This study

2.6 Oligonucleotide primers

Oligonucleotide primers were synthesised by Sigma (UK). Where necessary, restriction sites were incorporated into the 5' end of the primer to aid cloning of the PCR product. Primer sequences are listed in Table 2-3.

Table 2-3 Oligonucleotide primer sequences

Primer	Sequence (5' to 3')	Function
lasI3UF	CGGGTTCACCGAAATCTATC	Primer used to amplify *lasI* upstream region
lasI3DR	ACTCGCTTTACAGCGGATTC	Primer used to amplify *lasI* downstream region
lasR3UF	CCGAATCCATATTTGGCTGA	Primer used to amplify *lasR* upstream region
lasR3DR	GAGAACCTGCCCTTCCCTAT	Primer used to amplify *lasR* downstream region
lecAUF	TTGGAAAGGTGAGGTTCTGGCTAAT	Primer used to amplify *lecA* internal upstream region
lecADR	GACTGATCCTTTCCAATATTGACAC	Primer used to amplify *lecA* internal downstream region
lecBHpa1UR	AGGGTGTTAACTCCTTGTGTTGC	Primer used to sequence out from the 5' end of *lecB* to amplify the upstream flanking region and pBluescript plasmid DNA incorporating the Hpa1 restriction site
lecBHpa1DF	TGGTGGTTAACTGGCCGCTCGGC	Primer used to sequence out from the 3' end of *lecB* to amplify the downstream flanking region and pBluescript plasmid DNA incorporating the Hpa1 restriction site
lecBUF	TGGCAACACAAGGAGTGTTC	Primer used to amplify *lecB* internal upstream region
lecBDR	GCCAGTTGATCACCACGAC	Primer used to amplify *lecB* internal downstream region
Sac1lecBUF	TATGAGCTCAACCCAACGGGCAAATCGTTG	Primer used to amplify *lecB* and 500 bp upstream incorporating the Sac1 restriction site
Kpn1lecBDR	ATAGGTACCAGGTGGCGGTGATCAACTTC	Primer used to amplify *lecB* and 500 bp downstream incorporating the Kpn1 restriction site
*pqsH*intUF	CTGGCGCGCGAATTCACCAAGGCA	Primer used to amplify *pqsH* internal downstream region
*pqsH*intDR	AAGACGCTGGTGGAGGCGCCTGCG	Primer used to amplify *pqsH* internal upstream region
rhlIUF	ACGACACGGGGACTTGGT	Primer used to amplify *rhlI* upstream region
rhlIDR	GCAGGAGAAGCGAAAAA	Primer used to amplify *rhlI* downstream region
rhlRUF	TGCCATGATTTTGCCGTAT	Primer used to amplify *rhlR* upstream region
rhlRDR	CACACATGAGGGGGAAGACT	Primer used to amplify *rhlR* downstream region

2.7 Chemical reagents

2.7.1 General chemicals

Unless otherwise stated, all chemicals were obtained from Sigma (UK).

2.7.2 Antibiotics

Stock solutions of antibiotics were prepared according to Sambrook *et al*. (2003) and stored at -20 °C. Ampicillin (Amp) was used from a 50 mg/ml in 50 % (v/v) ethanol (EtOH) stock, carbenicillin (Cb) from a 50 mg/ml stock in deionised water (dH_2O) and gentamicin from a 50 mg/ml stock in dH_2O (Gibco, Invitrogen). The final concentration of antibiotics added to media for the selection and maintenance of plasmids was: Amp 100 μg/ml (*E. coli*); Cb 300 μg/ml (*P. aeruginosa*) and gentamicin 15 μg/ml (*E. coli*) and 100 μg/ml (*P. aeruginosa*). All reagents were filter sterilised before use (0.22 μm pore Minisart venting filter, Sartorius, Germany).

2.7.3 Synthetic AHLs

Synthetic 3-oxo-C12-HSL and C4-HSL were made by S. R. Chhabra at the Centre for Biomolecular Sciences, University of Nottingham (Chhabra *et al.*, 1993). Stocks of 1 μg/ml 3-oxo-C12-HSL and 10 μg/ml C4-HSL in acetonitrile were used. Compounds were stored at -20 ºC.

2.7.4 Synthetic AQs

Synthetic PQS and its HHQ were synthesised by S. R. Chhabra and stored at -20 ºC. Both were dissolved in methanol and PQS kept as 10 mM stock, HHQ as 5 mM stock.

2.7.5 Trace metals solution

Trace metals solution was prepared according to the formulation provided by the Center of Biomedical Microbiology (CBM), DTU. It consisted of iron (II) sulphate 2000 mg, manganese sulphate monohydrate 200 mg, copper sulphate 200 mg, zinc sulphate heptahydrate 200 mg, cobalt sulphate heptahydrate 100 mg, sodium molybdate dihydrate 120 mg and boric acid 50 mg in 1 L dH_2O, pH 4.5. The solution was autoclaved at 121 ºC for 20 min at 15 p.s.i. (which caused the iron to precipitate).

2.8 Growth media

Media was prepared using dH_2O and autoclaved at 121 ºC for 20 min at 15 p.s.i. The sterility of large volumes of media was ensured by running the autoclave cycle with 'load sensing' activated and placing the temperature probe in an equivalent volume of liquid. This often required a prolonged heating phase in excess of 90 min.

2.8.1 Luria Bertani medium

All bacterial strains were routinely grown in Luria Bertani (LB) broth unless otherwise stated. LB broth was prepared as described by Sambrook *et al*., (2003) and consisted of tryptone 10 g, yeast extract 5 g and sodium chloride 10 g in 1 L dH_2O.

LB agar was prepared by addition of 0.8 % (w/v) Technical Agar No. 3 (Oxoid) to LB broth.

2.8.2 Soft-top agar

LB soft-top agar consisted of tryptone 10 g, sodium chloride 5 g and Technical Agar No. 3 (Oxoid) 6.5 g in 1 L dH_2O.

2.8.3 Nutrient broth

Nutrient broth No. 2 (NB) (Oxoid) was prepared according to the manufacturer's instructions and consisted of 'Lab-lemco' powder 10 g, peptone 10 g and sodium chloride 5 g in 1 L dH_2O.

2.8.4 Nutrient yeast broth

Nutrient yeast broth (NYB) (Oxoid) was prepared according to the manufacturer's instructions and consisted of nutrient broth No. 1 25 g and yeast extract 5 g in 1 L dH_2O.

2.8.5 Sucrose/agar broth

Sucrose agar and broth were used in recombinant-selective experiments. A stock concentration of 50 % (w/v) sucrose was made by adding 100 g sucrose to 200 ml sterile dH_2O and filter sterilised. Sucrose broth consisted of tryptone peptone 10 g and yeast extract 5 g in 900 ml sterile dH_2O, pH 7. To this, 100 ml sucrose stock solution was added aspetically, giving a final sucrose concentration of 5 % (w/v).

2.8.6 PIA

PIA (Difco) was prepared according to the manufacturer's instructions and consisted of peptone 20 g, magnesium chloride 1.4 g, potassium sulphate 10 g, irgasan 0.025 g and agar 13.6 g in 1 L dH_2O containing 20 ml glycerol.

2.8.7 Jensen's minimal media

Jensen's minimal media was prepared according to a recipe provided by Luyan Ma, Wake Forest University School of Medicine, Winston-Salem (personal communication). Stock solutions of 10 x glucose (12.61 g in 100 ml dH_2O), 100 x magnesium sulphate heptahydrate (33 g in 100 ml dH_2O), calcium chloride dehydrate (210 mg in 100 ml dH_2O), Iron (II) sulphate heptahydrate (11 mg in 100 ml dH_2O) and zinc sulphate heptahydrate (24 mg in 100 ml dH_2O) were made and autoclaved to sterilise separately.

To the 100 ml 10 x glucose stock solution, valine 2.81 g and phenylalanine 1.32 g were added and the resulting solution filter sterilised.

For 1 L final volume 1 x solution, sodium chloride 5 g, dipotassium (hydrogen) phosphate 2.51 g and glutamic acid 15.56 g was added to 860 ml dH_2O and mixed thoroughly. The pH was raised to approximately 7 to enable the glutamic acid to dissolve and the solution autoclaved. To this, the 100 ml 10 x glucose stock solution containing valine and pheylalanine and 10 mls of each of the 100 x metal stock solutions were added aspectically.

When required, L-arabinose was added sterile to a final concentration of 2 % (w/v).

2.8.8 A-10 media

A-10 media was prepared according to Haagensen *et al*. (2006). It consisted of ammonium sulphate 20 g, disodium (hydrogen) phosphate dehydrate 60 g, potassium phosphate 30 g, sodium chloride 30 g in 1 L deionised water, pH 6.4 ± 0.1.

2.8.9 FB media

FB media (Haagensen *et al.*, 2006) consisted of 10 ml 1 M magnesium chloride, 1 ml 1 M calcium chloride, 1 ml trace metals solution (see 2.7.5) in 9 L Milli-Q water.

2.8.10 ABtrace minimal media

ABtrace minimal media was prepared by aseptically mixing 1 L A-10 media with 9 L FB media and adding glucose as the carbon source to a final concentration of 0.3 mM (Haagensen *et al.*, 2006).

2.9 Growth and storage of bacteria

2.9.1 Bacterial growth conditions

Liquid cultures were grown in LB broth with agitation at 200 revolutions per minute (rpm) on a Heidolph unimax 2010 shaker (SLS) in a 37 ºC warm room unless otherwise stated.

2.9.2 Monitoring of bacterial growth

Growth of bacterial cultures was monitored by absorbance at a wavelength of 600 nm (optical density, OD_{600}) using a Novaspec II visible spectrophotometer (Pharmacia LKB Ltd., Cambridge, UK).

For serial growth measures, OD_{600} was monitored in a 96 well microtitre plate (Black/Clear Isoplates, Perkin Elmer Life Sciences) using the GENios Pro spectrophotometer (Tecan, UK). Overnight cultures grown in LB were standardised to an OD_{600} of 1, diluted 1:100 in LB and 300 μl aliquots were inoculated into test wells. Assays were performed at 37 ºC and measurements were made from all wells every 30 min for 24 h. Readings were then analysed using Microsoft Excel 2002.

2.9.3 Long term storage of bacterial strains

For long-term storage of bacterial strains, 0.75 ml of an overnight bacterial culture was added to 0.75 ml 50 % (v/v) glycerol and mixed thoroughly in a 2 ml Micro tube (Sarstedt, Germany) before being flash frozen in liquid nitrogen. The tubes were then stored at -80 ºC.

2.9.4 Preparation of cultures for biofilm flowchamber system

To minimise variation between successive biofilm flowchamber experiments, bacterial cultures with the same history were used. A batch of cultures was prepared by inoculating 10 ml LB with a loopful of a single colony grown overnight on a LB agar plate streaked from a frozen stock. Following overnight incubation at 37 °C 200 rpm shaking, the OD_{600} of the culture was standardised to 0.1 in sterile 0.9 % (w/v) sodium chloride solution with 10 % (v/v) glycerol. Aliquots of 1.5 ml were flash frozen in liquid nitrogen and stored at -80 °C.

2.10 DNA manipulation

2.10.1 Isolation of chromosomal DNA

Chromosomal DNA was extracted from *P. aeruginosa* PAO1 using a method based on (Gamper *et al.*, 1992). Briefly, 1.5 ml of an overnight bacterial culture was centrifuged at 13,000 rpm for 3 min in a microfuge (Biofuge pico, Kendro, Germany). The supernatant was discarded and the pellet resuspended in 1.5 ml TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8). Following a second centrifugation at 13,000 rpm for 2 min, the bacterial pellet was resuspended in 300 µl TE buffer. The suspension was treated with 100 µl 5 % (w/v) sodium dodecyl sulphate (SDS) and 100 µl Proteinase K (2.5 mg/ml in dH_2O) and incubated for 1 h at 37 °C. The liquid was then repeatedly drawn up and expelled using a syringe needle to physically disrupt the cells. The DNA was extracted with an equal volume of 1:1 phenol:chloroform by vortexing to mix and then centrifuging at 14,000 rpm for 5 min. The upper aqueous phase was transferred to a fresh tube and the vortexing and centrifugation steps were repeated a further two times.

To precipitate the DNA, a $1/10^{th}$ volume of sodium acetate (3 M, pH 5.2) and two volumes of ice cold 100 % (v/v) ethanol were added and the solution was centrifuged at

13,000 rpm for 10 min. The supernatant was discarded and the pellet washed with 500 µl 70 % (v/v) ethanol. This was then centrifuged at 13,000 rpm for 10 min, the supernatant removed and the DNA pellet was dried in a centrifugal evaporator (Jouan). Finally, the pellet was resuspended in 500 µl HPLC grade H_2O (Fisher Scientific, UK), incubated at 50 °C for 1 h to enable gentle dissolution of the DNA and then stored at -20 °C.

2.10.2 Isolation of plasmid DNA

Plasmid DNA isolation was performed using the Qiagen Miniprep kit (Qiagen, UK) according to the manufacturer's protocol. Briefly, cells pelleted from 1-10 ml of an overnight bacterial culture were subjected to alkaline lysis, then neutralised and centrifuged in a microfuge (Biofuge pico, Kendro, Germany) at 13,000 rpm for 10 min to remove denatured and precipitated cellular debris. Lysates were then loaded onto a silica-gel filter, washed and plasmid DNA was eluted into 30-50 µl HPLC grade H_2O.

2.10.3 DNA agarose gel electrophoresis

DNA loading buffer (5 x stock: 1 ml glycerol; 2 ml 50 x TAE; bromophenol blue 0.002g; sterile dH_2O to 10 ml) was added to DNA samples which were analysed routinely on 0.8 % (w/v) agarose gels using a horizontal gel apparatus (Flowgen Instruments Ltd., UK). The gels were prepared using the method described by Sambrook *et al*., (2003) using analytical grade agarose (Promega, UK) in 1 x TAE buffer (40 mM Tris base, 1 mM EDTA pH 8.0, 0.1142 % [v/v] glacial acetic acid) with the addition of ethidium bromide to a final concentration of 10 µg/ml. The gels were run in 1 x TAE buffer and electrophoresis was performed at 80-120 V. DNA fragments were visualised on a UV transilluminator (UVP, USA).

2.10.4 DNA molecular weight markers

To establish the size of DNA fragments, 1 µg Ready-Load 1 kb DNA ladder (Invitrogen) was loaded onto agarose gels alongside the sample.

2.10.5 DNA restriction enzymes

Restriction enzymes were purchased from Promega and used according to the manufacturer's instructions. Restriction digests generally contained 0.5-1 μg plasmid DNA, 0.5-1 μl restriction endonuclease(s) and 1 x appropriate restriction buffer made to a final volume of 20 μl with sterile dH_2O. The reaction mixture was incubated at 37 °C for a minimum of 1 h or until the digestion was complete. Reactions were analysed on agarose gels and the appropriate sized bands cut out prior to DNA extraction using the Qiagen Gel Extraction Kit (Qiagen, UK).

2.10.6 Extraction and purification of DNA from agarose gels

DNA fragments were purified from agarose gel slices using a Qiagen Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. DNA was routinely eluted in 50 μl sterile dH_2O.

2.10.7 Ligation of DNA fragments

DNA ligations were routinely performed using 1:1 or 2:1 ratios of vector to insert. Reactions were carried out at 4 °C overnight using 0.5-1 μl T4 DNA ligase (Promega, USA) and 2 μl T4 ligation buffer in a final volume of 20 μl.

2.10.8 DNA sequence analysis

DNA sequencing was performed by the commercial company Geneservice (Cambridge, UK). Analysis of DNA sequences was performed using the FinchTV computer package (http://www.geospiza.com/index.shtml) in combination with the Basic Local Alignment Search Tool (BLAST) programme available from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Comparisons were made to the *P. aeruginosa* PAO1 reference genome sequence (http://www.pseudomonas.com). Any errors detected in the sequences of DNA templates were confirmed by repeat sequencing.

2.11 Polymerase chain reaction (PCR)

2.11.1 PCR amplification

PCR amplifications were performed according to the protocol of Saiki *et al*., (1988) (Saiki *et al.*, 1988) in a final volume of 50 µl. For general PCR reactions, 0.5 µl *Taq* DNA polymerase in 5 x buffer (GoTaq™ Promega, UK) or 0.5 µl Phusion high-fidelity DNA polymerase in 5 x Phusion HF buffer (New England Biolabs, Inc.) were used. Approximately 1 µg of each primer was included in the reaction mix containing 1.5 mM $MgCl_2$ and 2mM dNTPs. When amplifying *P. aeruginosa* DNA, 8 % (v/v) Dimethyl sulphoxide (DMSO) was added to the reaction mixture to aid DNA melting. The DNA template used was either from whole cells transferred from a fresh colony or 1 µg purified DNA from a chromosomal or plasmid preparation. Reactions were carried out in a Progene PCR Thermocycler (Techne) for a total of 30 cycles. Briefly, the DNA template was initially denatured at 94 °C for 5 min (hot start), followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55-58 °C for 30 s and then extension at 72 °C for 1-3 min depending on the length of the DNA to be amplified (1 min per kb). The last cycle finished with a final extension stage at 72 °C for 8 min to ensure completion of all strands. The annealing temperature was varied depending on the nature of the primers used and the stringency levels required. Reaction mixes were then incubated at 4 °C indefinitely to allow PCR reactions to be carried out overnight.

2.11.2 Inverse PCR

To introduce a deletion on a recombinant fragment of DNA from a plasmid, inverse PCR was performed using 0.5 µl *Taq* DNA polymerase in 5 x buffer (GoTaq™ Promega, UK). Primers were designed to sequence out from both the 5' and 3' ends of the gene of interest to amplify both upstream and downstream flanking regions and plasmid DNA, leaving a deleted gene. These primers incorporated a Hpa1 restriction site that, after subsequent digestion of the PCR products (section 2.10.5), produced blunt ended DNA strands which were subsequently self-ligated (section 2.10.7). The restriction site for this Hpa1 digest was manipulated to be in-frame from the start codon of the gene of interest, thereby producing in-frame gene deletions when self-ligated. The Inverse PCR reaction mix was set up as described in section 2.11.1.

2.11.3 Cloning of PCR products

PCR products were analysed on agarose gels and purified using a Qiagen Gel Extraction Kit (Qiagen, UK) according to the manufacturer's instructions. The product was then digested overnight at 37 °C with the relevant restriction enzyme(s) in the appropriate buffer. Digested PCR fragments were ligated into an appropriate vector and electroporated into *E. coli* DH5α or S-17 λ*pir*. Transformants containing recombinant clones were selected by growth on LB agar plates containing the appropriate antibiotics.

2.12 Introduction of DNA into bacterial strains

2.12.1 Preparation of electrocompetent *E. coli* cells

To prepare competent *E. coli* cells a 1 % (v/v) inoculum from an overnight *E. coli* culture was added to 100 ml of sterile NYB in a 1 L conical flask and grown at 37 °C, 200 rpm to an OD_{600} of 0.4-0.8 (approximately 6 h). Cells were harvested by centrifugation at 5,500 rpm (JA-14, Beckman) for 10 min at 4 °C and washed twice in sterile ice cold 10 % (v/v) glycerol containing 1 mM MOPS (4-morpholinepropanesulfonic acid) before being resuspended in 1 ml of the same buffer. Cells were aliquoted into 50 µl samples, flash frozen in liquid nitrogen and stored at -80 °C in microcentrifuge tubes.

2.12.2 Electroporation of electrocompetent *E. coli* cells

Electroporation was performed in 0.2 cm electrode gap Gene Pulser cuvettes (BioRad, UK) containing 50 µl of competent cells and 2 µl DNA. An electroporation pulse of 2.5 kV (200 Ω) was delivered using the BioRad Gene Pulsar connected to a BioRad pulse controller (BioRad, UK). A 1 ml aliquot of pre-warmed (37 °C) LB broth was added to the cells which were then incubated at 37 °C for 1 h in the absence of antibiotics. Aliquots of cells were subsequently plated onto LB agar plates containing appropriate antibiotics to select for transformants and grown overnight at 37 °C. Controls of electroporated cells with no plasmid were also similarly prepared.

2.12.3 Preparation of electrocompetent *P. aeruginosa* cells

Competent *P. aeruginosa* cells, for transformation with uncut vector, were prepared by centrifugation of 1.5 ml of overnight culture at 13,000 rpm for 5 min in a microfuge (Biofuge pico, Kendro, Germany). The cells were washed three times in 1 ml ice cold 10 % (v/v) glycerol with 1 mM MOPS. The pellet was then resuspended in 50 µl ice cold 10 % (v/v) glycerol with 1 mM MOPS. If necessary, the cells were flash frozen in liquid nitrogen and stored at -80 °C.

2.12.4 Electroporation of *P. aeruginosa*

Transformation of plasmids into electrocompetent *P. aeruginosa* cells was performed as for *E. coli* (section 2.12.2).

2.12.5 Conjugation of plasmid DNA into *P. aeruginosa*

Plasmid transfer from *E. coli* donor to *P. aeruginosa* recipient cells was carried out by bacterial conjugation. Donor and recipient cells were each grown in 5 ml of LB by overnight incubation. *P. aeruginosa* recipient strains were grown at 42 °C to inactivate a restriction enzyme system which degrades incoming foreign DNA, whilst *E. coli* donor strains were grown at 37 °C. Cells were harvested from 1.5 ml of each culture by centrifugation at 13,000 rpm for 1 min, washed with 1 ml NYB and then repelleted under the same conditions. The *Pseudomonas* pellet was resuspended in 1 ml NYB broth and this suspension was added to the *E. coli* pellet. The pellets were resuspended well and spun at 13,000 rpm for 1 min. The majority of the supernatant was removed, leaving approximately 100 µl, in which the mixed bacterial pellet was thoroughly resuspended. This was spotted as a drop onto a LB agar plate and allowed to air dry. It was then incubated at 37 °C for at least 3 h to allow bacterial mating to occur. For each conjugation, a *P. aeruginosa* and *E. coli* control were performed and treated in exactly the same way as the actual samples.

Cells were scraped from the plate using a sterile 10 µl inoculation loop and then resuspended in 0.5 ml NYB broth (controls were resuspended in 0.25 ml NYB broth). Aliquots of 100 µl were spread on to PIA plates containing the appropriate antibiotics to select for *P. aeruginosa* transconjugants and incubated overnight at 37 °C. No growth was expected on the control plates.

2.13 Construction of PAO1Δ*lecB* mutant

A *lecB* chromosomal deletion mutant in PAO1 was constructed as follows. Using PAO1 template DNA, a fragment containing the intact *lecB* gene (348 bp) plus 500 bp upstream and 500 bp downstream was amplified using the primer pair Sac1lecBUF and Kpn1lecBDR (Table 2-3). The resulting PCR product was cloned into pBLS resulting in the plasmid pBLS::*lecB*. To introduce a deletion of the recombinant *lecB* gene, the primer pair lecBHpa1UR and lecBHpa1DF (Table 2-3), possessing Hpa1 restriction sites, was used in conjunction with inverse PCR using pBLS::*lecB* DNA as the template. The resulting blunt-ended PCR product containing a deletion in *lecB* was self-ligated, resulting in the plasmid pBLSΔ*lecB*. The PCR product was excised from the vector using Sac1 and Kpn1 and cloned into the similarly digested suicide vector pEX18Gm (Hoang *et al.*, 1998), resulting in the plasmid pEX18GmΔ*lecB*. Allelic exchange using pEX18GmΔ*lecB* contained in *E. coli* S17-1 *λ-pir* with PAO1 resulted in a *P. aeruginosa* strain (PAO1Δ*lecB*) containing an in-frame deletion of the *lecB* gene. This deletion was confirmed by PCR and sequence analysis.

2.14 RNA analysis

2.14.1 Stabilisation of RNA and preparation of bacterial lysate

Total RNA was stabilised and a bacterial lysate prepared as per manufacturer's instructions using the RNeasy Mini Kit (Qiagen, UK). Briefly, 0.5 ml of an overnight culture of *P. aeruginosa* cells grown in LB (~ 1 x 10^9 cells) was mixed by vortexing with 1 ml RNAprotect Bacterial Reagent (Qiagen, UK) and then incubated at room temperature for 5 min. Cells were harvested by centrifugation at 5,000 x *g* for 10 min in a benchtop microfuge and the supernatant decanted. The bacteria were then lysed by resuspending in 200 µl TE buffer containing 1 mg/ml lysozyme, vortexing and incubating for 5 min at room temperature with agitation. 700 µl Buffer RLT (containing 10 µl β-mercaptoethanol per 1 ml buffer RLT) was added, the mixture vortexed and then centrifuged for 2 min at 13,000 rpm to pellet any particulate material. The supernatant was transferred to a fresh microtube and mixed with 500 µl 100 % (v/v) EtOH by pipetting.

2.14.2 Purification of total RNA from bacterial lystate

Up to 700 µl bacterial lysate at a time was transferred to an RNEasy Mini spin column (Qiagen, UK) which was placed in a collection tube and centrifuged for 15 s at ≥ 10,000 rpm. The flowthrough was discarded and 700 µl Buffer RW1 was added to the column which was centrifuged for 15 s at ≥ 10,000 rpm to wash the membrane. The column was placed in a fresh collection tube to which 500 µl Buffer RPE was added. The column was centrifuged for 15 s at ≥ 10,000 rpm and the flow-through was discarded. A further 500 µl Buffer RPE was added followed by a 2 min centrifugation step at ≥ 10,000. The spin column was placed in a new collection tube and spun at full speed for 1 min to remove all residual EtOH. The column was then placed in a new 1.5 ml collection tube and the RNA eluted with 50 µl RNAse-free water and stored immediately at – 80 °C until used.

2.14.3 Removal of contaminating genomic DNA from total RNA

20 µl of purified RNA (containing ~ 1 µg/µl RNA) was mixed with 50 µl TURBO DNase and 10 µl 10 x TURBO DNase buffer (Ambion) and the reaction volume made up to 100 µl with RNAse-free water. This was incubated at 37 °C for 1 h to enable digestion of contaminating genomic DNA.

2.14.4 DNase treated RNA sample clean up

The DNase treated RNA sample was cleaned up using the RNeasy MiniElute kit (Qiagen, UK) as per the manufacturer's instructions. Briefly, 100 µl sample was mixed with 350 µl RLT buffer (without additional β-mercaptoethanol) and mixed thoroughly. 250 µl 100 % (v/v) EtOH was added to the diluted RNA and mixed by pipetting. The whole volume was then applied to a spin column, which was placed in a collection tube and centrifuged for 15 s at ≥ 10,000 rpm. The column was transferred to a new collection tube and 500 µl Bufffer RPE was added. Following repeat centrifugation, the flow-through was discarded and 500 µl 80 % (v/v) EtOH was added. The column was centrifuged for 2 min at ≥ 10,000 rpm and then transferred to a new collection tube and spun at full speed for 5 min with the cap open to dry the silica-gel membrane. Elution was performed with 14 µl RNAase-free water and the resulting 12 µl eluate was stored at – 80 °C until use.

2.14.5 One-step reverse transcription PCR

Reverse transcription PCR (RT-PCR) was performed using the OneStep RT-PCR Kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, template RNA (2 µl cleaned up RNA), primer solutions, dNTP Mix, 5 x QIAGEN OneStep RT-PCR Buffer and RNase-free water were thawed and placed on ice. A master-mix was prepared based on the volumes of components required for a single reaction listed in Table 2-4.

Table 2-4 Reaction components for one-step RT-PCR

Component	Volume/reaction	Final concentration
Master mix		
RNase-free water	20 µl	-
5 x QIAGEN OneStep RT-PCR Buffer	10 µl	1 x
dNTP Mix (containing 10 mM of each dNTP)	2 µl	400 µM of each dNTP
Primer A (10 mM)	3 µl	0.6 µM
Primer b (10 mM)	3 µl	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	2 µl	-
Template RNA		
Template RNA, added separately	2 µl	1 pg – 2 µg/reaction
Total volume	50 µl	-

Working on ice, 48 µl aliquots of mastermix were dispensed in to individual PCR tubes and 2 µl of the template RNA was added to each tube. Control reactions were performed where the RNA template was exchanged for dH_2O (negative control) or DNA template (positive control). Samples were maintained on ice whilst the T3000 Thermocycler (Biometra) was started. See Table 2-5. When the thermal cycler had reached 50 °C the programme was paused and the samples loaded. Following amplification, the samples were stored at – 20 °C until analysed by agarose gel electrophoresis.

Table 2-5 Thermal cycler conditions

Reverse transcription:	30 min	50 °C
Initial PCR activation step	15 min	95 °C
3-step cycling		
Denaturation	0.5 min	94 °C
Annealing	0.5 min	55-68 °C (approx 5 °C below T_m of primers)
Extension	1 min	72 °C
Number of cycles	30	
Final extension	10 min	72 °C

2.15 Protein analysis

2.15.1 Preparation of whole cell protein extracts

Whole cell protein extracts were prepared by subculturing 250 µl of an overnight bacterial culture in 25 ml LB broth and incubating for a further 12 to 16 h. The OD_{600} of the culture was measured and then a 100 µl sample was centrifuged at 13,000 rpm in a microfuge (Biofuge pico, Kendro, Germany) for 2 min and the supernatant removed. In order to standardise the OD_{600} to 1, the cells were resuspended in an appropriate volume of dithiothreitol (DTT) sample loading buffer (50 mM Trizma base pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue and 100 mM DTT) and boiled for 10 min to denature the proteins.

2.15.2 Preparation of sputum protein extracts

An equivalent volume of phosphate buffered saline (PBS) or sputolysin (DTT, Calbiochem) was added to neat sputum and vortexed vigorously to liquify the sputum. 100 µl of the resulting suspension was centrifuged at 13,000 rpm for 15 min in a microfuge (Biofuge pico, Kendro, Germany). The supernatant was mixed with an appropriate volume of 5 x DTT sample loading buffer and boiled. Additionally, the sputum pellet was resuspended in 100 µl 1 x DTT sample loading buffer and then boiled for 10 min.

2.15.3 SDS-polyacrylamide gel electrophoresis

For the analysis of proteins, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was carried out using the method of Laemmli (1970). 18 % acrylamide resolving gels were cast in a Bio-Rad Mini-Protean II casting tray and overlaid with 100 µl of water saturated with butanol. When set, a 4 % acrylamide stacking gel was prepared and added to the separating gel. See Table 2-6.

Table 2-6 Composition of resolving and stacking gels

Component	18 % resolving gel	4 % stacking gel
Gel buffer (3 M Trizma base, 0.5 % (w/v) SDS, pH 8.45)	3.3 ml	1.25 ml
30 % acrylamide:bisacrylamide	6 ml	670 µl
dH_2O	0.7 ml	3.03 ml
10 % (w/v) ammonium persulphate (APS)	50 µl	50 µl
N, N, N', N'-tetramethylethylenediamine (TEMED)	10 µl	5 µl

Sample aliquots of 5 µl were loaded onto the gel. Electrophoresis was performed in an outer anode buffer (0.1 M Trizma base, pH 8.9) and inner cathode buffer (0.1 M Trizma base, 0.1 M Tricine and 0.1 % SDS) at a constant voltage of 150 V until the sample buffer had migrated to the bottom of the gel. Precision plus protein standard (BioRad) was used as a molecular weight marker in the gels.

2.15.4 Western blotting

Proteins to be analysed by Western blotting were first separated by SDS-PAGE as described in section 2.15.3. Proteins were then transferred to a nitro-cellulose membrane (Hybond ECL, Amersham Biosciences) using a Biorad Mini Trans Blot apparatus. Transfer was carried out in Western blot buffer (0.24 % [w/v] Trizma base, 1.14 % [w/v] glycine, 20 % [v/v] methanol, in dH_2O) at 100 V for 2 h.

The blot was removed from the apparatus and blocked in PBS containing 0.5 % (v/v) Tween 20 (PBST) and 5 % (w/v) dried skimmed milk powder for 1 h at room temperature with rocking. The blot was then incubated with the primary anti-lectin antibody (1:500 dilution) in PBST containing 5 % skimmed milk for 1 h. The blot was washed for 3 x 20 min in PBST and incubated with the secondary antibody (1:3000 anti-rabbit IgG-horseradish peroxidase conjugate, Amersham Biosciences) in PBST containing 5 % (w/v) skimmed milk for 1 h. After washing (3 x 20 min in PBST) the blot was developed using the ECL plus Western blotting detection system (Amersham Biosciences) according to the manufacturer's instructions. Following this, the blot was exposed to Hyperfilm ECL (Amersham Biosciences), a high performance chemiluminescence film for 5 to 20 min. The film was developed in Ilford PQ universal developer for 1-2 min and washed in water. The film was fixed in Ilford Hypam rapid fixer, rinsed in water and air-dried.

2.15.5 Raising of antibodies used in Western blotting

A polyclonal antibody was raised against the lectin LecA (PA-IL, Sigma) by K. Bishop (Institute of Infection, Immunity and Inflammation, University of Nottingham) in a New Zealand White rabbit. The rabbit was immunised with 50 µg LecA in 250 µl PBS, administered subcutaneously, four times at two weekly intervals. Primary immunisation used Freunds Complete adjuvant and the three subsequent immunisations used Freunds Incomplete adjuvant. Serum was obtained from the rabbit at week 5 (test bleed) and the final bleed was taken two weeks after the fourth immunisation.

2.16 AHL extraction and detection from broth cultures

2.16.1 Extraction of AHLs

Overnight bacterial cultures were standardised to OD_{600} 1.0 before being diluted 1 in 100 in 25 ml LB and grown in 250 ml flasks at 37 °C with shaking at 200 rpm overnight. A 900 µl aliquot of this was spun at 13,000 rpm for 5 min in a microfuge (Biofuge pico, Kendro, Germany). The supernatant was mixed thoroughly with 100 µl 1 M hydrochloric acid and incubated at 37 ºC overnight. Samples were stored at -20 ºC.

2.16.2 Detection of AHLs using thin layer chromatography and a biological reporter

Extracted AHLs were analysed by thin layer chromatography (TLC) based on the methods of Shaw *et al*. (1997) and McClean *et al*. (1997). For the detection of the long chain 3-oxo-C12-HSL, 1 µl of each sample was spotted onto a 20 x 20 cm Alugram reverse phase RP-2/UV254 plate (Macherey-Nagel) along with 1 µl of the synthetic standard. The plate was run in 45 % (v/v) MeOH overnight. It was then overlaid with soft top agar (100 ml) containing 1 ml of an overnight culture of *E. coli* S17 *λpir* [pSB1142]. Following incubation at 37 ºC for 3 h the plate was analysed for light production using a Berthold Luminograph LB980.

For the detection of the short-chain C4-HSL, 5 µl of each sample and 1 µl of the standard were loaded onto a 20 x 20 cm aluminium reverse phase RP-18 F2545 plate (Merck). The plate was run in 60 % (v/v) MeOH for 1.5 to 2.5 h and then overlaid with 100 ml soft top agar containing 1 ml of an overnight culture of *E. coli* JM109 [pSB536]. The plate was incubated and viewed as above.

2.17 PQS extraction and detection from broth cultures

2.17.1 Extraction of PQS

PQS was extracted from culture supernatants based on the method of Pesci *et al*. (1999). Overnight bacterial cultures were standardised to OD_{600} 1.0 before being diluted 1 in 100 in 100 ml LB and grown overnight. 10 ml of the resulting culture was mixed thoroughly with 10 ml acidified ethyl acetate (0.01 % [v/v] glacial acetic acid in 1 L ethyl acetate) and allowed to separate. The uppermost organic ethyl acetate layer was dried to completion using a rotary evaporator (Buchi, Rotovapor R-114) at 45 ºC. This was then re-suspended in 1.2 ml acidified ethyl acetate before being evaporated to dryness under a stream of nitrogen gas. Samples were then re-suspended in 50 µl MeOH and stored at -20 ºC.

2.17.2 Detection of PQS using TLC

A 20 x 20 cm aluminium plate (silica gel 60 F254, Merck) was prepared by soaking in 5 % KH_2PO_4 for 30 min before being air-dried and activated in a hybridisation oven (Stuart Scientific) at 100 ºC for 1 h. 2 µl of the PQS and 1 µl of the HHQ synthetic

standards were spotted onto a plate along with 5 µl of each sample. The plate was run in a solvent system of dichloromethane (DCM) to MeOH 95:5 for 1.5 to 2 h. The plate was viewed immediately using a UV transilluminator (UVP, USA) and photographed using a Nikon Coolpix995 digital camera.

2.17.3 Detection of PQS using a biological reporter

5 ml of an overnight culture of PAO1 *lecA*::*lux*Δ*pqsA* was added to 100 ml soft top agar and this was overlaid onto the plate run in 2.17.2. Following incubation at 37 ºC for a minimum of 6 h, lectin production was measured as light production using the Berthold luminograph.

2.18 Detection of QS signal molecules from CF sputum

2.18.1 Extraction of signal molecules from sputum

An equivalent volume of 0.9 % (w/v) sodium chloride was added to each sputum sample and the mixture vortexed vigorously for 3 min to produce a 50 % (v/v) sputum suspension. From this, a 3 ml aliquot was mixed with 3 ml acidified ethyl acetate (see 2.17.1) and vortexed for 1 min, then centrifuged for 2 min at 3,000 rpm in a microfuge (Biofuge pico, Kendro, Germany). The upper organic phase was transferred to a fresh glass tube and the remaining aqueous phase was re-extracted a further two times with 3 ml of solvent on each occasion. The organic phases were combined and dried to completion in a rotorvap (Jouan). The solute was dissolved in 50 µl MeOH by vortexing for 30 s and then transferred to a fresh 0.1 ml eppendorf. The solution was spun at 13,000 rpm for 5 min and the supernatant was removed and stored at -20 °C. As a negative control, six 3 ml volumes of 0.9 % (w/v) sodium chloride were each extracted with acidified ethyl acetate.

2.18.2 Preparation of standards

Six 3 ml volumes of 0.9 % (w/v) sodium chloride were each spiked with the appropriate volumes of synthetic standards in order to achieve a final concentration of 0.1 µM for the AHLs and 0.5 µM for the AQs in 50 µl MeOH following extraction with acidified ethyl acetate as described in section 2.18.1. In addition, unextracted volumes of MeOH containing the standards at the same concentration were analysed.

2.18.3 Liquid chromatography – Mass spectroscopy (LC-MS) analysis of extracted sputum samples

LC-MS analysis was performed on 5 µl aliquots of extracted sputum. Reverse phase liquid chromatography (RP-HPLC) using a Phenomenex Gemini (C18, 5 µM, 250 x 2.00 mm) column was coupled with mass spectroscopy (MS) (Applied Biosystems 4000 Q-TRAP) and eluted with a gradient based on 0.1 % (v/v) formic acid. The fragmentation ions of each of the anticipated AHLs and AQs were recorded using Multiple Reaction Monitoring Triggered Traps (MRMTT). The majority of compounds were detected by positive electrospray. The detection of PQS was performed as a separate run with the addition of a chelating agent, which served to "block" metals present in the column and prevent PQS binding to these. The system was additionally run in negative for the identification of the acidic ring open forms of 3-oxo-C12-HSL and C4-HSL. The threshold for detection for each compound was set by the instrument at five times the value of baseline noise unless otherwise stated.

2.19 Biofilm assays

2.19.1 Flowchamber biofilm system

For detailed methodology describing the local establishment of a flowchamber biofilm system and the associated COMSTAT and IMARIS image analysis tools, please refer to Appendix 4.

2.19.2 Biofilm microtitre assays

A modified version of the method described by Stepanovic *et al.* (2000) was employed. Sterilised, clear, ninety-six well, U-bottomed polystyrene microtitre plates (Costar) were prepared by adding 300 µl sterile dH_2O to the peripheral wells to decrease evaporation from test wells. Aliquots of 270 µl culture medium (0.05 % [v/v] nutrient broth) were added to internal wells. 30 µl samples of overnight cultures, pre-washed in 0.05% (v/v) nutrient broth and normalised to an OD_{600} 1.0, were inoculated into the test wells. Plates were incubated in a humid environment for 24 h at 37 °C. The contents of the wells were aspirated and the wells were washed three times with dH_2O. They were then fixed with 300 µl MeOH for 5 min, before staining with 150 µl 0.1 % (w/v) crystal violet for 5 min. After rinsing the wells with dH_2O, the contents were resolubilised in

33 % (v/v) glacial acetic acid and transferred to a polystyrene flat bottomed 96 well plate (Costar). Optical densities of the microtitre wells were determined using a plate reader (MRX, Dynex Technologies) at 540 nm.

2.19.3 Stainless steel coupon biofilm assay

Biofilms were grown on stainless steel coupons (surgical grade 316L) under conditions similar to those described by Dhir and Dodd (1995). Sterile steel coupons were placed at the edges of Petri dishes with 10 ml 0.05 % (v/v) nutrient broth containing the appropriate concentration of the test compound. Inocula of *P. aeruginosa* test strains were prepared from 5 ml of overnight cultures grown in LB broth. The OD_{600} of these cultures was adjusted to 1.0 with sterile LB broth and aliquots of 100 µl were used to inoculate the medium contained within the Petri dishes. The Petri dishes were incubated at 37 °C on a rotary shaker (60 rpm) for 24 h. An additional 100 µl LB broth was added and the Petri dishes were incubated for a further 24 h. The test was performed in triplicate for each strain.

2.19.4 Acridine orange staining of stainless steel coupon biofilms

The inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons. 20 ml sterile PBS was then added and the dish agitated at 60 rpm for 5 minutes to rinse the coupons. The PBS was removed and this rinse step was repeated. Following this, the coupons were air-dried and then heat-fixed by passing them through a Bunsen flame three times. The surface attached biofilms were then stained by applying 200 µl 0.1 % (w/v) acridine orange to the upper surface of each coupon and leaving for 2.5 min. To remove unbound acridine orange, 20 ml of sterile PBS was then added to the Petri dish and the dish gently agitated as before for 5 minutes. The PBS was removed and this wash step was repeated two further times. The coupons were then air-dried and fixed with tape along their edges to a glass microscope slide.

2.19.5 Visualisation of acridine orange stained steel coupon biofilms

The coupons were examined for bacterial attachment with an inverted fluorescent microscope (Nikon Eclipse TE200) using the x 10 objective lens and green filter. Ten images were collected per coupon using a JVC KY-F58 video camera. Sampling was

conducted at random from each coupon, avoiding areas at the edges. With the 'red' and 'low' options set to the maximum level of 255, the area fraction for each image was calculated using the Lucia G/Comet software (Nikon UK) with a threshold set at between 175 and 185.

2.19.6 Capturing of confocal images

Coupons were placed in the universal slide holder of an inverted Zeiss Axiovert100M microscope and confocal images and Z-stacks were captured with a Zeiss LSM510uv META Kombi confocal system. The objective used was a Zeiss Plan-NeoFluar 40x/1.3 oil immersion lens and the acridine orange was excited with an Argon laser at 488 nm and emission collected with a LP560 filter.

2.19.7 Stainless steel coupon dispersion assay

Biofilms were grown on stainless steel coupons as described in 1.19.2. Following incubation, the inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons and the coupons were rinsed with 10 ml 0.05 % nutrient broth. Then, 10 ml fresh 0.05 % (v/v) nutrient broth containing the appropriate concentration of the test compound was added to each Petri dish. To the control dish, 0.05 % nutrient broth alone was added. The dishes were incubated at 37 °C on a rotary shaker (60 rpm) for 2 h. The media was collected, vortexed and serially diluted to a final dilution of 1:10000. Aliquots of 100 µl were spread onto LB agar plates in triplicate and incubated at 37 °C overnight. The resulting colonies were counted and a calculation of the viable bacterial count made.

2.20 Conjugation of LecA with Cy5 cyanine fluorescent dye

1 mg purified LecA protein (PA-IL, Sigma) was dissolved in 1 ml PBS to give a concentration of 1 mg/ml protein. Cy5 mono-reactive NHS ester (Amersham) was dissolved in DMSO to a final concentration of 5 µg/µl. 500 µl protein (500 µg) was mixed with 40 µl carbonate buffer ($NaHCO_3$ 142 mM, Na_2CO_3 8 mM, pH 9.4) and 10 µl Cy5 solution and the mixture was incubated for 2 h at RT.

Fluorescent dye removal columns (ThermoScientific) were used to remove free dye after the labelling reaction according to the manufacturer's instructions. Briefly, 250 µl

Dye Removal Resin was added to each of 2 spin columns each placed within a collection tube. These were centrifuged for 30 s at 1,000 x g in a microfuge (Biofuge pico, Kendro, Germany) to remove the storage solution. The used collection tubes were discarded and the columns placed in new tubes. The labelling reaction was divided in to two 250 µl aliquots and each was added to a prepared Spin column and mixed briefly by vortexing. The column was centrifuged for 30 s at 1,000 x g to collect the purified protein. The labelled protein was quantified using the Nanodrop ND-1000 Spectrophotometer (Labtech) with the protein A-280 option.

2.21 Labelling of bacterial cells with lectin-fluorophore conjugates

Lectin staining was undertaken based on the method described by Ma *et al*. (2007). The binding of fluorescently labelled lectins was evaluated by staining planktonically grown bacterial strains. Following overnight growth in Jensen's media, the OD_{600} of cultures was corrected to 1 and the cells were rinsed with PBS before staining with 100 µg/ml fluorescein isothiocyanate (FITC) labelled *Marasmium oreades agglutinin* (MOA) mushroom lectin (EY laboratories, Inc.) or Cy5 labelled *P. aeruginosa* lectin LecA. Staining was allowed to progress for 4 h at room temperature with agitation and then samples were washed twice with PBS and finally resuspended in 400 µl PBS. This entire volume was applied to a poly-D-lysine (PDL) coated round microscope dish (Mattek) and then imaged with a Zeiss LSM 510 Axiovert 100M ConfoCor 2 microscope (Carl Zeiss, Switzerland). The 63 x 1.3 water objective in conjunction with the 633 nm Argon laser was used for all image acquisition.

2.22 Enzyme-linked immunoSorbent assay (ELISA) investigation of bacterial-protein interactions

2.22.1 Preparation of wells

Purified LecA (PA-IL, Sigma) was prepared at a concentration of 10 µg/ml in PBS. 100 µl aliquots of protein were introduced to each test well of a 96 well Immoblizer Amino plate (Nunc) and allowed to covalently couple to the polymer surface by incubation for 2 h at room temperature with agitation. The control wells were treated with 100 µl 1 % (w/v) bovine serum albumin (BSA) in PBS. The wells were then emptied and the remaining active sites blocked with 100 µg/ml BSA in PBS for 1 h at room temperature

with agitation. This solution was then discarded and the wells washed x 3 with PBST detergent solution to remove excess non covalently-bound lectin.

IPTG was made up at the test concentrations in 1 % (w/v) BSA in PBS (0.2 mM and 2 mM) and the wells were inoculated and allowed to incubate overnight at 4 °C with agitation. Untreated wells were simply incubated with 1 % (w/v) BSA in PBS.

2.22.2 Digoxigenin (DIG-NHS) labelling of bacterial cells

Overnight cultures of the test strains were grown in Jensen's minimal media. 1 ml volumes of cultures were spun to pellet at 4,000 rpm for 5 min in a microfuge (Biofuge pico, Kendro, Germany). The supernatant was removed and the cells were resuspended in PBS to wash. Following a second centrifugation, the cells were resuspended in carbonate buffer (section 2.20) and the OD_{600} adjusted to 0.2. Two microlitres of digoxigenin-3-0-succinyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (DIG-NHS, Roche) at a concentration of 10 µg/µl in DMSO was added to each 1 ml volume of cells and this was allowed to incubate for 1 h at room temperature with agitation.

Following this, the cells were spun and rinsed once with PBS. They were finally resuspended in PBS containing 1 % (w/v) BSA and diluted to OD_{600} of 0.02. For the test wells blocked with IPTG, the bacterial cells were resuspended in 1 % (w/v) BSA in PBS containing the test concentrations of IPTG.

2.22.3 Inoculation of wells with labelled bacterial cells

The contents of the wells were discarded and the wells washed x 3 with PBST. Then, 100 µl aliquots of DIG-NHS-labelled bacterial cells were added. Each strain was tested in triplicate. To the control wells, 100 µl 1 % (w/v) BSA in PBS alone was added. The wells were incubated at 4 °C overnight with agitation at 125 rpm.

2.22.4 Washing of wells; substrate labelling of bound cells and measurement of absorption

The incubating bacterial culture was removed from the wells and the wells were rinsed 3 x with PBST. The wells were then incubated for 1 h at room temperature with 100 µl enzyme conjugated anti-DIG-NHS monoclonal antibody (Roche) at a dilution of 1:5000 in 1 % (w/v) BSA in PBS which reacted with the DIG-labelled bacterial cells.

Following this, wells were rinsed 4 x with PBST. Then, the complexed enzyme was visualised with 100 µl of the soluble substrate ABTS (2,2h-Azino-di-[3-ethylbenzthiazoline-sulfonate (6)], Roche) added to each well. After 2-4 h incubation at room temperature, absorbance was read with an ELISA plate reader (EMS Reader MF, Labsystems) at a wavelength of 405 nm.

2.23 **LDH cytotoxicity assay**

All work was performed aseptically in a class 2 microbiological safety cabinet.

2.23.1 Preparation of multiwell plate

Human 293T kidney-embryonic cells were prepared by overnight incubation in DMEM media (Gibco). The incubating media was removed and the adherent cells harvested by the addition of the protease trypsin (Sigma). The cell count was determined using a counting chamber and then 200 µl aliquots of cell suspension at 1 x 10^5 cells/ml were added to individual wells of a microtitre plate (Nunc). The plate was incubated overnight at 37 ˚C, 5 % CO_2, 90 % humidity to allow the cells to adhere tightly.

2.23.2 Addition of test compounds

The test compound (denrimer D20) was titrated to the appropriate test concentrations (0.1 mM, 0.05 mM, 0.025 mM and 0.0125 mM) in the assay medium in a separate multiwell plate by serial dilutions.

The assay medium was removed from the adherent cells (to remove LDH activity released from the cells during the overnight incubation) and 100 µl fresh assay medium was added to each well. To these, 100 µl of the test substance dilutions were added, with each concentration tested in triplicate and the plate was incubated overnight. Control wells consisted of assay medium alone (‘background’); cells incubated in assay medium (‘low’); dendrimer 20 at each test concentration in assay medium (‘substance control I’) and cells incubated in 2 % Triton X-100 in assay medium (‘high’).

2.23.3 Determination of LDH activity

The microplate was centrifuged at 250 x *g* for 10 min and then 100 µl/well cell-free supernatant was collected and transferred into the corresponding wells of an optically clear 96-well flat bottom microplate. 100 µl freshly prepared LDH detection Reaction mixture (Roche) was added to each well and incubated for 30 min at room temperature protected from light. The absorbance of the samples was measured at 492 nm using an ELISA plate reader (Labsytems Multiskan Ex).

Chapter 3: Isolation and characterisation of clinical strains from CF sputum

3.1 Introduction

Research over the past decades has revealed that bacteria preferentially live in communities in which the behaviour of individual cells is coordinated by cell-cell communication, termed quorum sensing (QS). This is of great importance in enabling the interaction of bacterial cells with each other, their environment and higher organisms.

P. aeruginosa is probably the most thoroughly studied bacterium in terms of QS and laboratory strains are known to contain two interacting QS systems termed *las* and *rhl*. These are responsible for the production of the major AHL signals 3-oxo-C12-HSL and C4-HSL respectively. In addition, an AQ-based QS system uses PQS (whose immediate precursor is HHQ) as its major cognate signal molecule. The *las* and the *rhl* systems are organised in a hierarchical manner (Latifi *et al.*, 1996) and PQS is interspaced between the two (Pesci *et al.*, 1999). These systems are integrated within a complex regulatory network in the cell and are under the transcriptional and post-transcriptional control of a large number of other regulators (Venturi, 2006). Refer back to Figure 1.2 for an overview of *P. aeruginosa* QS systems.

These signal molecules bind to transcriptional regulator proteins that specifically induce numerous virulence genes. For example, 3-oxo-C12-HSL, together with the transcriptional regulator LasR, regulates the production of elastase, alkaline protease and exotoxin A (Cámara *et al.*, 2002). Similarly, C4-HSL activates RhlR such that RhlR/C4-HSL induces the production of rhamnolipid, elastase, LasA protease, cyanide, pyocyanin and siderophores as well as the lectins LecA and LecB (Winzer *et al.*, 2000) (Winson *et al.*, 1995). In addition, PQS is known to be essential for both LecA and pyocyanin production (Diggle *et al.*, 2003). To date, more than thirty QS-regulated virulence factors have been described (Bjarnsholt and Givskov, 2007).

The *in vivo* significance of this regulation is also being elucidated. Studies in a number of animal models including mice, nematodes and insects have demonstrated the importance of a fully functional QS-circuit for the full virulence of *P. aeruginosa* (Rumbaugh *et al.*, 1999c) (Mahajan-Miklos *et al.*, 2000). In terms of specific pathogenic mechanisms, QS has been implicated in the development of bacterial biofilms, a key virulence strategy for the establishment of chronic infections (Davies *et al.*, 1998). In addition, there are reports of QS-deficient *P. aeruginosa* biofilms showing reduced tolerance to antimicrobial agents (Bjarnsholt *et al.*, 2005) (Hentzer *et al.*, 2003).

The recognition that different bacterial species make similar signal molecules has led the study of their role in interactions between bacterial species (interspecies) as well as between bacteria and eukaryotic (interkingdom) organisms sharing a common environment. This has been referred to as 'cross-talk' (Riedel *et al.*, 2001) and is also understood to mediate pathogenesis, for example by host immunomodulation (Pritchard, 2006), with the QS molecules acting as virulence determinants in their own right.

Given the *in vitro* and animal model evidence for the multiple roles of QS signal molecules and signalling in pathogenesis, attention has turned from the well-studied behaviour of laboratory strains to understanding the QS-status of isolates of *P. aeruginosa* derived from clinical sources. Indeed, the occurrence of clinical isolates deficient in the production of extracellular virulence factors regulated by QS, such as elastase and exotoxin A, has been shown (Hamood *et al.*, 1996) (Rumbaugh *et al.*, 1999b). There are also increasing reports describing both the proficiency and deficiency of AHL production by clinical isolates recovered from a number of different sites (Hamood *et al.*, 1996) (Geisenberger *et al.*, 2000) (Sokurenko *et al.*, 2001) (Cabrol *et al.*, 2003) (Denervaud *et al.*, 2004). Furthermore, measurement of PQS production by *P. aeruginosa* isolates from asymptomatic children with CF indicated that strains with increased synthesis of PQS were present during early colonisation of CF patient airways (Guina *et al.*, 2003).

The further characterisation of these isolates has demonstrated that the relative *in vitro* production of 3-oxo-C12-HSL and C4-HSL as well as the associated QS-dependent phenotypes (such as elastase production) varied according to their site of isolation, suggesting that the host microenvironment selects for different phenotypic behaviours (Favre-Bonte *et al.*, 2007). Indeed, growth in low magnesium, which can destabilise the

Gram-negative outer cell membrane, was associated with increased induction of proteins essential for the production of PQS (Guina *et al.*, 2003). Mutations affecting the AHL cell-signalling circuit were found to be located preferentially in the response regulator genes (Denervaud *et al.*, 2004) and despite deficiency in AHL production, such isolates were still capable of causing clinically significant infections (Bosgelmez-Tinaz and Ulusoy, 2008) (Schaber *et al.*, 2004).

The aim of this first chapter was to establish the QS signal molecule profile of a cohort of clinical isolates of *P. aeruginosa* obtained from CF sputum. Given the contribution of *P. aeruginosa* biofilm formation to pathogenesis in CF, the conservation of the QS-regulated lectin LecA was investigated amongst these isolates with a view to exploring further its contribution to biofilm development in subsequent chapters.

3.2 Results

3.2.1 Patient recruitment and sample collection

To enable the characterisation of QS signal molecule production by clinical isolates and the detection of QS molecules in sputum from CF patients, a clinical study was undertaken. Between May 2004 and July 2005, 37 adult and 25 paediatric patients were recruited from the CF outpatients' clinics at Nottingham City Hospital, UK. A further 3 patients were approached but declined participation in the study. An additional 6 patients were excluded from the study; 4 of these were not sputum producers, one had grown *B. cepacia* and one 39 year old female adult patient was found to be colonised with *Burkholderia pseudomallei* following travel to South East Asia a number of years previously. This organism was suspected when an aminoglycoside and colistin resistant Gram-negative oxidase positive rod was detected following routine clinical sputum culture.

Of the 62 patients recruited, at least one sputum sample was provided by 39 (64 %). The genotype of both alleles at the CFTR locus was known for 33 patients and the average age of diagnosis was 3 years 10 months. The ratio of males to females was 35:27 and the median age at entry to the study was 20 years (range 6-52). See Table 3-1 for details of the recruited patients.

Table 3-1 Recruited patient details.

Study number	Sex	Age at diagnosis (y) year (m) month	Age at entry to study (y) year (m) month	Allele 1 at CFTR locus	Allele 2 at CFTR locus	*P. aeruginosa* established
A001	M	43y 2m	46y 6m	Not done	Not done	Yes
A002	F	3m	27y 5m	phe508del	phe508del	Yes
A003	F	0	17y 11m	Not done	Not done	Yes
A004	F	0	18y 8m	phe508del	Q493X	Yes
A005	F	50y 4m	52y 7m	phe508del	Not identified	Yes
A006	M	1m	22y 3m	phe508del	phe508del	Yes
A007	M	2y	19y 5m	Not done	Not done	Yes
A008	M	9m	26y	phe508del	Not identified	Yes
A009	M	5m	22y 3m	phe508del	Not identified	Yes
A010	M	2y 6m	24y 4m	phe508del	Not identified	Yes
A011	F	31y	40y 11m	phe508del	2789+5G→A	Yes
A012	M	5y 2m	19y 4m	phe508del	phe508del	Yes
A013	F	16y	25y 11m	phe508del	phe508del	Yes
A014	F	6y	23y 3m	Not done	Not done	Yes
A016	M	2y	31y 7m	phe508del	phe508del	Yes
A017	M	5m	24y	Not done	Not done	Yes
A018	M	0	27y 4m	Not done	Not done	Yes
A019	M	0	20y 11m	phe508del	Not identified	Yes
A021	M	0	28y 6m	phe508del	Not identified	Yes
A022	F	14y	21y 7m	phe508del	Not identified	No
A023		F	21y 1m	phe508del	phe508del	Yes
A024	F	Not known	32y 1m	phe508del	phe508del	Yes
A025	M	6y	46y 8m	phe508del	phe508del	Yes
A026	F	1m	32y 8m	phe508del	phe508del	Yes
A027	M	21y	41y 5m	phe508del	Not identified	No
A028	F	5m	25y 2m	phe508del	Q493X	Yes
A029	M	0	25y 8m	Not done	Not done	No
A030	F	8y	20y 9m	Not done	Not done	No
A031	F	2m	20y 10m	phe508del	phe508del	Yes
A032	M	2m	22y 3m	Not done	Not done	Yes
A033	M	7m	22y 4m	Not done	Not done	Yes
A034	M	0	25y 8m	Not done	Not done	Yes
A035	M	6m	21y	phe508del	phe508del	Yes
A036	F	6m	21y 5m	Not done	Not done	Yes
A037	M	2y	24y 4m	phe508del	phe508del	Yes
A038	F	Not known	30y 10m	Not done	Not done	Yes
A039	F	Not known	28y 2m	phe508del	phe508del	Yes
P001	M	1m	9y 5m	phe508del	phe508del	Yes
P002	M	0	14y 2m	phe508del	R347P	Yes

Study number	Sex	Age at diagnosis (y) year (m) month	Age at entry to study (y) year (m) month	Allele 1 at CFTR locus	Allele 2 at CFTR locus	*P. aeruginosa* established
P003	F	11m	15y 9m	phe508del	phe508del	Yes
P004	F	1m	13y 6m	phe508del	phe508del	Yes
P005	M	9y 5m	14y 11m	phe508del	Y563D	Yes
P006	F	2m	10y 1m	phe508del	Not identified	Yes
P007	F	1m	14y 2m	phe508del	phe508del	Yes
P008	M	3y 9m	10y 3m	phe508del	phe508del	Yes
P009	M	1m	15y	phe508del	G542X	Yes
P010	M	Not known	16y 5m	Not done	Not done	Yes
P011	M	1m	13y 8m	Not done	Not done	Yes
P012	F	0	17y 6m	621+1G>T	Not identified	Yes
P013	F	6m	17y 10m	phe508del	Not identified	Yes
P014	M	0	6y 11m	phe508del	phe508del	Yes
P015	F	2y	16y 2m	phe508del	phe508del	Yes
P01	M	1m	8y 8m	phe508del	Not identified	Yes
P017	M	0	7y 11m	phe508del	G542X	Yes
P018	F	6m	16y 11m	3850-1(G>A	3659delC	Yes
P019	F	1m	15y 4m	phe508del	phe508del	Yes
P020	M	2y 9m	15y 7m	Not known	Not known	Yes
P021	M	0	8y 4m	phe508del	phe508del	Yes
P022	F	1m	10y 10m	phe508del	Not identified	Yes
P023	M	1y 3m	16y 5m	V520F	3659delC	Yes
P024	M	4m	16y 9m	phe508del	phe508del	Yes
P025	M	5m	15y 7m	phe508del	phe508del	Yes

3.2.2 Recovery of *P. aeruginosa* clinical isolates

In order to study the QS signal molecule production of clinical *P. aeruginosa* isolates, 43 isolates were recovered from a cohort of 36 individual patient sputum samples. Of these isolates, 16 came from samples provided by paediatric patients. Each sputum sample was plated onto selective agar as described in 2.4.2. The resulting isolates were identified by the same code as the sputum sample, with the addition of the letter A or B if more than one colony type was present. This was the case for six sputum samples, in each of which a pair of phenotypically different isolates was recovered.

All isolates were confirmed to be rapidly oxidase positive Gram-negative rods. Twelve (28 %) grew as mucoid colonies on PIA after incubation at 37 °C for 24 to 48 h. The remaining 31 (72 %) were non-mucoid. The proportion of mucoid isolates was similar for the subset of isolates obtained from adult patients and for those obtained from paediatric patients at 26 % and 31 % respectively. Of the 6 cases in which a pair of isolates was recovered from a single sputum sample, 5 of these consisted of a non-mucoid isolate co-existing with a mucoid isolate. The majority of isolates (31; 72 %) produced the green pigment pyocyanin. Of those remaining, 5 (12 %) produced a pink pigment, likely to be pyorubin and 7 (16 %) were colourless, suggesting no pigment production. See Table 3-2 for phenotypic characteristics of the isolates and Figure 3-1 for light-phase photographs of a selection of isolates plated on PIA.

Table 3-2 Phenotypic characteristics of clinical isolates.
Strain codes start with A or B depending on whether they were from sputum samples obtained from adult or paediatric patients respectively. The code ends in a letter A or B if more than one colony type was isolated.

Strain	Mucoid (m) Non-mucoid (nm)	Pigment
A001-200804	nm	none
A002-051104	m	none
A003-280504	nm	green
A004-130804	m	pink
A005-100904	nm	green
A007-110604	nm	green
A009-110604	nm	green
A012-180604A	nm	green
A012-180604B	m	none
A014-291004	nm	pink
A017-081004	m	green
A018-151004	nm	green
A019-040205	nm	green
A021-101204A	nm	green
A021-101204B	nm	green
A023-200804	nm	green
A024-270804	nm	green
A025-221004	m	pink
A026-130804	nm	green
A029-110305	nm	green
A031-030904	nm	green
A032-200804	nm	green
A033-200804	nm	green
A035-051104A	m	pink
A035-051104B	nm	green
A037-230205A	m	pink
A037-230205B	nm	green
P003-170804	nm	green
P004-010205	nm	green
P006-170804	nm	green
P007-280904	nm	green
P009-280904	nm	green
P010-191004	nm	green
P010-211204	m	green
P013-101204A	m	none
P013-101204B	nm	green
P015-170804A	m	none
P015-170804B	nm	green
P016-280904	nm	none
P018-161104	nm	green
P020-191004	m	none
P021-211204	nm	green
P024-070904	m	green

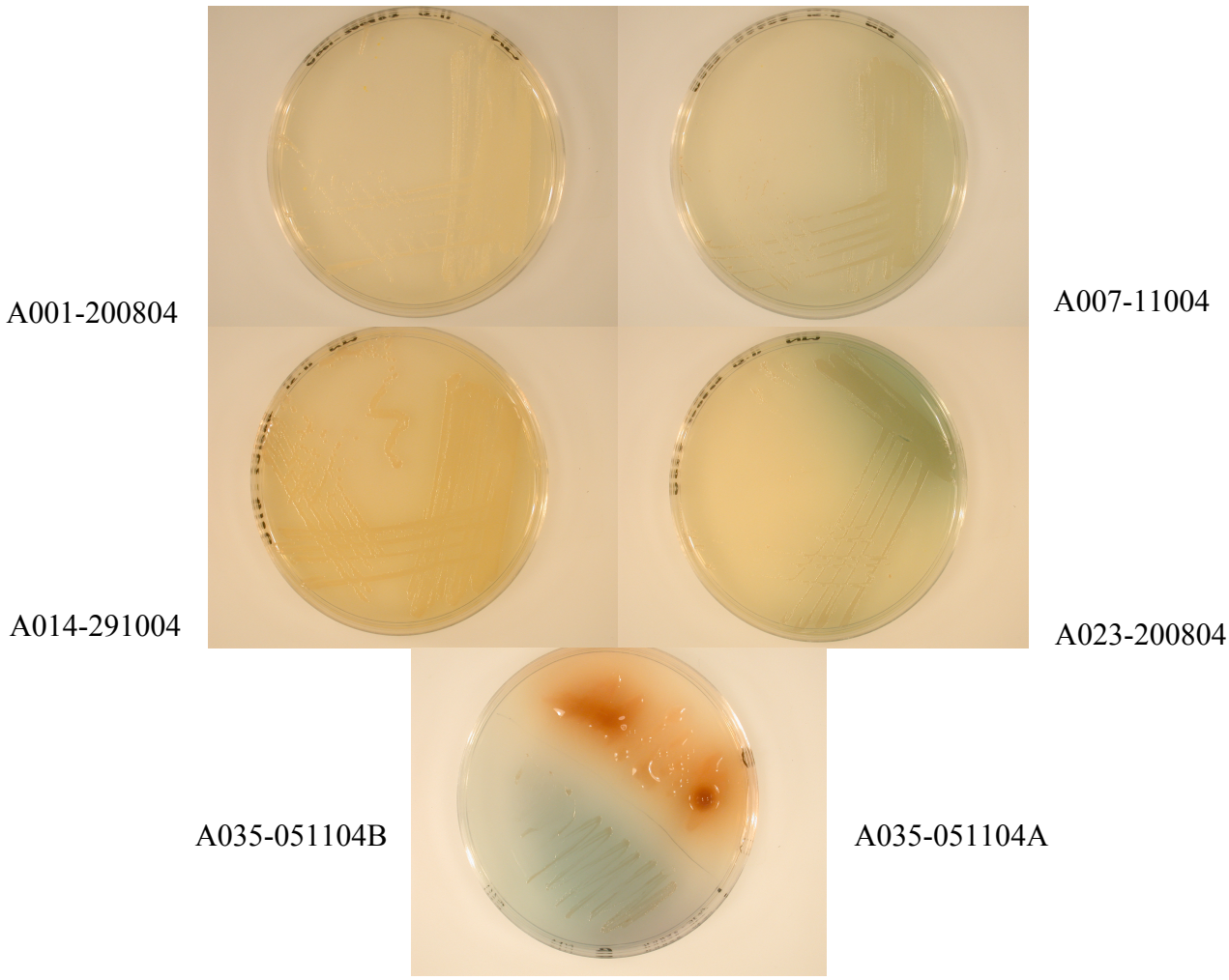


Figure 3-1 Light-phase photographs of a selection of clinical strains plated on PIA.

3.2.3 QS signal molecule production by clinical isolates

The cohort of 43 clinical isolates was investigated for their ability to produce the QS signal molecules 3-oxo-C12-HSL, C4-HSL, HHQ and PQS when grown as broth cultures. TLC analysis was undertaken in conjunction with specific signal molecule *lux*-based bioreporter bacteria. TLC plates were selected to allow the optimal separation of each signal molecule in an appropriate solvent system and then overlaid with a lawn of bioreporter bacteria. The *E. coli* reporter strains S17-1 λ*pir* [pSB1142] and JM109 [pSB536] were used to detect the presence of 3-oxo-C12-HSL and C4-HSL respectively. For the detection of PQS and HHQ, PAO1 *lecA'*::*lux*Δ*pqsA* was used.

Activation of the *E. coli* JM109 [pSB536] bioreporter by extracted broth cultures indicated the presence of C4-HSL for all isolates bar one obtained from an adult patient, which was mucoid and coexisted with a non-mucoid C4-HSL producing isolate. Using the *E. coli* S17-1 λ*pir* [pSB1142] bioreporter, 3-oxo-C12-HSL was not detected for either of these isolates, nor so from paired isolates from two paediatric patients. In total, 22 (51 %) isolates were deficient in 3-oxo-C12-HSL. Of these, 17 (77 %) were non-mucoid. Examining the distribution of these signal molecule-deficient isolates, it was apparent that 37 % (10/27) of adult isolates lacked 3-oxo-C12-HSL compared to 75 % (12/16) of isolates obtained from paediatric patients.

The PAO1 *lecA'*::*lux*Δ*pqsA* bioreporter indicated the presence of HHQ for all isolates and in 95 % (41) of isolates, PQS was also detected. The 2 isolates in which PQS was not detected were obtained from non-mucoid, pyocyanin-producing paediatric isolates which both made low levels of C4-HSL and lacked 3-oxo-C12-HSL. In this instance, the production of pyocyanin may have been achieved by the substitution of PQS by HHQ. See Figure 3-2 to Figure 3-4 for representative images of QS signal molecule detection by TLC and signal molecule bioreporter bacteria and Table 3-3 for a summary of clinical isolate QS signal molecule production.

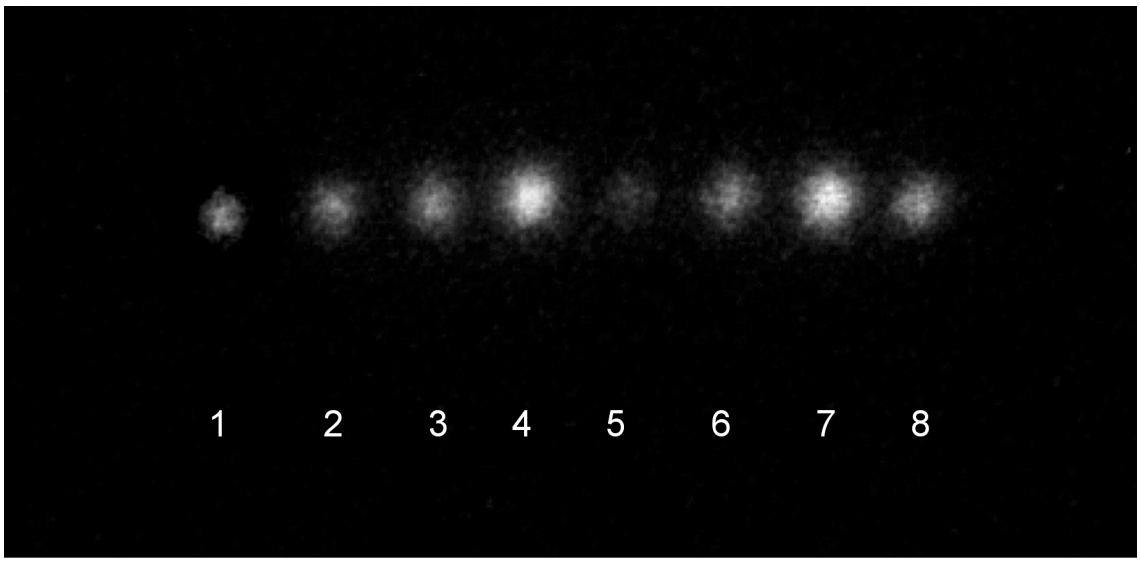


Figure 3-2 Detection of 3-oxo-C12-HSL in a selection of clinical isolates using TLC and the *E. coli* S17-1 λ*pir* [pSB1142] bioreporter strain.
3-oxo-C12-HSL detection on reverse phase RP-2 TLC plates after 3 h incubation at 37 ºC. 1 µl 1 µg/ml 3-oxo-C12-HSL was run as a standard and PAO1 extract as a control.

1 3-oxo-C12-HSL standard (1 µg/ml)
2 PAO1
3 PAO1Δ*lecA*
4 A001-200804
5 A007-110604
6 A014-291004
7 A023-200804
8 A035-051104B

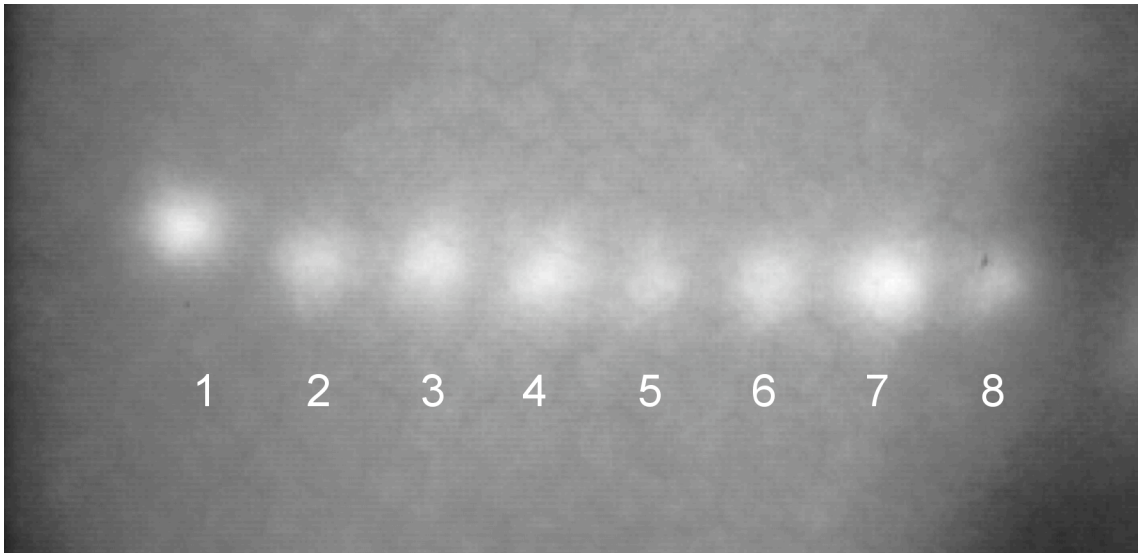
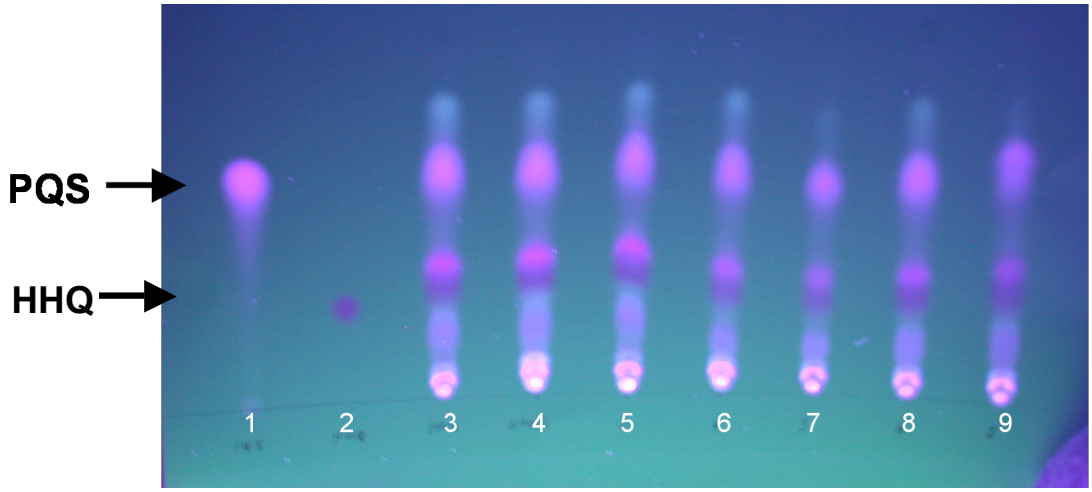


Figure 3-3 Detection of C4-HSL in a selection of clinical isolates using TLC and the *E. coli* JM109 [pSB536] bioreporter strain.
C4-HSL detection on reverse phase RP-18 TLC plates after 3 h incubation at 37 ºC. 1 µl 10 µg/ml C4-HSL was run as a standard and PAO1 extract as a control.

1 C4-HSL standard (10 µg/ml)
2 PAO1
3 PAO1Δ*lecA*
4 A001-200804
5 A007-110604
6 A014-291004
7 A023-200804
8 A035-051104B

(A)



(B)

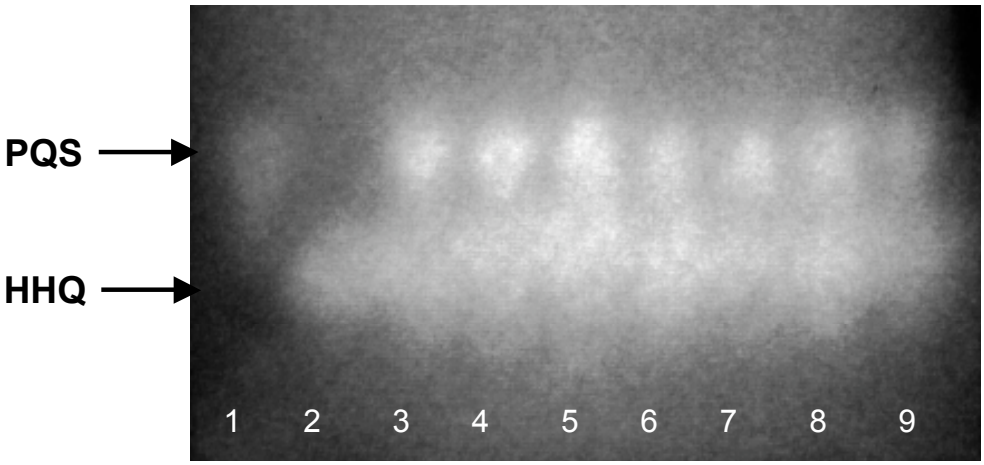


Figure 3-4 Detection of PQS and HHQ in a selection of clinical isolates.
(A) Normal phase TLC plates of extracts of the clinical isolates viewed under a UV transilluminator. 1 µl 5 mM HHQ and 2 µl 10 mM PQS were run as standards and PAO1 extract was used as a control. (B) *lecA*::*lux* expression by PAO1 *lecA'*::*lux*Δ*pqsA* overlaid on TLC plates after 5.5 h incubation at 37 ºC, indicating presence of both PQS and HHQ in a selection of clinical isolates.

1 PQS standard (10 mM)
2 HHQ standard (5 mM)
3 PAO1
4 PAO1Δ*lecA*
5 A001-200804
6 A007-110604
7 A014-291004
8 A023-200804
9 A035-051104B

Table 3-3 Clinical isolate QS signal molecule production.

Strain	C4	C12	HHQ	PQS	Strain	C4	C12	HHQ	PQS
A001-200804	y	y	y	y	A033-200804	y	y	y	y
A002-051104	y	y	y	y	A035-051104A	y	y	y	y
A003-280504	y	y low	y	y	A035-051104B	y	y	y	y
A004-130804	y	y	y	y	A037-230205A	y	y	y	y
A005-100904	y	y low	y	y	A037-230205B	y	y	y	y
A007-110604	y	y	y	y	P003-170804	y low	**n**	y	**n**
A009-110604	y	y low	y	y	P004-010205	y	y low	y	y
A012-180604A	y	**n**	y	y	P006-170804	y low	**n**	y	**n**
A012-180604B	**n**	**n**	y	y	P007-280904	y	**n**	y	y
A014-291004	y	y	y	y	P009-280904	y	**n**	y	y
A017-081004	y	**n**	y	y	P010-191004	y	y	y	y
A018-151004	y	y	y	y	P010-211204	y	**n**	y	y
A019-040205	y	**n**	y	y	P013-101204A	y	**n**	y	y
A021-101204A	y	y	y	y	P013-101204B	y	**n**	y	y
A021-101204B	y	**n**	y	y	P015-170804A	y	**n**	y	y
A023-200804	y	y	y	y	P015-170804B	y	**n**	y	y
A024-270804	y	**n**	y	y	P016-280904	y	**n**	y	y
A025-221004	y	y	y	y	P018-161104	y	**n**	y	y
A026-130804	y	**n**	y	y	P020-191004	y	y	y	y
A029-110305	y	**n**	y	y	P021-211204	y	**n**	y	y
A031-030904	y	**n**	y	y	P024-070904	y	y	y	y
A032-200804	y	**n**	y	y					

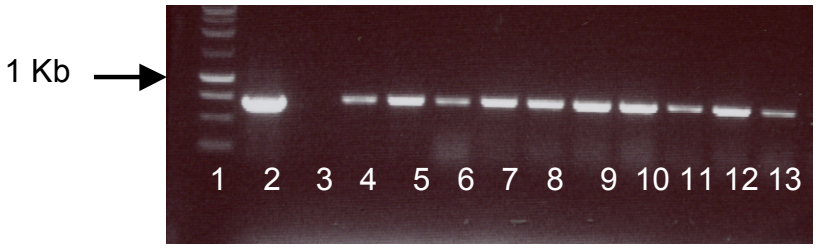
3.2.4 Investigation of signal molecule deficient isolates

Previously it has been shown that QS signal molecule deficient *P. aeruginosa* strains have been recovered from clinical sources and mutations were found to be preferentially located in the *lasR* and *rhlR* response regulator genes (Dénervaud *et al.*, 2004). In addition, Fox *et al* (2008) have recently reported the spontaneous occurrence of mutants in the *vfr* gene which is known to positively control the expression of *lasR*. Hence, further investigations were conducted on the 22 3-oxo-C12-HSL deficient isolates, the 1 C4-HSL deficient isolate and the 2 PQS deficient isolates recovered in this study in order to characterise whether their deficiency in signal production was due to a defect in the synthase or transcriptional regulator genes themselves or in their expression.

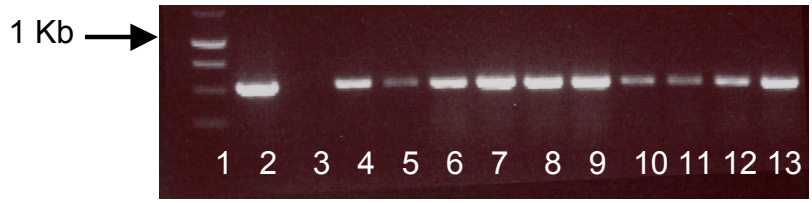
3.2.4.1 Investigation of 22 isolates deficient in 3-oxo-C12-HSL

As an initial screen for their presence, primers were designed to amplify *lasR* and *lasI* and when applied to the 3-oxo-C12-HSL signal-deficient isolate templates, a product of the appropriate size was obtained for both genes in all cases (data not shown). Following this, RT-PCR was undertaken to investigate the expression of *lasR* and *lasI*, which was found to occur for all isolates. See Figure 3.5.

(A)



(B)



1 1 Kb DNA ladder
2 PAO1 chromosomal DNA template
3 Blank well
4 PAO1 RNA
5 A017-081004
6 A019-040205
7 A021-101204B
8 A024-270804
9 A026-130804
10 A029-110305
11 A031-030904
12 A032-200804
13 P003-170804

Figure 3-5 Detection of (A) *lasR* and (B) *lasI* gene transcripts by RT-PCR in a selection of clinical isolates.
One-step RT-PCR was undertaken using the primers pairs (A) lasR2UF/lasR2DR and (B) lasI2UF/lasI2DR yielding products of the expected sizes when examined on agarose gel electrophoresis.

Given this, it was hypothesised that the deficiency in 3-oxo-C12-HSL production may be due to a defect in the sequence of the synthase or transcriptional regulator genes themselves, affecting their translation into a functional protein product. Hence, the initial PCR products of the synthase and transcriptional activator genes were sequenced as described in section 2.10.8. BLAST comparisons against the published sequence for PAO1 identified 8 isolates with a defect in the *lasR* gene alone and one isolate with a defect in both *lasR* and *lasI*. See Table 3-4 and Table 3-5 for a summary of the position, nature and consequent amino acid changes of the gene defects detected.

Table 3-4 Summary of mutation detected in *lasI* of clinical isolate deficient in 3-oxo-C12-HSL.

Isolate	Codon number	Nature of error	Original codon (amino acid)	Mutated codon (amino acid)
A017-081004	144	substitution	ACC (threonine)	ACT (threonine)

Table 3-5 Summary of mutations detected in *lasR* of clinical isolates deficient in 3-oxo-C12-HSL.

Isolate	Codon number	Nature of error	Original codon (amino acid)	Mutated codon (amino acid)
A012-180604A	222	substitution	ACC (threonine)	ATC (isoleucine)
A012-180604B	222	substitution	ACC (threonine)	ATC (isoleucine)
A017-081004	45	substitution	CAG (glutamine)	TAG (stop)
A024-270804	126	substitution	GGC (glycine)	AGC (serine)
A026-130804	77	Deletion and frameshift	AGT (serine)	AGC (serine)
A031-030904	89	substitution	GAA (glutamate)	GAG (glutamate)
	120	substitution	GGT (glycine)	GGC (glycine)
	121	substitution	GCT (alanine)	GCC (alanine)
	162	substitution	GGT (glycine)	GGC (glycine)
	192	substitution	AGA (arginine)	AAA (lysine)
	224	substitution	CGC (arginine)	CAC (histidine)
	225	substitution	CGC (arginine)	CGT (arginine)
P006-170804	235	substitution	GGT (glycine)	GTT (valine)
P013-101204B	209	substitution	AAC (asparagine)	AAG (lysine)
P015-170804B	222	substitution	ACC (threonine)	ATC (isoleucine)

In almost all cases, a single point substitution occurred, leading to the incorporation of a different amino acid and hence an alteration to the LasR protein sequence. In 3 of the 3-oxo-C12-HSL deficient isolates, there was conservation of the error, with a replacement of the base cytosine by thymine in codon 222, leading to the incorporation of isoleucine rather than threonine. In one isolate (A026-130804), there was a base deletion (thymine) and subsequent shift of the reading frame. Isolate A031-030904 was found to have a number of defects, however only 3 of these actually coded for a different amino acid.

Only one isolate (A017-081004) was found to have an error in the sequence of *lasI*. Indeed, this single base substitution did not lead to a consequent change in amino acid and its lack of 3-oxo-C12-HSL was most likely due to the substitution of cysteine by thymine in codon 45 of its *lasR* gene, creating a stop codon and hence truncating the amino acid chain of the regulator protein.

3.2.4.2 Investigation of 1 isolate deficient in C4-HSL

The same set of experiments was conducted to investigate the C4-HSL deficient isolate, A012-180604B. Primers were designed to amplify the entire region of the genes *rhlR* and *rhlI*, yielding PCR products of the appropriate size and RT PCR confirmed the expression of both gene transcripts (data not shown). Subsequent sequence analysis and BLAST comparison with the genome of PAO1 revealed that both genes were mutated as detailed in Table 3-6 and Table 3-7.

Table 3-6 Summary of mutations detected in *rhlI* of clinical isolate deficient in C4-HSL.

Isolate	Codon number	Nature of error	Original codon (amino acid)	Mutated codon (amino acid)
A012-180604B	31	substitution	GAA (glutamate)	GAG (glutamate)
	47	substitution	AGC (serine)	GGC (glycine)
	50	substitution	GGC (glycine)	GGT (glycine)
	54	substitution	TGC (cysteine)	TGT (cysteine)
	58	substitution	CTG (leucine)	TTG (leucine)
	68	substitution	GAC (aspartate)	GAA (glutamate)
	79	substitution	CGA (arginine)	CCA (proline)
	84	substitution	TCT (serine)	TAT (tyrosine)
	99	substitution	AGC (serine)	AAC (asparagine)

Table 3-7 Summary of mutations detected in *rhlR* of clinical isolate deficient in C4-HSL.

Isolate	Codon number	Nature of error	Original codon (amino acid)	Mutated codon (amino acid)
A012-180604B	49	substitution	CAC (histidine)	CAT (histidine)
	197	substitution	AGT (serine)	AGC (serine)

The two base substitutions in *rhlR* did not lead to subsequent amino acid changes, hence it can be assumed that this protein was functional. However, of the 9 substitution errors detected in *rhlI*, 5 lead to the incorporation of a different amino acid and hence it could be deduced that the failure to make C4-HSL was due to a problem with the structure and hence function of the RhlI synthase protein.

3.2.4.3 Investigation of the 2 PQS deficient isolates

It is known that conversion of HHQ into PQS requires the product of the *pqsH* gene (Gallagher *et al.*, 2002). Therefore to further investigate the two PQS deficient isolates, primers were designed to amplify *pqsH*. These yielded PCR products of the appropriate size, confirmed to be the intact internal portions of the *pqsH* gene by sequence analysis. However, the ensuing RT-PCR failed to detect the gene transcripts, indicating that the deficiency in PQS production was due to failure of *pqsH* expression.

3.2.5 The conservation of *lecA* and production of LecA by clinical isolates

The *lecA* gene (and its corresponding protein product, LecA) is an example of a highly QS regulated gene because it is both AHL (Winzer *et al.*, 2000) and PQS (Diggle *et al.*, 2003) dependent. In addition, this 47 kDa carbohydrate-binding protein is known to be an important virulence determinant of *P. aeruginosa* (Bajolet-Laudinat *et al.*, 1994) and a contributor to biofilm formation by this organism (Diggle *et al.*, 2006b).

To determine whether *lecA* (369 bp) was conserved in the cohort of non-mucoid and mucoid *P. aeruginosa* isolates recovered from CF sputum, the strains were subjected to PCR analysis for the *lecA* gene. As it was not known whether there was variation in the flanking regions of this gene in strains other than the sequenced strain PAO1, primers were designed to amplify an internal fragment of *lecA*. PAO1 and PAO1Δ*lecA* (containing a chromosomal deletion of *lecA*) were the positive and negative controls respectively.

Figure 3-6 depicts an agarose gel of the appropriate size PCR products obtained for the wildtype control and a selection of clinical isolates. The wildtype PAO1 PCR product was subsequently purified and sequenced. A BLAST analysis against the published sequence for *lecA* demonstrated an intact alignment of nucleotides, implying that this primer set was indeed detecting the *lecA* gene.

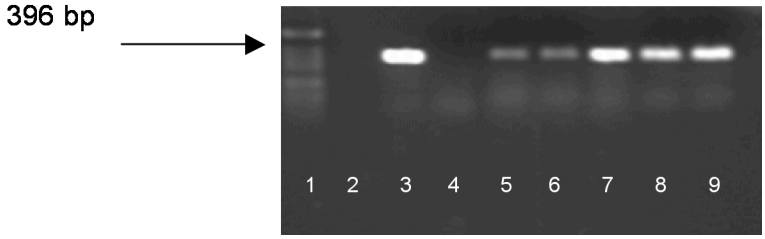


Figure 3-6 Example of the detection of *lecA* by PCR in a selection of clinical isolates.
Amplification of an internal fragment of *lecA* using the primers lecAUF and lecADR. A product of between 344 and 396 bp was detected in all isolates except PAO1Δ*lecA* (negative control).

1 1 Kb DNA ladder
2 Empty lane
3 PAO1
4 PAO1Δ*lecA*
5 A002-051104
6 A004-130804
7 A035-051104A
8 P024-070904
9 A033-200804

To determine whether the LecA protein was produced *in vitro* by the clinical isolates of *P. aeruginosa*, Western blot analysis of SDS-PAGE gels was performed using a polyclonal antibody raised against LecA. This was used to detect the presence or absence of the lectin in the controls: purified LecA protein (PA-IL, Sigma) and PAO1 (positive), PAO1Δ*lecA* and PAO1 *lecA*::*lux* (negative).

Figure 3-7 shows the presence of a band running between the 10 and 15 kDa standards, which was present in the positive controls and absent in the negative controls, and likely represented the 12.5 kDa monomers of LecA. Further bands were seen around 25 and 50 kDa which could represent binding to the dimer and tetramer state of the protein respectively. Other identical bands were seen in all the *P. aeruginosa* isolates as a result of polyclonal antibody binding to other bacterial proteins (data not shown). See Figure 3.8 for LecA detection by Western blotting in a selection of clinical isolates.

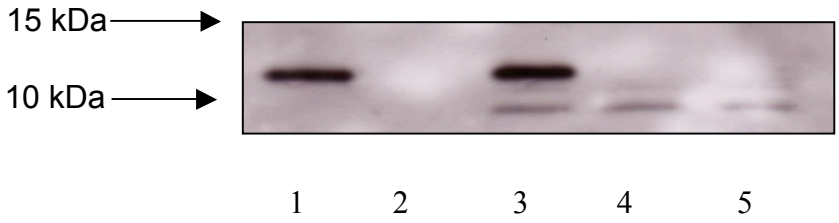


Figure 3-7 Western blot analysis of LecA production by laboratory strains.
P. aeruginosa whole cell protein extracts were prepared and analysed by SDS-PAGE and Western blotting as described in sections 2.15.3 and 2.15.4 respectively. Anti-LecA was added at a dilution of 1:1000.

1 Purified LecA (10^{-4} mg/ml)
2 Empty lane
3 PAO1
4 PAO1Δ*lecA*
5 PAO1 *lecA*::*lux*

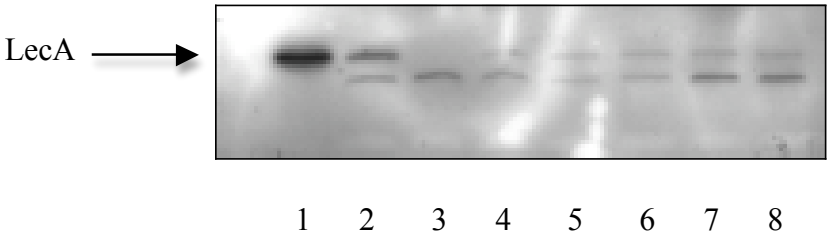


Figure 3-8 Western blot analysis of LecA production in a selection of clinical isolates.
P. aeruginosa whole cell protein extracts were prepared and analysed by SDS-PAGE and Western blotting as described in sections 2.15.3 and 2.15.4 respectively. Anti-LecA was added at a dilution of 1:500.

1 Purified LecA (10^{-4} mg/ml)
2 PA01
3 PAO1Δ*lecA*
4 A002-051104
5 A004-130804
6 A035-051104A
7 P024-070904
8 A033-200804

In 6 of the cohort of 43 clinical isolates (14 %), there was failure to detect the *lecA* gene and as expected, no LecA protein was made. A full QS signal molecule profile was observed for all of these isolates, except A031-030904, which lacked 3-oxo-C12-HSL. See Table 3-8.

Table 3-8 The QS signal molecule profile and *lecA* status of clinical isolates deficient in LecA production.

Strain	Mucoid/ nonmucoid	Pigment	*lecA*	LecA	C4	C12	HHQ	PQS
A014-291004	nm	pink	**n**	**n**	y	y	y	y
A018-151004	nm	green	**n**	**n**	y	y	y	y
A025-221004	m	pink	**n**	**n**	y	y	y	y
A031-030904	nm	green	**n**	**n**	y	**n**	y	y
P010-191004	nm	green	**n**	**n**	y	y	y	y
P020-191004	m	none	**n**	**n**	y	y	y	y
P021-211204	nm	green	y	**n**	y	**n**	y	y

The non-mucoid, pyocyanin-producing isolate P021-211204 appeared to possess and express *lecA* (as determined by PCR and RT-PCR respectively), however did not make the protein (see Figure 3-9). Sequence analysis of the gene indicated two redundant substitution errors, which did not result in changes to the amino acid chain. This isolate was noted to be deficient in 3-oxo-C12-HSL production and there was failure to restore protein production when a culture was grown in the presence of biologically active levels (20 μM) of this signal (data not shown) suggesting that the failure of protein production was not due to a disrupted QS circuitry. Other possible reasons for the lack of LecA production by this isolate could be problems with the translation or stability of the protein.

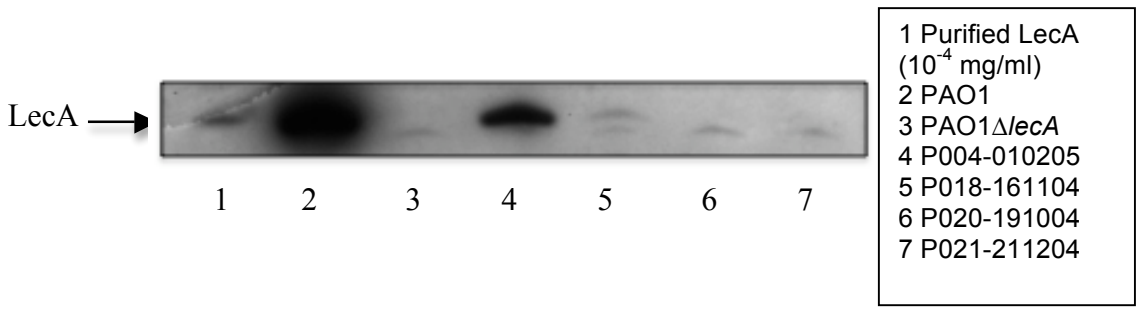


Figure 3-9 Isolate P021-211204 does not produce LecA.
P. aeruginosa whole cell protein extracts were prepared for a selection of clinical isolates and analysed by SDS-PAGE and Western blotting. Anti-LecA was added at a dilution of 1:500.

A table detailing the conservation of *lecA* and production of LecA by the full cohort of clinical isolates in conjunction with their QS signal molecule profile can be found in Appendix 5.

3.2.5.1 The detection of LecA in sputum

In addition to this characterisation of the LecA-status of the clinical isolates, attempts were made to detect LecA itself in sputum samples by SDS-PAGE and Western blotting. Following repeated experimentation (see section 2.15.2), there was failure to detect the protein, possibly because of the presence of only low levels in sputum.

3.3 Discussion

The analysis of clinical isolates described in this chapter was achieved by conducting a clinical study of CF patients. The establishment and implementation of such a study was a rigorous process, necessitating the full approval of the Ethics and R&D departments, the assistance of the CF clinical team and clinical microbiology department. The high recruitment rate and regular provision of sputum samples by the study participants reflects the high motivation and commitment of this relatively young patient group, their families and their allied clinical team to participate in and facilitate clinical research.

A cohort of 43 clinical *P. aeruginosa* isolates was recovered from individual sputum samples by the use of selective agar plates and laboratory identification methods. In addition, there was access to information on the identification of strains in the clinical laboratory from parallel sputum samples provided by each patient. Phenotypic differences, principally in mucoidy, were used to further distinguish the putative presence of more than one strain from a given sample. It is know however, that phenotypic identification of Gram-negative bacteria from CF patients carries a risk of misidentification (Wellinghausen *et al.*, 2005) and that differences in morphology may reflect the diversification of a single clone (Tummler and Kiewitz, 1999). Such an approach, however, was deemed pragmatic in terms of the further laboratory analysis planned. Furthermore, the detection of PQS, which to date has only been shown to be made by *P. aeruginosa*, was an independent confirmation of species identification.

Twelve (28 %) of the recovered isolates grew as mucoid colonies. This striking phenotype is rarely observed outside the CF lung, suggesting that this unique environmental niche selects for the occurrence and survival of mucoid mutants. This has significance in that the appearance of such mucoid colonies in CF lung disease correlates with impairment in lung function and the clinical decline of the patient (Parad *et al.*, 1999).

Other marked differences were noted in pigment production, with the intensity of the colours sometimes deepening dramatically as the plated colonies aged. A defining characteristic of *P. aeurginosa* is the production of diffusible fluorescent pigments such as pyoverdin, the soluble phenazine pigment pyocanin as well as pyorubin and pyomelanin (dark red and black respectively) (Pollack *et al.*, 2000). Indeed, these simple observations of the diversity of strains on agar plates reinforced the importance of studying clinical isolates. These phenotypic variations reflect the adaptation and survival of individual strains within the same hosts, often for a number of years and hence differences are likely to occur in other pathogenic mechanisms such as QS systems.

The investigation of the ability of this cohort of clinical isolates to produce four major QS signal molecules when grown as broth cultures demonstrated that the majority produced C4-HSL (98%), HHQ (100 %) and PQS (95 %). However, 51 % of the isolates were deficient in 3-oxo-C12-HSL production. Further investigation of these 22 isolates revealed that *lasR* and *lasI* were detected and expressed in all cases. Subsequently, 9 isolates were found to have a sequence error in *lasR*, which coded for the incorporation of a different amino acid (7 isolates), a stop codon (1 isolate) and for a shift in the open reading frame (1 isolate). One mutation (substitution of cytosine by thymine in codon 222), which was seen in three isolates, was also detected in a 20 year old patient as part of the analysis of a cohort of patients with CF conducted by Smith *et al.* (2006), yet their computational prediction suggested that the mutation would be tolerated and thus not have an effect on LasR protein function.

Thus the failure to detect 3-oxo-C12-HSL production by these and the remaining 13 isolates could be for a number of reasons. These include the sensitivity of the assay itself and possible failure to detect low levels of signal production, mutations in other regulators such as *vfr* (Fox *et al.*, 2008) or problems with the translation or stability of the response regulator and synthase proteins themselves.

Overall, these findings are in keeping with previous studies which have shown that QS deficient strains mostly carry mutations in the *lasR* regulator gene, which impairs the response to signal molecules ((Denervaud *et al.*, 2004) (Smith *et al.*, 2006) (Salunkhe *et al.*, 2005). There are a number of possible explanations for this. Firstly, QS may not be important for growth in these environments and therefore Darwinian selection results in QS loss over time. Given that inactivation of *lasR* reduces *P. aeruginosa* virulence in diverse model hosts (Rumbaugh *et al.*, 1999a), the occurrence of *lasR* mutants in the CF airway could be due to selection against a strategy for acute virulence in favour of the opportunity to survive and persist in a chronic state. Additionally, it has been suggested that a *lasR* mutation confers a growth advantage on particular carbon and nitrogen sources, which may offer a selective advantage over QS-positive strains (D'Argenio *et al.*, 2007).

In this study, a pair of isolates was recovered in which one was found to make 3-oxo-C12-HSL (A021-101204A) and the other was deficient (A021-101204B). Indeed, another plausible explanation for the prevalence of *lasR* mutants is that bacteria can 'cheat' or 'freeload' on QS cooperating populations (West *et al.*, 2006). In *P. aeruginosa* many QS-regulated products are released into the extracellular environment and benefit not only the producing cell but also its neighbours. Mutants that do not respond to QS signals do not incur the cost of producing these 'public goods' but gain the benefit of production by neighbours (Diggle *et al.*, 2007b) (Sandoz *et al.*, 2007). Put another way, *lasR* cheaters have a social fitness advantage over QS-positive strains. This cooperation and cheating principle has been demonstrated for QS *in vitro* (Sandoz et al., 2007) (Diggle *et al.*, 2007b) and also for the production of other public goods, such as siderophores, which in turn influence the virulence of an infection (Harrison *et al.*, 2006) (Buckling *et al.*, 2007). Social cheating could reconcile why *lasR* mutants are repeatedly found in clinical isolates from CF patients and yet QS-deficient mutants of *P. aeruginosa* have reduced virulence when grown in monocultures (Wu *et al.*, 2001).

Despite the occurrence of signal deficient isolates, the demonstration that in general, QS is conserved amongst these CF airway-derived clinical isolates supports its role as a mediator of virulence strategies *in vivo*. The clear implication of this is that these molecules and QS systems themselves represent potential targets for novel and specific antipathogenic therapies. For example, antibody interference with AHL-mediated QS

systems has been proposed (Kaufmann *et al.*, 2006), along with enzymatic degradation of the signal molecule (Dong *et al.*, 2001) and inhibition or blockage of the signalling circuit (Hentzer *et al.*, 2003). Such an approach is attractive as it does not exert the same strong selective pressure as agents which kill bacteria and would not be expected to disturb the communities of beneficial normal flora in the host.

To characterise further the QS-regulated behaviour of *P. aeruginosa* and as a prelude to the later investigation of biofilm formation, an analysis of the conservation of the highly QS-regulated gene *lecA* and the production of the LecA protein amongst the clinical isolates was undertaken. In addition to the laboratory strain, the gene was detected and the protein expressed in the majority of isolates, implicating a role for this protein in the *in vivo* pathogenesis of *P. aeruginosa* in the CF lung. Given its known cytotoxic effects on human peripheral lymphocytes (Sharabi, 1979) and respiratory epithelial cells (Bajolet-Laudinat *et al.*, 1994) and its role in biofilm formation (Diggle *et al*., 2006b) the detection of this protein could therefore serve as a useful marker of lung damage as well as an indicator of the biofilm state. However, attempts to recover the protein by Western blot analysis *ex-vivo* from sputum samples were unsuccessful, possibly due to its presence at low levels.

Despite this and given the evidence presented in this chapter for the production of QS signal molecules by clinical isolates, the principle of this approach prompted an investigation of the potential role of QS molecules as novel biomarkers of lung infection and tissue damage. Furthermore, the production of LecA amongst clinical isolates coupled with the report from our laboratory for its role in biofilm development (Diggle *et al.*, 2006b) paved the way for ensuing chapters investigating the role of lectins in biofilm formation by laboratory and clinical isolates in a flowchamber biofilm system.

Chapter 4: Investigation of QS signal molecules as biomarkers of lung infection and damage

4.1 Introduction

The demonstration that clinical isolates of *P. aeruginosa* make QS signalling molecules when grown as broth cultures raises the question of whether these compounds can be detected in clinical samples derived from the lungs of CF patients. Indeed, evidence that QS is functional *in vivo* dates to 1998 when Storey *et al.* (1998) demonstrated the presence of gene transcripts for the transcriptional regulator protein LasR in CF sputum. The first direct detection of AHLs in CF sputum was performed by Middleton *et al.* (2002) using *lux*-based *E. coli* AHL biosensors. Both short chain AHLs and a molecule which co-migrated with 3-oxo-C12-HSL on thin layer chromatograms were detected. Similarly, using the induction of β-galactosidase activity in reporter plasmids, low levels of the principal AHLs 3-oxo-C12-HSL and C4-HSL have been detected in sputum (Erickson *et al.*, 2002). The use of biosensor strains has been further applied to demonstrate the presence of AHLs in other clinical sources of lung material such as lung tissue samples from CF patients (Favre-Bonte *et al.*, 2002), material from human lung allograft recipients, including subjects with no apparent infection (Ward *et al.*, 2003), mucopurulent respiratory secretions harvested from portions of lungs from CF patients removed at transplant (Chambers *et al.*, 2005) as well as from biofilms forming on the cuffs of endotracheal intubation devices (Favre-Bonte *et al.*, 2007).

In addition to bacterial biosensors, other laboratory approaches have been utilised. For example, following TLC separation, UV illumination was used to detect the presence of PQS in a range of clinical samples obtained from the CF lung (Collier *et al.*, 2002). The use of more sophisticated physical-chemical methods has also been applied to enable the separation and unequivocal determination of structures based on their spectroscopic properties. Middleton *et al*. (2002) used LC coupled with high-resolution MS to identify C6-HSL and 3-oxo-C12-HSL in pooled sputum samples from CF patients. A recent report describes the use of LC-MS-MS to demonstrate the presence of a range of AHLs

in the saliva of a small group of healthy subjects and those with gastrointestinal disorders (Kumari *et al.*, 2008).

The detection of *P. aeruginosa* QS signalling molecules by the analytical approaches described provides evidence for the occurrence of QS *in vivo*. This is a key mechanism employed by this organism to regulate virulence and biofilm maturation and enable it to adapt and thrive in the particular environmental niche of the CF lung. There is therefore the potential to utilise these signals as novel and specific biomarkers of infection, which not only enable identification, but also offer specific insight into pathogenic mechanisms.

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention. There is increasing interest in the use of sputum as a rich, non-invasive source of biomarkers of inflammation and infection in CF (Sagel *et al.*, 2007). To date, a range of host-derived candidate biomarkers have been proposed, such as free neutrophil elastase and interleukin 8. Whilst the bacterial densities of *P. aeruginosa* and *S. aureus* from sputum have also been examined in relation to FEV_1 (Mayer-Hamblett *et al.*, 2007), actual bacterial-derived markers have not yet been studied in great detail. The application of such markers alone or in conjunction with other candidates is an important and novel approach given the complex relationship between airway infection and inflammation in CF.

This chapter describes the application of LC-MS to detect *P. aeruginosa* QS signal molecules from serial sputum samples from a cohort of CF patients and the interpretation of findings in conjunction with patient clinical data. This approach, which aimed to estimate associations between the recovery of QS signal molecules and clinical status, paves the way for formal testing of the hypothesis that these molecules are clinically and biologically relevant, reproducible, sensitive, specific and feasible biomarkers of infection and tissue damage by *P. aeruginosa* in the CF lung.

4.2 Results

4.2.1 Description of study cohort

As described in section 2.1, a cohort of 62 patients with CF was recruited and at least one sputum sample provided by 39 (64 %). From these individuals, a subset of 10 patients (4 males and 6 females) was chosen for further analysis based on the criteria that they had produced at least two sputum samples, each being greater than 1 ml in volume. Eight of the patients were diagnosed with CF during their first two years of life. Six of the patients were recruited from the adult clinic (study number starting with A) with a mean age of 27 years 1 month at the time their first sputum sample was collected. Four patients were recruited from the paediatric clinic (study number starting with P) with a mean age of 16 years and 8 months. The full genotype of 7 patients was known and all had established infection with *P. aeruginosa*. A median of 6 sputum samples was collected from each patient (range 2 to 7). The FEV_1 % predicted varied from 20 to 90 enabling a classification of disease severity based on Erickson *et al*. (2002). See Table 4-1 for a summary of the baseline characteristics of the study cohort.

Table 4-1 Characteristics of study cohort
Classification of disease severity is based on Erickson *et al.* (2002) with patients defined as suffering from mild pulmonary disease having a forced expiratory volume in 1 s (FEV_1) of > 70 % of expected values. Patients classified as having moderate disease had a FEV_1 which fell in the range > 40 % and < 70 % of predicted; severe were those patients whose FEV_1 was < 40 % of expected values.

Study number	Sex	Age at diagnosis	Age when first sample collected	Genotype 1	Genotype 2	Disease severity	Number of sputum samples provided
A001	M	43y 2m	46y 11m	Not done	Not done	Information unavailable	7
A002	F	3m	28y 2m	phe508del	phe508del	Information unavailable	6
A004	F	0	19y 2m	phe508del	Q493X	moderate	5
A014	F	6y	24y	Not done	Not done	severe-moderate	7
A035	M	6m	21y 3m	phe508del	phe508del	severe	6
A037	M	2y	24y 11m	phe508del	phe508del	severe	6
P003	F	11m	16y 8m	phe508del	phe508del	moderate	5
P013	F	6m	18y 4m	phe508del	Not identified	mild	7
P015	F	2y	16y 11m	phe508del	phe508del	moderate	2
P025	M	5m	16y 9m	phe508del	phe508del	moderate	2

4.2.2 Synthetic QS molecules detected by LC-MS

A full spectrum of synthetic AHL and AQ synthetic standards of varying side chain length and substitution, at a concentration of 0.5 μM, were examined by LC-MS as described in section 2.18.4. This was undertaken in order to determine the relative peak area counts (or relative response) that could be detected at this concentration for these compounds using this particular method of extraction and analysis by Multiple Reaction Monitoring Triggered Traps (MRMTT). This is designed for sensitivity and relative quantification, enabling identification of analytes based on expected elution times, precursor ion and product ion. Analytes with longer carbon side chains were detected at higher peak area counts as were the unsubstituted AQs in comparison to the AHLs (Figure 4-1).

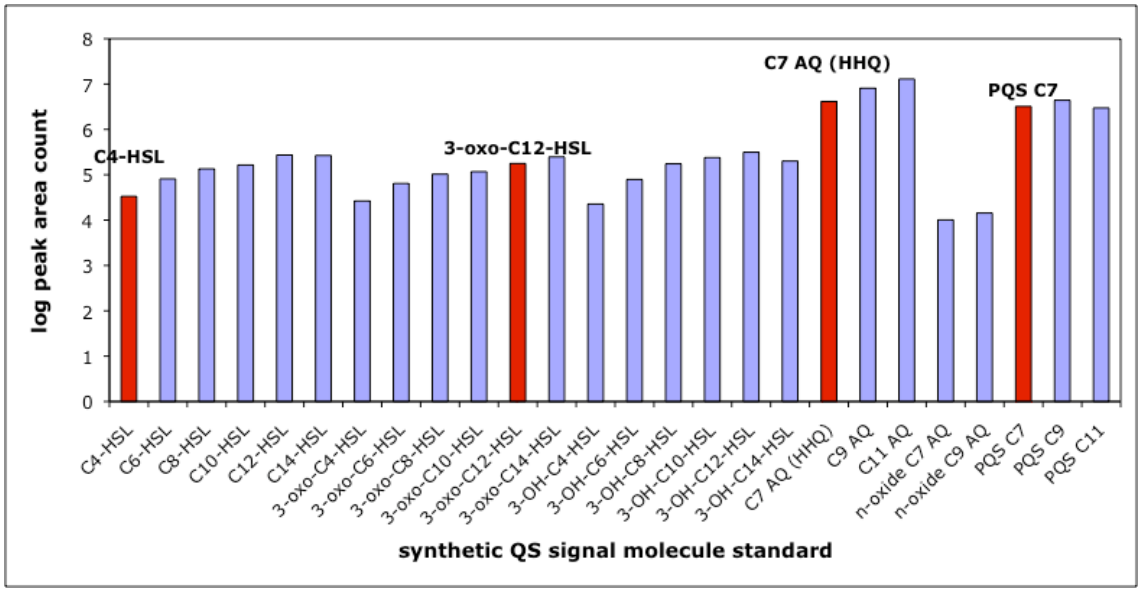


Figure 4-1 Relative response of synthetic standards.
LC-MS detection of 0.5 μM synthetic standards of QS molecules using MRMTT. Values represent logarithmically transformed peak area counts of a single reading. The key biologically significant *P. aeruginosa* QS signal molecules C4-HSL, 3-oxo-C12-HSL, HHQ and PQS are highlighted in red.

To enable relative quantification of the key biologically significant molecules C4-HSL, 3-oxo-C12-HSL, HHQ and PQS, synthetic signal molecule standards at concentrations of 0.1 to 0.5 μM were analysed (Figure 4-2).

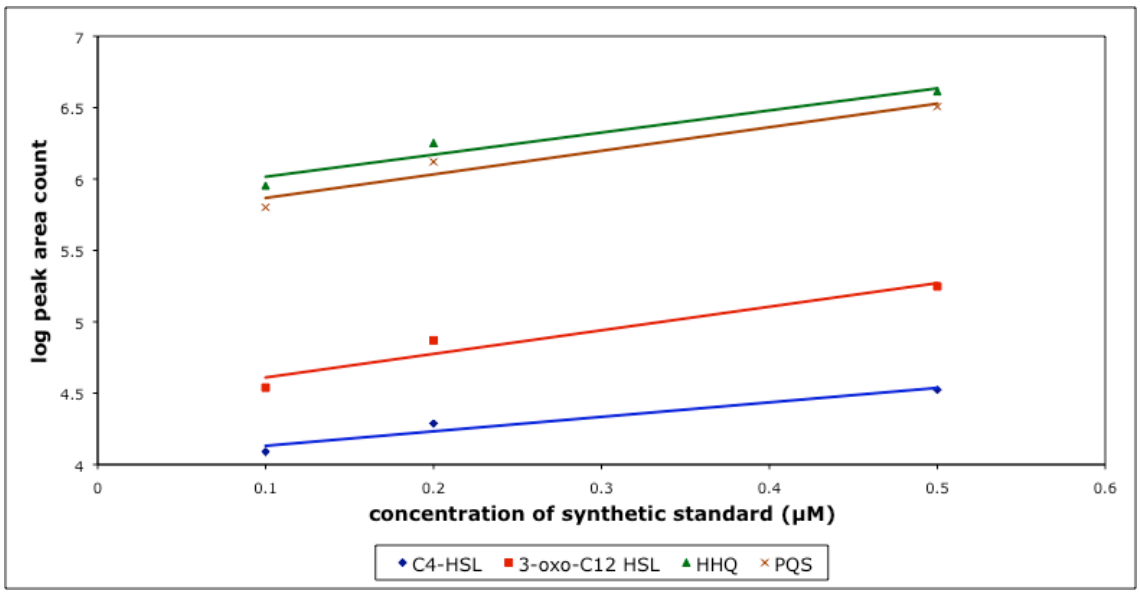


Figure 4-2 Dose-response of QS synthetic standards.
C4-HSL, 3-oxo-C12-HSL, HHQ and PQS at concentrations ranging from 0.1 to 0.5 μM were analysed by LC-MS. Values represent logarithmically transformed peak area counts of a single reading.

4.2.3 Sputum QS molecules detected by LC-MS

The total cohort of 53 sputum samples provided by the study participants were individually extracted and analysed by LC-MS as described in section 2.18. A wide range of *P. aeruginosa*-relevant molecules was recovered at varying frequencies and relative amounts. C4-HSL, HHQ and PQS were detected in almost all samples yet the recovery of 3-oxo-C12-HSL was lower at just over 50 %. The open ring form of this compound was detected in 37 (70 %) samples, 10 of which did not contain intact 3-oxo-C12-HSL. An additional breakdown compound, tetramic acid, was detected in 12 samples (5 of which did not contain intact 3-oxo-C12-HSL). The failure to detect 3-oxo-C4-HSL served as a useful negative control as this compound is not known to be produced by *P. aeruginosa*. See Table 4-2.

Table 4-2 Range aned frequency of QS signal molecules detected in sputum
A total of 53 sputum samples from the cohort of 10 patients were individually extracted and analysed by LC-MS. A wide range of *P. aeruginosa* relevant compounds was detected in varying frequencies.

Signal molecule	Number of samples in which signal detected (total 53)	% of samples in which signal detected
C4-HSL	**52**	**98**
open ring C4-HSL	**19**	**36**
C6-HSL	44	83
C8-HSL	19	36
C10-HSL	3	6
C12-HSL	25	47
C14-HSL	16	30
3-oxo-C4-HSL	**0**	**0**
3-oxo-C6-HSL	6	11
3-oxo-C8-HSL	4	8
3-oxo-C10-HSL	11	21
3-oxo-C12-HSL	**28**	**53**
open ring 3-oxo-C12-HSL	**37**	**70**
3-oxo-C14-HSL	19	36
tetramic acid	**12**	**23**
3-OH C4-HSL	3	6
3-OH C6-HSL	4	8
3-OH C8-HSL	1	2
3-OH C10-HSL	25	47
3-OH C12-HSL	28	53
3-OH C14-HSL	33	62
C7 AQ (HHQ)	**53**	**100**
C9 AQ	53	100
C11 AQ	52	98
n-oxide C7 AQ (HHQNO)	53	100
n-oxide C9 AQ	51	96
C7 PQS	**52**	**98**
C9 PQS	53	100
C11 PQS	46	87

Further detailed examination of select molecules known to be important for virulence and biofilms was undertaken to achieve an estimation of their relative spread of recovery. See Table 4-3. In keeping with the differing relative responses of these analytes (refer back to Figure 4-1), it can be seen that the AHLs are mainly recovered at the lower to mid ranges (peak area counts up to 10^5) whilst the AQs are in the mid to high end of the spectrum of peak area counts. These values correspond to concentrations of approximately 0.1 to 0.5 μM (refer back to Figure 4-2).

Table 4-3 Frequency of detection over a range of peak area counts for select compounds
Values represent the number of sputum samples from which each compound was detected with the percentage in brackets. The range of peak area counts are depicted as **0**/+ (counts less than 10^4); + (counts of 10^4-10^5) and ++ (counts of 10^6-10^7) and approximately correspond to concentrations of between 0.1 to 0.5 μM.

Analyte	**Peak area count**		
	<10^4 **0**/+	**10^4-10^5** +	**10^6-10^7** ++
C4-HSL	1 (1 %)	44 (83 %)	8 (15 %)
3-oxo-C12-HSL	40 (75 %)	13 (25 %)	0
tetramic acid	45 (85 %)	8 (15 %)	0
C7 AQ	0	6 (11 %)	47 (89 %)
C9 AQ	0	9 (17 %)	44 (83 %)
n-oxide C7 AQ	0	22 (42 %)	31 (58 %)
n-oxide C9 AQ	2 (4 %)	24 (45 %)	27 (51 %)
PQS	0	22 (42 %)	31 (58 %)

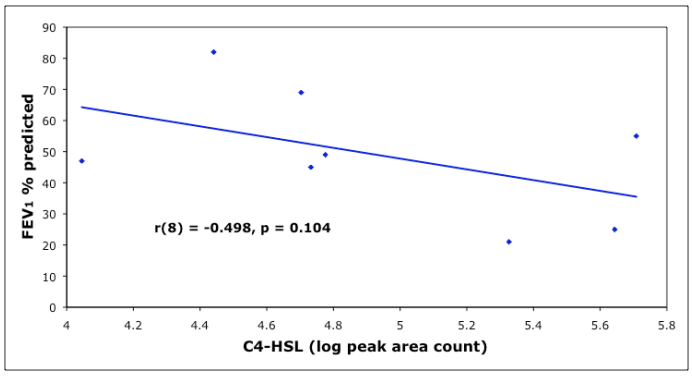
4.2.4 Cross sectional association between FEV_1 and sputum QS signal molecules

In order to determine whether the recovery of sputum QS molecules related to patient clinical status, a number of associations were investigated. Figure 4-3 displays scatterplots of each of the major *P. aeruginosa* QS signal molecules versus FEV_1 % predicted for the first sputum sample collected from each patient. Any values of zero (indicating a peak area count below the threshold of detection) for the QS signal molecule level were disregarded.

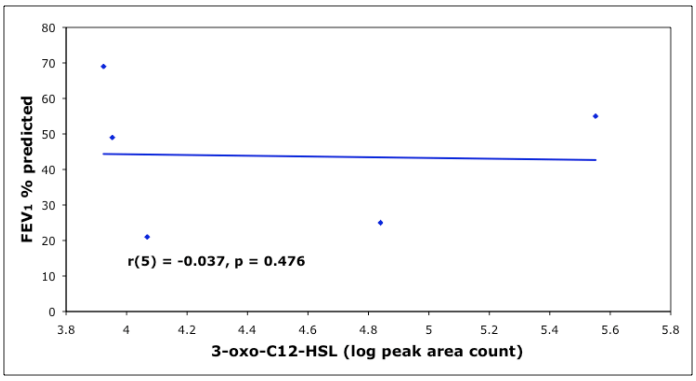
The null hypothesis (H_0) was that the data were not correlated and the alternative hypothesis (H_a) was that the data were negatively correlated (ie that a fall in FEV_1 was

associated with a rise in signal molecule level). The Pearson's correlation coefficient (r) was determined using Microsoft excel and for a one-tailed test with a cut-off p value of 0.05, none of the sample correlation coefficients reached the minimum values needed in order to be deemed significant. However a trend for a negative association between C4-HSL and FEV_1 was noted.

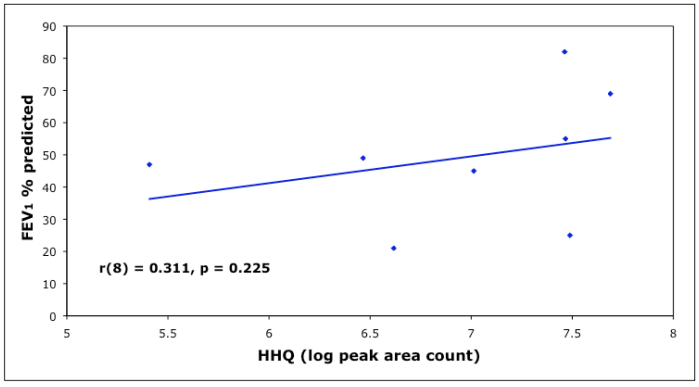
(A)



(B)



(C)



(D)

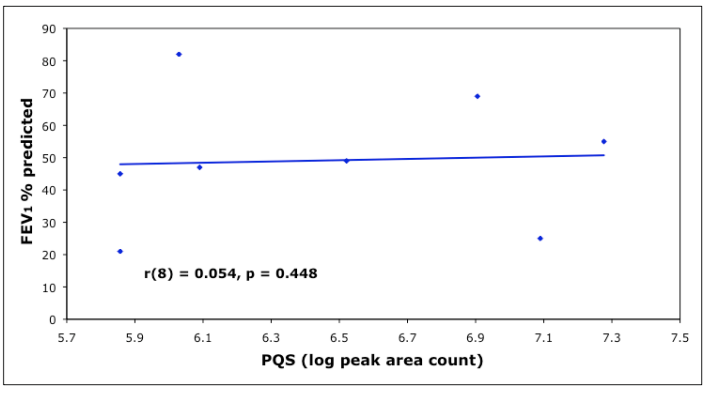


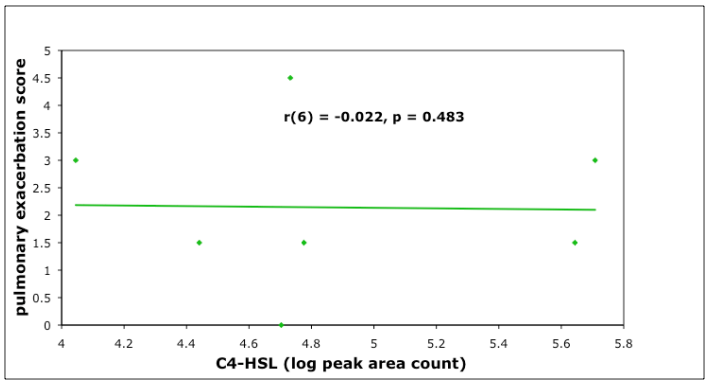
Figure 4-3 Correlation between FEV_1 % predicted and each of the major QS signal molecules recovered from sputum (A) C4-HSL (B) 3-oxo-C12-HSL (C) HHQ (D) PQS.
Data plotted represent logarithmically transformed peak area counts from the first sputum sample provided by each patient against the corresponding FEV_1 % predicted.

4.2.5 Cross-sectional association between pulmonary exacerbation score and sputum QS signal molecules

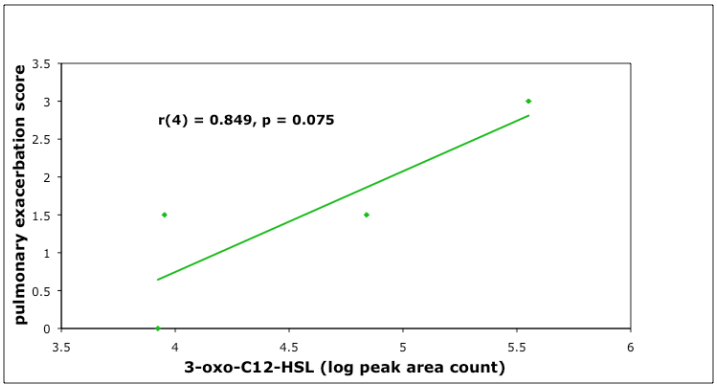
Pulmonary exacerbation score is a clinical outcome measure, which serves to reflect acute changes in pulmonary status over weeks. Based on a scoring system devised by Rosenfeld *et al*. (2001), which assesses for the presence of clinical features of an exacerbation, all patients were assigned a numerical score (see Appendix 3). Figure 4-4 depicts scatterplots of each major QS signal molecule from the first sputum sample provided by each patient against their corresponding pulmonary exacerbation score.

In this instance, the H_0 was the data were not correlated and the H_a was that the data were correlated (ie that a rise in pulmonary exacerbation score was associated with a rise in signal molecule level). Again, r values represent the Pearson's correlation coefficient and using a one-tailed test with a cut-off of 0.05, none of the sample correlation coefficients reached the minimum values needed in order to be significant. Despite this it was interesting to note that the trend of the data suggested a positive association between 3-oxo-C12-HSL and pulmonary exacerbation score, with a p value (0.075) approaching significance.

(A)



(B)



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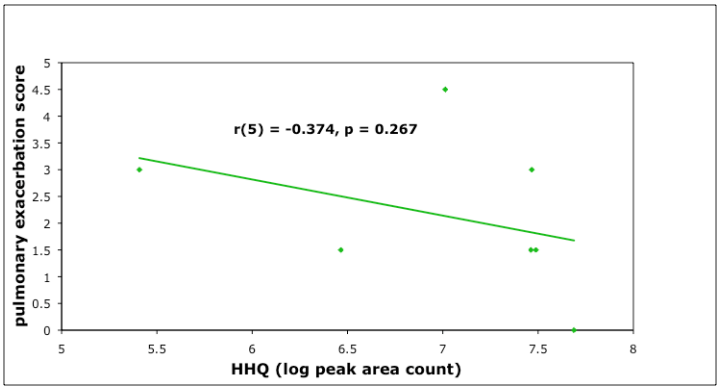

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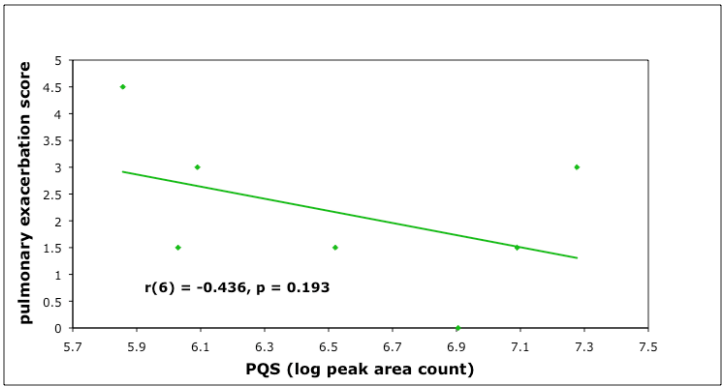


Figure 4-4 Correlation between pulmonary exacerbation score and each of the major QS signal molecules recovered from sputum (A) C4-HSL (B) 3-oxo-C12-HSL (C) HHQ (D) PQS.

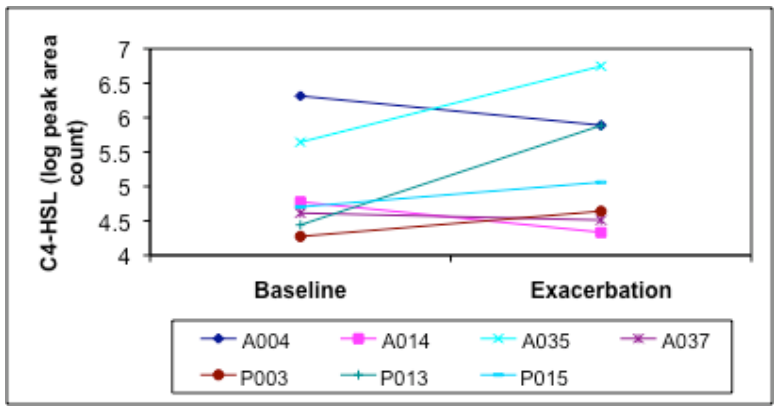
Data plotted represent logarithmically transformed peak area counts from the first sputum sample provided by each patient against the corresponding pulmonary exacerbation score based on Rosenfeld *et al*. (2001).

4.2.6 Longitudinal association between QS signal molecule level and pulmonary exacerbation

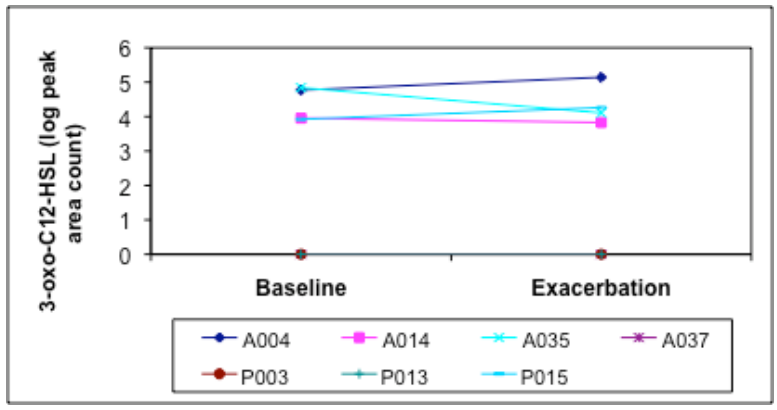
In order to examine the relationship between patient clinical status and sputum QS signal molecule level over time, two-point comparisons of sputum baseline signal levels with those at the time of an exacerbation were plotted (Figure 4-5). For this within patient comparison, baseline was taken as the first sputum sample collected and an exacerbation was defined as the first time that the pulmonary exacerbation score rose above 2.6. If the first sample had a score above 2.6, the next sample for which the score had dropped (ie lung function had stabilised) was taken as the baseline.

For certain patients there was a rise in the level of signal molecule at the time of an exacerbation. For example, an increase in C4-HSL, HHQ and PQS was seen for patient P013. However this trend was not observed for all patients and for all signal molecules.

(A)



(B)



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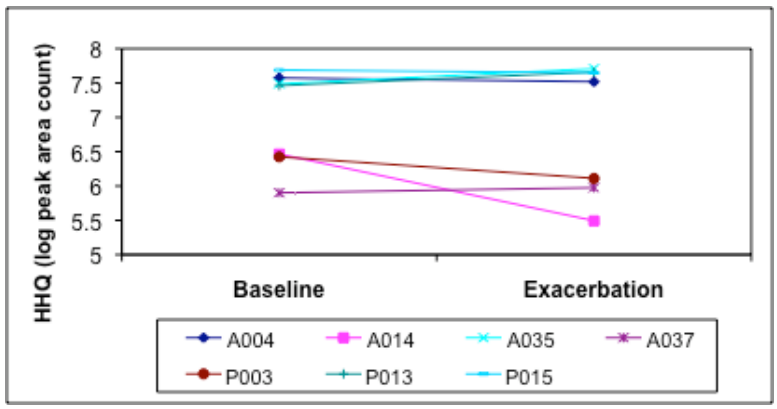

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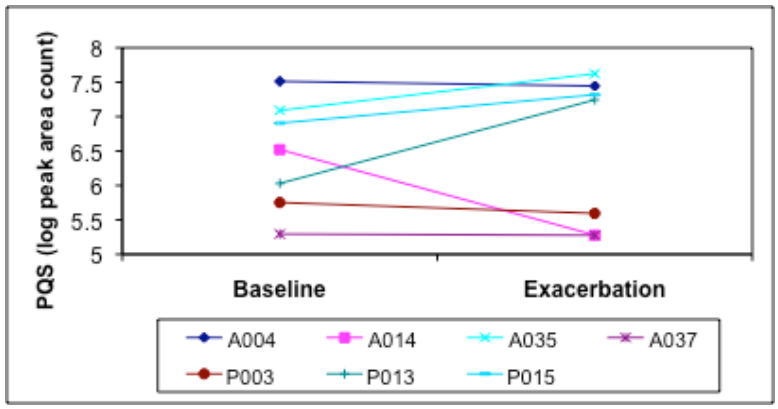


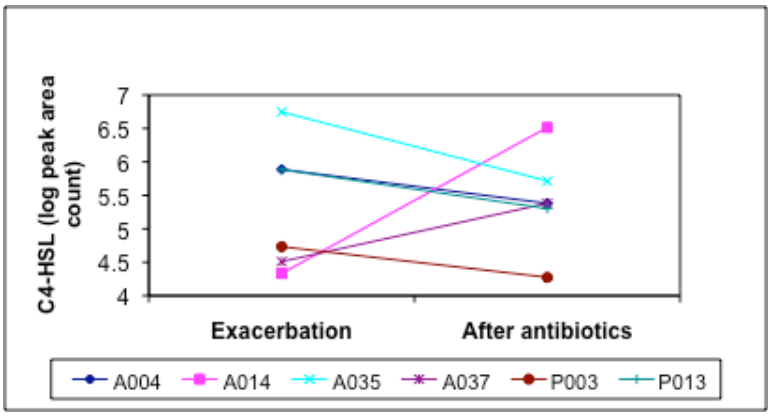
Figure 4-5 The change in sputum QS signal molecule peak area count during a pulmonary exacerbation (A) C4-HSL (B) 3-oxo-C12-HSL (C) HHQ (D) PQS.
Baseline corresponds to the first sputum sample with a pulmonary exacerbation score below 2.6 and exacerbation is the next sample for which the score has risen above 2.6.

4.2.7 The effect of IV antibiotic treatment on sputum QS molecules

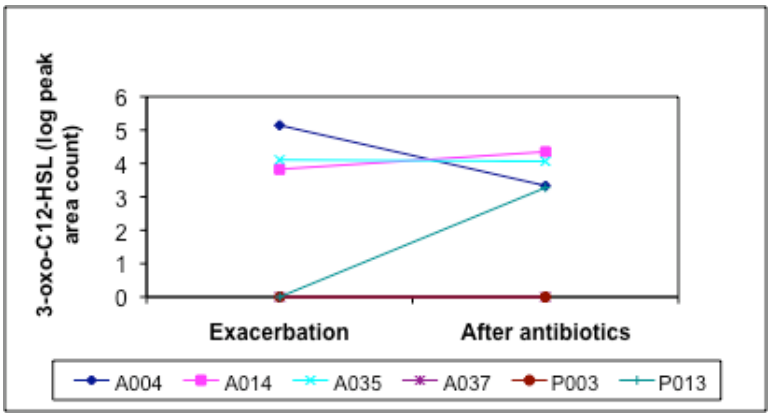
To examine the effect of IV antibiotic treatment on sputum QS molecule levels, the change in levels from a period of pulmonary exacerbation (pulmonary exacerbation score >2.6) compared to a sequential sample following a course of IV antibiotic treatment was examined. See Figure 4-6.

For patient P013, in whom a rise in C4-HSL, HHQ and PQS was previously seen in association with a pulmonary exacerbation, there was a corresponding fall in the levels of these signal molecules following treatment with IV antibiotics. However, as before, this trend was not seen consistently for all patients.

(A)



(B)



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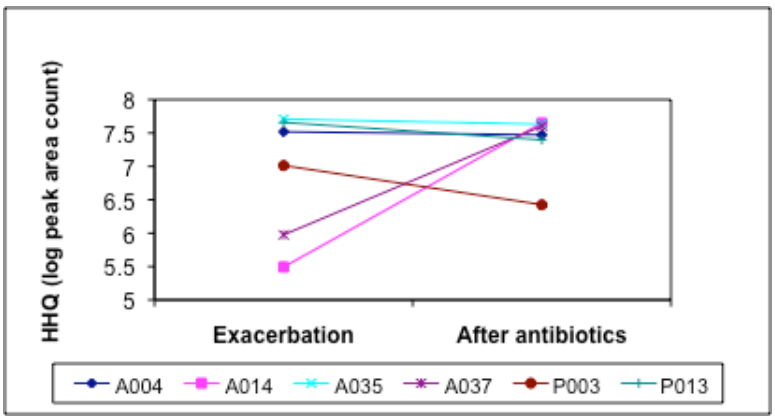

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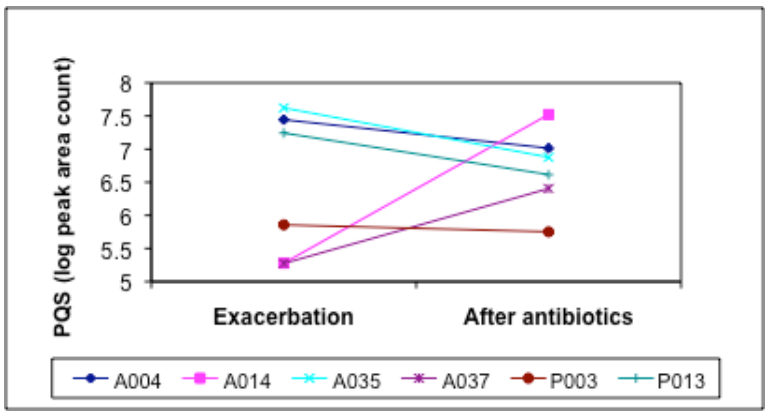


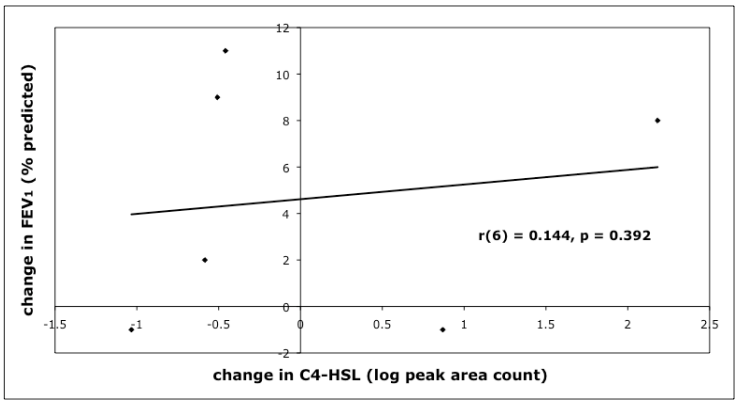
Figure 4-6 The change in sputum QS signal molecule peak area count following an exacerbation treated with IV antibiotics (A) C4-HSL (B) 3-oxo-C12-HSL (C) HHQ (D) PQS.

4.2.8 Change in FEV_1 versus change in QS signal molecule after IV antibiotics

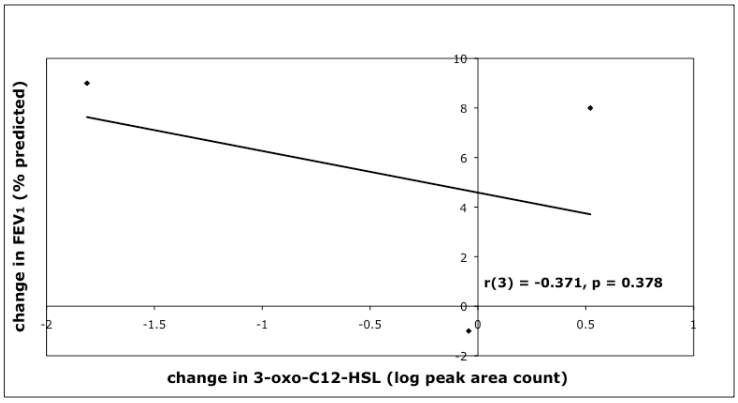
The difference in FEV_1 between the time of an exacerbation (defined as the first time the score was > 2.6) and following a course of IV antibiotics was compared to the difference in sputum QS signal molecule peak area counts for the same time-points across all patients (Figure 4-7).

It was hypothesised that an improvement in FEV_1 following IV antibiotics would be associated with a fall in QS signal molecule level (ie a negative correlation). Using a one-tailed test with a cut-off of 0.05, none of the Pearson's correlation coefficient r values were significant, a likely reflection of the small sample size of this preliminary study. Yet in a similar fashion to the data presented in Figure 4-3 and Figure 4-4, C4-HSL showed a trend to a positive correlation, suggesting a return to a chronic disease state. In contrast, 3-oxo-C12-HSL demonstrated a negative correlation, implying that it may indicate the resolution of an acute disease state. This data is very intriguing and certainly merits further work.

(A)



(B)



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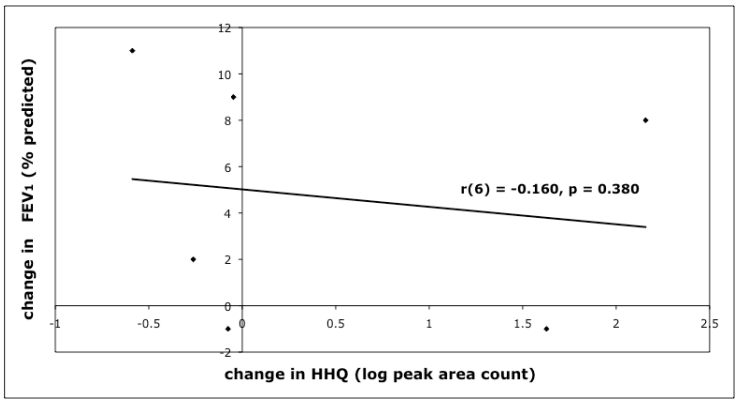


(D)

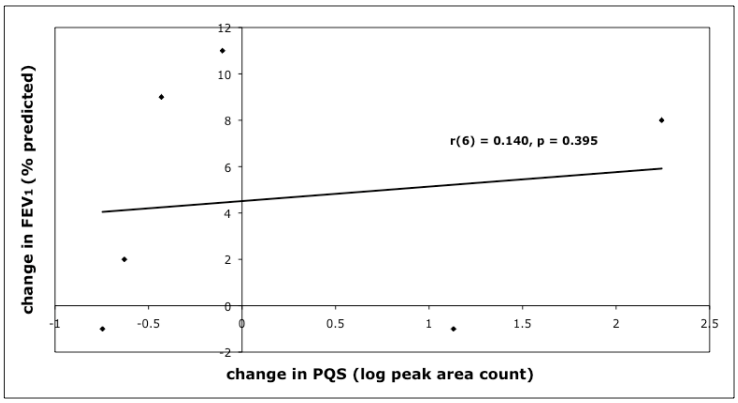
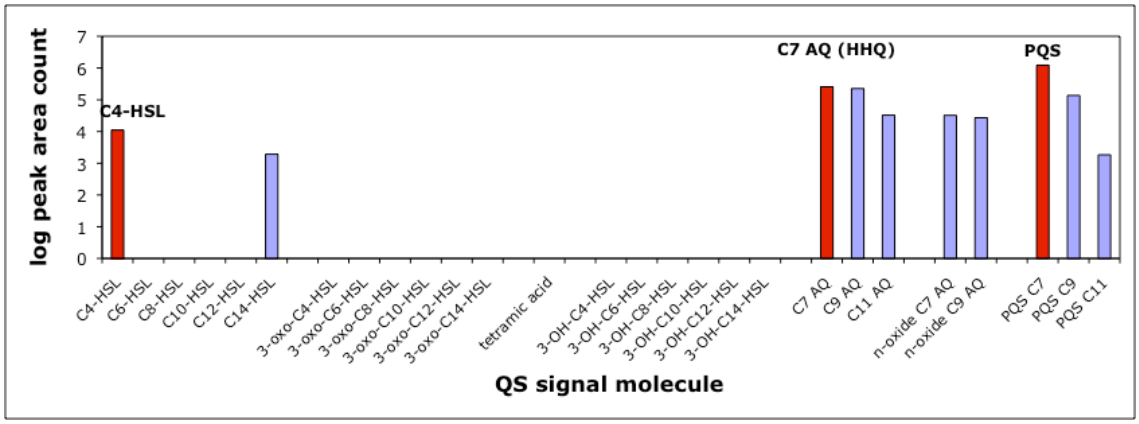


Figure 4-7 Change in FEV_1 versus change in sputum QS signal molecule peak area count (A) C4-HSL (B) 3-oxo-C12-HSL (C) HHQ (D) PQS.
The difference in FEV_1 % predicted from the time of an exacerbation (first score > 2.6) and following IV antibiotics was compared to the corresponding change in sputum QS molecule logarithmically transformed peak area count.

4.2.9 The diagnostic potential of sputum QS signal molecules

All ten patients included in the study were known to have established *P. aeruginosa* lung infection. The routine clinical microbiological culture of corresponding sputum samples collected at the same time as the study samples usually recovered one or more strains of *P. aeruginosa*, distinguished by differences in mucoidy and antibiotic sensitivity pattern. Of interest were the two samples provided by paediatric patient P025. LC-MS analysis of these extracted samples revealed a paucity of QS signal molecules and in sample P025-010306, there was failure to recover PQS. Routine clinical culture of the corresponding sputum samples detected the presence of *S. aureus* but no strains of *P. aeruginosa*, suggesting that the recovery of these signal molecules could be used to indicate the presence of non-cultured organism. See Figure 4-8.

(A)



(B)

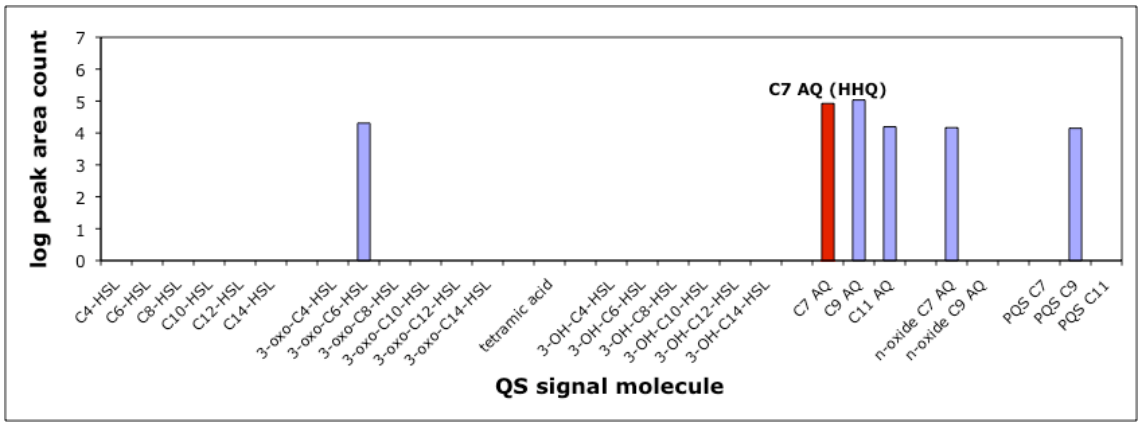


Figure 4-8 Detection of QS signal molecules in individual sputum samples provided by patients P025 (A) Sample P025-161104 (B) Sample P025-010306.
(A) Sample P025-161104, provided in November 2004, contains C4-HSL, HHQ and PQS, with 3-oxo-C12-HSL below the level of detection. (B) In sample P025-010306 (obtained in March 2006) of the key signal molecules, only HHQ is detected above the threshold level. Routine clinical culture of corresponding samples indicated the presence of *S. aureus* in each sample but no *P. aeruginosa*.

4.3 Discussion

Thus far, data have been presented which demonstrated that a range of *P. aeruginosa*-specific QS signal molecules was detected from individually extracted sputum samples from CF patients by LC-MS, corresponding to micromolar quantities. The biological relevance of these molecules for the pathogenesis of this organism is well documented and undisputed. In addition to their major role as regulators of virulence factor production, they may mediate bacterial interspecies communication, termed 'crosstalk' (Riedel *et al.*, 2001). In addition, they may function as virulence determinants in their own right, with effects on other prokaryotes as well as eukaryotic host tissue itself. In order to assess their clinical relevance as sputum biomarkers of infection and tissue damage, their relationship with other accepted clinical outcome measures was examined.

When applied to laboratory synthetic standards this method of solvent extraction and LC-MS analysis detected a full range of *P. aeruginosa*-relevant AHLs and AQs at concentrations of 0.1 μM and above. Differences in relative response were observed, with higher detection of the AQs compared to the AHLs. The analysis of individually extracted patient sputum samples indicated that whilst C4-HSL, HHQ and PQS were recovered in over 98 % of the cohort of 53 samples, the major signalling molecule 3-oxo-C12-HSL was only detected in 53 %.

There are a number of possible reasons for the failure to detect 3-oxo-C12-HSL. As discussed in the previous chapter, the analysis of *P. aeruginosa* clinical isolates recovered from the CF lung indicates that a high proportion is deficient in 3-oxo-C12-HSL production. It is also known that QS signal molecules can be degraded by the process of pH-dependent lactonolysis (Yates *et al.*, 2002). The pH of CF sputum is reported to range from 5.9 to 7 (Perricone *et al.*, 2000) and some AHL hydrolysis would be expected to occur under these conditions. In addition, the action of AHL-degrading enzymes such as the paraoxonase (PON) enzymes, which are produced by human airway epithelial cells, has been reported (Chun *et al.*, 2004). Indeed, in this analysis, the detection of the open ring forms of both C4-HSL (36 % samples) and 3-oxo-C12-HSL (70 % samples) was evidence for the occurrence of these physiological processes *in vivo*.

A further reason for the failure to detect 3-oxo-C12-HSL from sputum could be due to its breakdown into tetramic acid. This nonenzymatically formed compound was detected in 23 % of samples and together with 3-oxo-C12-HSL itself, has been reported to have potent bactericidal activity against Gram-positive strains (Kaufmann *et al.*, 2005). In the complex environment of the CF lung, this could represent a virulence strategy to out-compete other key pathogens such as *S. aureus*.

An important reason for the failure to detect 3-oxo-C12-HSL in just over half the samples could relate to its role in pathogenesis with the failure to detect low levels being the consequence of low production on the part of the organism. Previously, Singh *et al.* (2000) reported that planktonic cells produce more 3-oxo-C12-HSL than C4-HSL and that for biofilm organisms, the ratio is reversed. Importantly, they found that sputum samples from patients with CF produced higher rates of C4-HSL and took this as evidence for the occurrence of *P. aeruginosa* biofilms in the CF lung. In addition, previous reports have indicated that C4-HSL is important during the maturation stage of biofilm development (Sauer *et al.*, 2002), for the total amount of biofilm formed (Favre-Bonte *et al.*, 2003) and for the maintenance of biofilm architecture (Davey *et al.*, 2003).

The cross-sectional analysis of FEV_1 and sputum QS molecules did not yield any significant correlations, yet it was interesting to note that the trend of the data suggested a negative association between C4-HSL and FEV_1. Conversely, whilst no significant correlations were observed for the association between pulmonary exacerbation score and signal molecule level, a possible positive correlation for 3-oxo-C12-HSL could exist. Hence, if biofilms represent a chronic infectious strategy leading to chronic lung decline, a high sputum C4-HSL level could represent severe disease progression secondary to biofilm maturation and maintenance. In contrast, a high 3-oxo-C12-HSL could be due to planktonic organisms leaving the biofilm and causing an acute exacerbation in symptoms.

Whilst there is no conclusive evidence from this study to support these theories, such an application of sputum QS signal molecule detection could have major implications for planning treatment strategies specifically aimed at bacterial virulence strategies. For example, high dose, potent IV antibiotics could be administered at times of high sputum 3-oxo-C12-HSL in an attempt to target planktonic phase organisms. However such a strategy may not be effective at times of high sputum C4-HSL when there may be a need for novel anti-biofilm measures instead.

As serial sputum samples were collected for each patient, a longitudinal analysis was undertaken to investigate the change in sputum signal molecule level with clinical events such as the occurrence of a clinical exacerbation or the effect of a treatment course of IV antibiotics. Such an approach yields information on the sensitivity and specificity of changes in signal molecule levels to changes in clinical status or treatment effects. It was apparent that for some patients, a rise in signal molecule from baseline did occur at the time of an exacerbation and a fall was observed following treatment. For example, for paediatric patient P013, there was a rise in the levels of C4-HSL, HHQ and PQS with an exacerbation and a fall in these molecules following a course of IV antibiotics. However this pattern was not observed consistently for all patients for whom data was available.

Despite this, it is worth considering the potential clinical use of monitoring sputum QS molecules in this manner. Very much like serum CRP and WCC, the change in profile in sputum signal molecule levels could be used in conjunction with clinical symptoms and signs, as well as other biomarkers, to guide the initiation and termination of treatment courses. Such practice could help to rationalise antibiotic use and reduce such economic and patient burdens as cost of agent, cost of administration and the incidence of side-effects. There would also be the wider health impact of reducing the selective pressure for the rise in antibiotic resistance, a particular problem surrounding the treatment of *P. aeruginosa*.

The very fact that these QS signal molecules are synthesised by the infecting organisms themselves raises the question of their utility as a diagnostic test of infection with *P. aeruginosa*. Indeed, for patient P025, *S. aureus* was recovered from sputum by traditional laboratory methods, including Gram-stain and culture. It was very interesting to observe that a paucity of *P. aeruginosa* signal molecules was recovered from corresponding samples. In this scenario, was the presence of some, but not a full spectrum of, QS signal molecules (eg C4-HSL, HHQ and PQS in sample P025-161104) an indicator of the presence of *P. aeruginosa* that was not detected on routine culture? Or in fact, was the failure to detect some key *P. aeruginosa* QS signal molecules (eg lack of C4-HSL, 3-oxo-C12-HSL and PQS in sample P025-010306) due to the fact that indeed, this organism was not present in the lungs of this patient. It is very unlikely that this latter suggestion is true as all patients enrolled in the study were known to have established lung infection with *P. aeruginosa*.

However, the application of this potential diagnostic use of QS molecules may be more relevant in patients who are not yet chronically colonised with *P. aeruginosa*. Indeed, Ward *et al*. (2003) have reported the detection of AHLs in human lung allograft recipients even in the absence of apparent infection. Indeed, PQS would be very relevant in this context as to date, it has only been shown to be made by *P. aeruginosa*. Hence, the detection of *P. aeruginosa*-specific QS signal molecules could be used as an early sensitive and specific indicator of the presence of the organism before it is detected by traditional methods. This would allow the rapid initiation of targeted and individualised therapies aimed at eradication, before established infection can occur.

This study is limited by its small sample size and the retrospective nature of the clinical data collection. Despite the lack of conclusive outcomes, this preliminary analysis has provided evidence to support the application of sputum *P. aeruginosa* QS signal molecules as novel biomarkers of lung infection in CF, with key insights into organism-specific pathogenic mechanisms. Clearly a much larger clinical trial with scheduled clinical data collection is needed to validate this role.

This study has provided a rationale for focusing on four key signal molecules: C4-HSL, 3-oxo-C12-HSL, HHQ and PQS. Using the report of Sagel *et al*. (2007) as a guide, it is clear that more information is needed on their short- and long-term variability in conjunction with data on sputum bacterial density and the QS profile of individually isolated strains. This needs to be performed in a large number of CF subjects of varying ages who are characterised in terms of age, sex, genotype, modifier genes, lung function, lung structure and the presence of other microbial pathogens. The association between sputum QS signal molecules and other clinical endpoints such as quality of life or patient-reported symptoms needs exploring. In addition, given the complex relationship between airway infection and inflammation, their role as members of a panel of biomarkers of lung injury should be considered.

The reproducibility of these measures in terms of their variability and repeatability needs investigation. This raises the issue of the nature, collection and analysis of sputum as a source of biomarkers. A reason for the inconsistency between the detection levels of QS signal molecules and patient clinical status could be the variation in bacterial load between different sputum samples. This could be addressed by correcting the signal molecule level for the number of bacterial colony forming units per volume of sputum. Whilst non-invasively and inexpensively obtained, sputum is not produced

by all subjects, particularly children and may only reflect pathogenic activity in pockets of the lung. Methods of collection, storage and processing may vary between centres. Whilst the adoption of standardised approaches may partly address this, the analysis of other sources of lung material (eg induced sputum, BAL fluid, even saliva) and clinical specimens that may reflect more systemic processes, such as serum and urine, should be considered.

The feasibility of the current approach is an important factor. LC-MS is an expensive resource necessitating dedicated expertise. For the purposes of this study, this method has provided direct chemical confirmation of the presence of QS signal molecules in sputum. However, in terms of the future clinical application of biomarker technology, less resource-demanding methods such as the use of sensitive and specific biosensor strains in a high-throughput microtitre plate format should be considered. These currently exist for a number of molecules including the AHLs (Winson *et al.*, 1998) and PQS/HHQ (Fletcher *et al.*, 2007).

In addition to the many potential uses discussed, there are a number of important and exciting future applications of QS signal molecules as biomarkers of *P. aeruginosa* lung infection and tissue damage. A recent report has high-lighted the significance of this organism in chronic obstructive pulmonary disease (Murphy *et al.*, 2008), a debilitating respiratory condition that affects approximately 5-15 % of adults in industrialised countries (Anto *et al.*, 2001). Other applications outside the CF community include more acute clinical settings such as ventilator-associated pneumonia, community acquired pneumonia and infections in the immunocompromised host.

Finally, the relentless rise of resistance to antibiotics in many species of bacteria, an inevitable evolutionary consequence of the huge selective pressure exerted by conventional antibiotics, necessitates alternative therapeutic strategies. QS inhibitor therapy is a novel and promising route, which aims to disable pathogenic behaviour rather than destroy the organism and therefore does not promote the acquisition and spread of resistance. Cleary, the detection of QS signal molecules in host tissues will allow the screening and evaluation of future therapies in terms of their mechanism of action and the response to treatment. They will also have a role in patient stratification and could serve as surrogate endpoints in clinical trials of novel therapies. Such future

approaches include specifically targeting bacterial biofilms and will be considered in the following chapter.

Chapter 5: The contribution of lectins to biofilm maturation

5.1 Introduction

This study of the molecular mechanisms governing the pathogenesis of *P. aeruginosa* in the CF lung has focused on QS, with the demonstration that clinical isolates produced QS signal molecules and that their detection in sputum indicated a role as biomarkers of lung infection and damage. Furthermore, the conservation of the QS-dependent gene *lecA* and consequent production of the virulence determinant LecA amongst these clinical isolates, suggested a role for this lectin in the pathogenesis of *P. aeruginosa in vivo* in the CF lung.

This takes on particular significance when considering that QS has been implicated in biofilm formation (Davies *et al.*, 1998), a major virulence strategy employed by *P. aeruginosa* in the CF lung and that exopolysaccharides are considered to be key components of the biofilm matrix. Lectins, as a special class of multivalent carbohydrate-binding proteins could potentially mediate the binding of bacterial cells to each other (cohesion), the anchoring of biofilms to the substratum (adhesion) as well as cross-linking components of the inert biofilm scaffold via site-specific binding to exopolysaccharides.

In addition to LecA, *P. aeruginosa* synthesises a second lectin, LecB (Gilboa-Garber *et al.*, 1977). The production of both lectins is regulated by the RhlR/C4-HSL QS system (Winzer *et al.*, 2000). LecA is growth phase-dependent and is also subject to regulation by other factors, including the stationary phase sigma factor RpoS, the transcriptional regulator MvaT and the post-transcriptional regulator RsmA (Diggle *et al.*, 2002) (Winzer *et al.*, 2000) (Pessi *et al.*, 2001). The expression of *lecA* is also positively influenced the presence of PQS (Diggle *et al.*, 2003).

Both lectins are multivalent tetramers showing carbohydrate specificity and affinity. LecA (51 kDa) is composed of four subunits of 121 amino acids and binds D-galactose plus its derivatives (Gilboa-Garber *et al.*, 1972), whereas LecB (47 kDa) is composed of

four subunits of 114 amino acids and binds L-fucose and other monosaccharides (Gilboa-Garber *et al.*, 2000). Examination of this capacity for specific glycan recognition in biofilm formation has been undertaken in separate studies, which have focused on each lectin individually. Diggle *et al.* reported that a *lecA* negative mutant (PAO1 *lecA*::*lux*) formed biofilms with reduced depth and surface coverage compared to the parent strain PAO1 and a LecA-overproducing strain PAO-P47 (Diggle *et al.*, 2006b). Biofilm assays were performed in polystyrene microtitre plate wells and on stainless steel coupons under static conditions and laminar flow. In addition, Tielker and colleagues (Tielker *et al.*, 2005) showed that a *P. aeruginosa lecB* mutant was impaired in biofilm formation on glass slides under static conditions when compared to the wild-type strain.

A focus of recent research into *P. aeruginosa* biofilm development and pathogenesis has been the nature of the biofilm matrix. Whilst known to consist of protein, polysaccharide and nucleic acid, a more complete understanding of its composition has been advocated since this matrix represents an important interface between the organism and host tissues (Ryder *et al.*, 2007). Past investigation of the polysaccharide components of this matrix concentrated on the role of alginate, giving rise to the mucoid phenotype (Davies and Geesey, 1995). However the demonstration that alginate is not essential for *P. aeruginosa* biofilm development (Nivens *et al.*, 2001) led to the identification of two alternative polysaccharide loci, namely *pel* and *psl* (Friedman and Kolter, 2004b). The *psl* locus is an operon composed of 15 genes encoding the Psl biosynthetic machinery. Carbohydrate and lectin staining analyses indicate that Psl is a mannose and galactose-rich polysaccharide, yet its full structure is yet to be elucidated (Ma *et al.*, 2007). Using an inducible *psl* construct it was found that in addition to being required for cell-surface and cell-cell interactions, *psl* is also needed for maintenance of the biofilm structure post attachment, suggesting a role as a biofilm scaffold (Ma *et al.*, 2006). In addition, the demonstration that *psl* expression is localised at the centre of developing microcolonies implies a role in biofilm differentiation (Overhage *et al.*, 2005).

The very clear aim of characterising lectin-carbohydrate interactions within *P. aeruginosa* biofilms is to identify novel targets for therapies aimed at tackling this problematic disease state, which persists in the CF lung in the face of aggressive antimicrobial therapy and a robust immune response. Indeed, lectins by their very

nature lend themselves to site-specific inhibition and hence biofilm disruption. Two aspects of this include the inhibition of lectins to prevent biofilm formation and the inhibition of lectins to disperse pre-formed established biofilms. This was illustrated by Diggle *et al*. (2006) who demonstrated the effect of hydrophobic galactosides against LecA. As proof of principle for this novel therapeutic strategy, there is one case report in the literature of inhalation therapy consisting of a combination of tobramycin, fucose and galactose which was used to successfully treat a *P. aeruginosa* respiratory infection where tobramycin alone had been unsuccessful (von Bismarck *et al.*, 2001).

The search for suitable lectin-inhibitory ligands has been furthered by organic chemistry techniques, reflecting the collaborative and multidisciplinary approach to CF research. Glycopeptide dendrimers are polymers with a regular branched structure, which can be synthesised with targeted physiochemical or biological properties. A new approach to dendrimer property tuning based on the combinatorial variation of dendrimer branch length and multivalency has identified ligands which bind to LecB (Johansson, 2007). Additional work is underway to identify suitable targets for LecA and together, these structures are a promising lead for the development of novel antibiofilm agents against *P. aeruginosa*.

Hence, this final results chapter utilises the flowchamber biofilm system and other biofilm assays to further explore the role of lectins in biofilm formation in laboratory strains and clinical isolates. Experiments were undertaken to define the LecA putative target ligand and the action of novel anti-LecB dendrimers to prevent and disperse biofilms was examined.

5.2 Results

5.2.1 Investigation of the role of LecA in biofilm development by laboratory strains in the flowchamber biofilm system

The 2006 report by Diggle *et al*. proposed a role for LecA in biofilm maturation rather than in the initial attachment to the substratum based on simple laboratory biofilm assays. To further explore and define this role more fully, a flowchamber biofilm system was established, modified and utilised as described in detail in Appendix 4. This closed, continuously flowing system is widely regarded as the gold-standard laboratory biofilm model and is applicable to direct live, non-destructive high power microscopy.

Hence, the ability of the parental wildtype strain PAO1 to form biofilms in the flowchamber system was compared to that of a *lecA* deletion mutant PAO1Δ*lecA* (Stacey, 2003). All test strains were fluorescently labelled by transformation with the plasmid pUCP18::gfpmut3.1. Incubation of planktonic cells in a microtitre plate assay at 37 ºC demonstrated comparable growth rates and maximal cell densities, indicating that any differences in biofilm formation were not due to a general growth defect. See Figure 5-1 for growth curves of PAO1 and PAO1Δ*lecA* over 24 h.

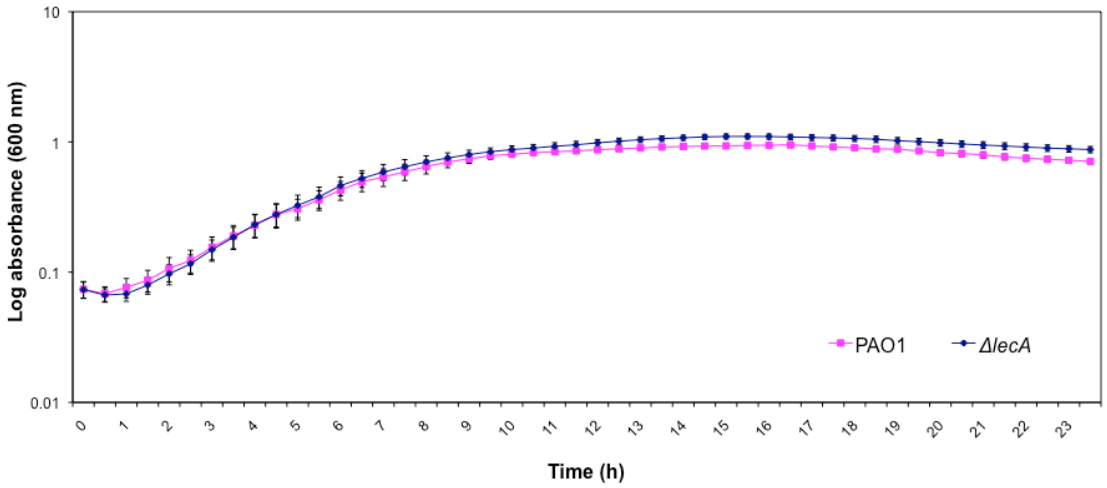


Figure 5-1 Comparison of growth of fluorescently labelled *P. aeruginosa* strains PAO1 and PAO1Δ*lecA*.
Growth of normalised cultures in a 96 well plate over 24 h at 37 °C was measured in a spectrophotometer. Mean value ± SEM of 20 independent readings plotted.

Strains were then grown at 37 °C within separate channels of a flowchamber biofilm system in replicate (4 channels per strain). The biofilms, which formed on the glass coverslip substrata, were imaged on days 4, 5 and 6 of incubation. The pooled confocal data from 3 independent experimental rounds was analysed using the COMSTAT programme to yield mean biofilm quantitative parameters. Both the wild type PAO1 and *lecA* mutant strains formed surface-attached biofilms, which increased in their mean biomass and mean average and maximum thickness over subsequent incubation days. By day 4, the wildtype had established biofilms around 12 µm deep with additional growth of around 5 µm in thickness each day thereafter. In contrast, the *lecA* mutant formed significantly smaller biomass, thinner biofilms in comparison to the wild type ($p < 0.05$). An exception was the mean maximum biofilm thickness on day 5 incubation in

which the difference between the wildtype (35.5 μm) and the *lecA* mutant (31.3 μm) had an associated p value of 0.098. No clear trend of difference in biofilm heterogeneity, quantified by the dimensionless roughness coefficient, was detected between the two strains. See Table 5-1 and Figure 5-2 for COMSTAT quantitative comparative biofilm data for PAO1 and PAO1Δ*lecA*.

Table 5-1 COMSTAT Quantitative analysis of 4-, 5- and 6-day old biofilms formed by PAO1 and PAO1Δ*lecA*.
Each strain was inoculated in 4 separate flow channels and 5 image stacks were collected per channel. Mean ± SEM of 3 independent experimental rounds plotted.

Biofilm parameter	Day	Strain		P value
		PAO1	PAO1Δ*lecA*	
Biomass ($\mu m^3/\mu m^2$)	4	12.42 ± 0.70	9.93 ± 0.49	0.0047
	5	14.31 ± 0.57	9.75 ± 0.57	< 0.0001
	6	17.81 ± 0.90	11.76 ± 0.55	< 0.0001
Average thickness (µm)	4	12.27 ± 0.78	9.52 ± 0.53	0.0047
	5	15.42 ± 0.63	10.37 ± 0.70	< 0.0001
	6	21.13 ± 1.09	13.07 ± 0.80	< 0.0001
Maximum thickness (µm)	4	32.26 ± 1.28	26.70 ± 1.47	0.0051
	5	35.53 ± 1.24	31.59 ± 1.91	0.098
	6	45.81 ± 1.61	34.46 ± 2.01	< 0.0001
Roughness coefficient	4	0.39 ± 0.02	0.38 ± 0.02	0.79
	5	0.31 ± 0.02	0.48 ± 0.04	0.0007
	6	0.32 ± 0.02	0.37 ± 0.02	0.072

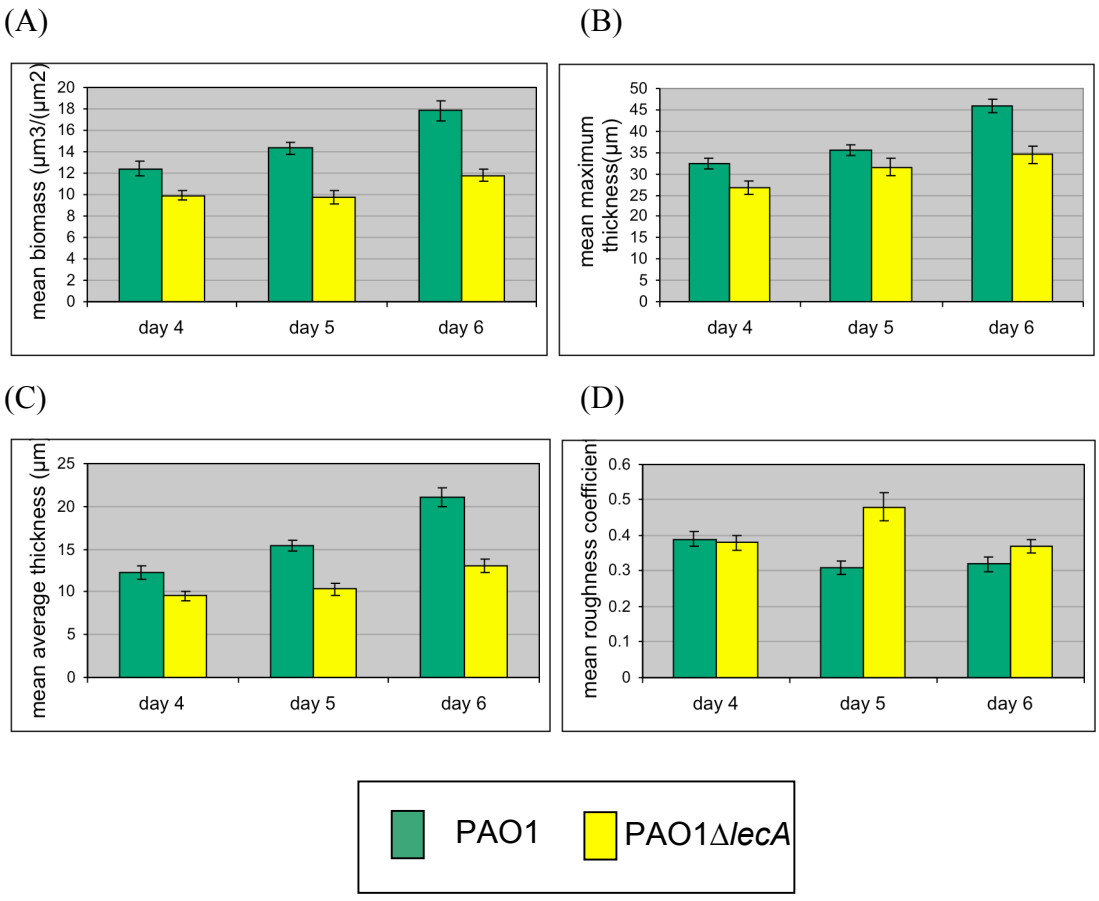


Figure 5-2 Comparison of PAO1 and PAO1Δ*lecA* biofilm formation.
(A) Biomass (B) Maximum thickness (C) Average thickness (D) Roughness coefficient. Mean values ± SEM are plotted for each biofilm parameter over successive incubation days.

Representative CLSM generated z-stack data for each strain were further subjected to IMARIS image rendering to produce a visual representation of the 3D biofilm structures formed. See Figure 5-3 and Figure 5-4. The wildtype PAO1 formed differentiated biofilms with evident mound-like microcolonies in this system. In contrast and bearing out the COMSTAT generated data, the *lecA* mutant strain formed flatter, thinner biofilms, with less pronounced topographical variation.

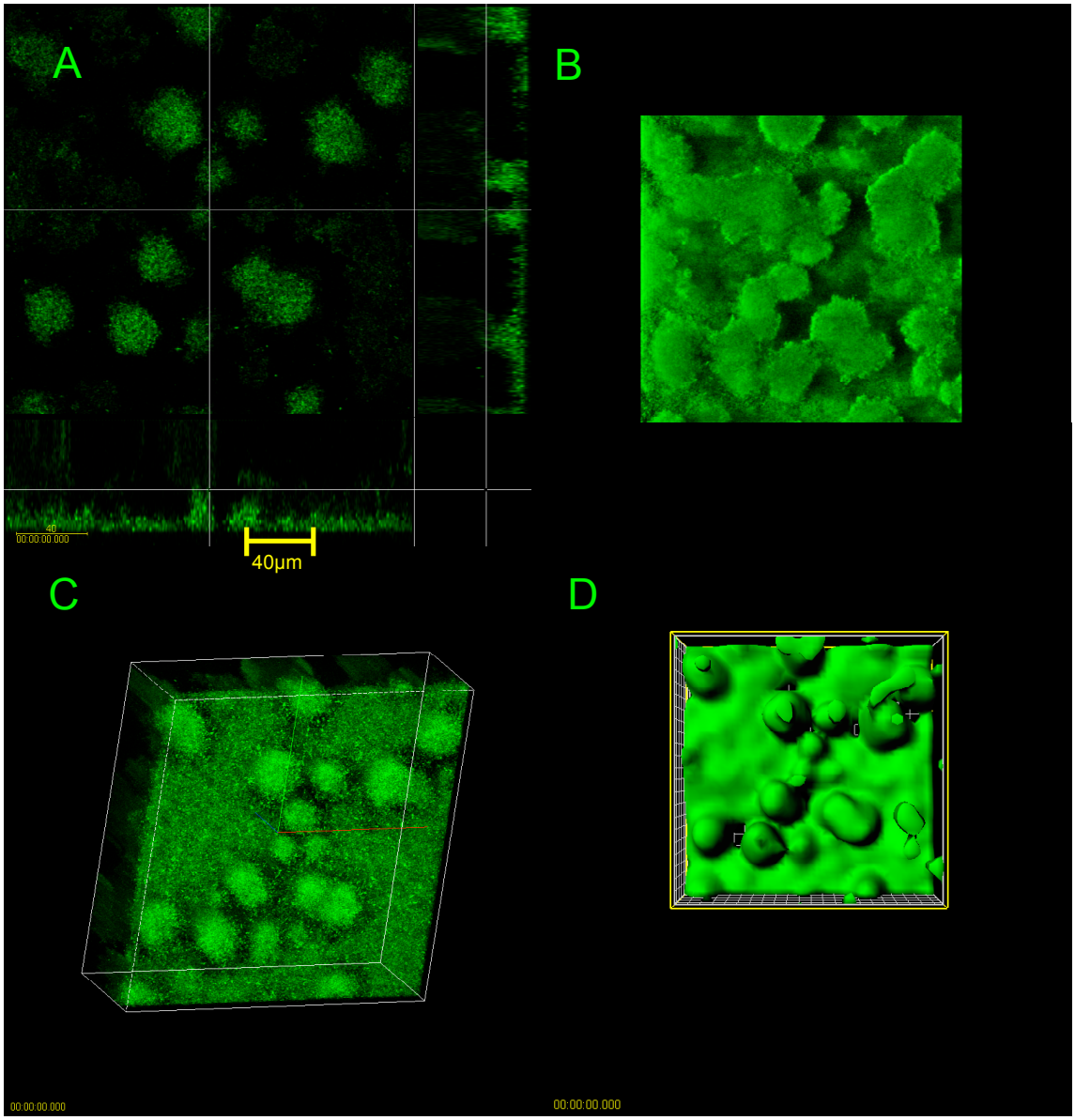


Figure 5-3 Representative IMARIS rendered images of 4-day old biofilm formed by PAO1.

(A) xy section through biofilm with side panels depicting microcolony profiles through z
(B) 3D rendered image viewed from above
(C) Full 3D rendered image
(D) 'Isosurface' image, with space-filling and surface smoothing

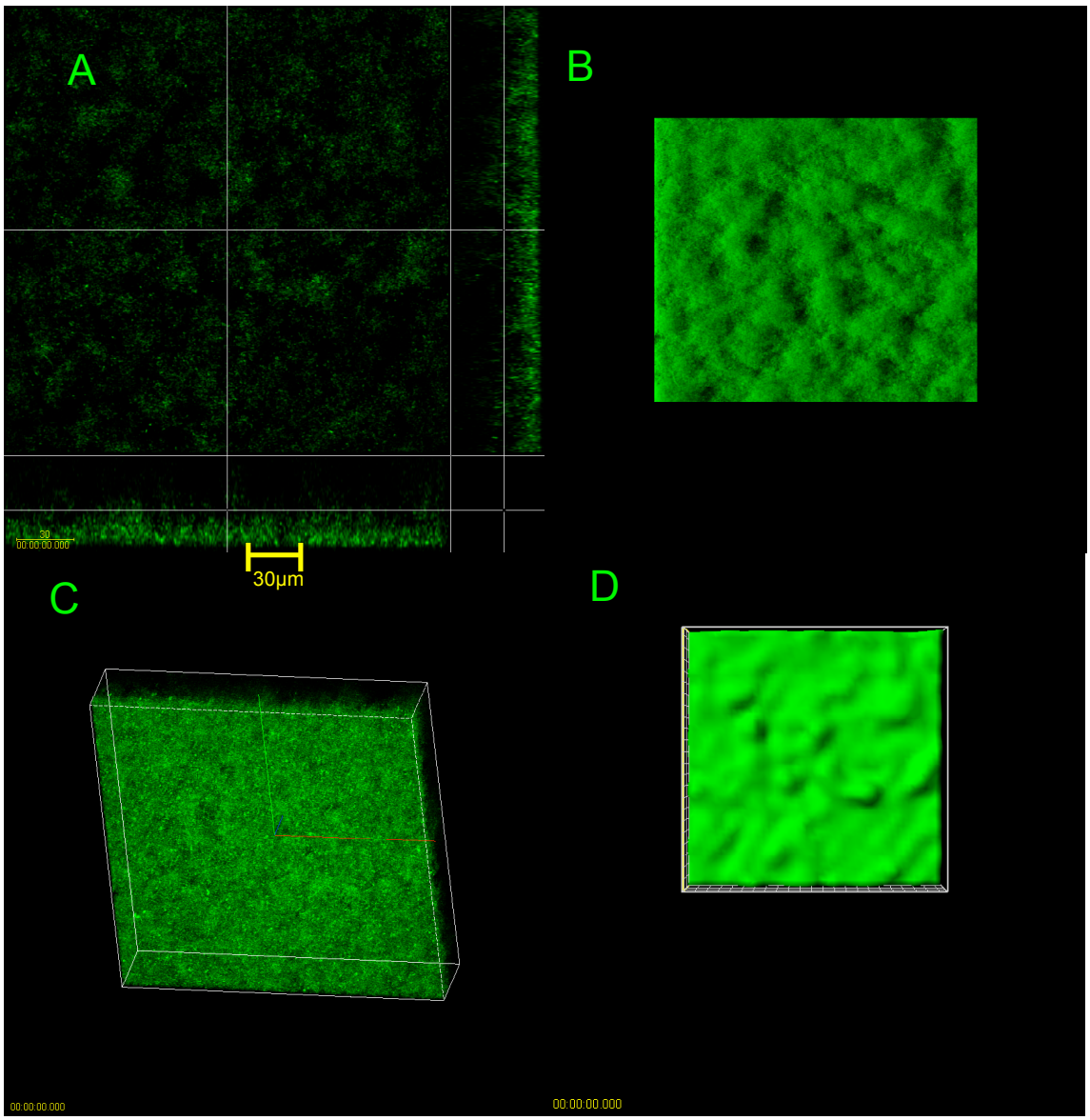


Figure 5-4 Representative IMARIS rendered images of 4-day old biofilm formed by PAO1Δ*lecA*.

(A) xy section through biofilm with side panels depicting microcolony profiles through z
(B) 3D rendered image viewed from above
(C) Full 3D rendered image
(D) 'Isosurface' image, with space-filling and surface smoothing

5.2.2 The effect of IPTG on flowchamber biofilm development by laboratory strains

Planktonic growth of *P. aeruginosa* PAO1 is known to be unaffected by the presence of isopropyl-β-D-thiogalactoside (IPTG), a hydrophobic galactoside with a strong affinity for LecA (Diggle *et al*., 2006b). This was confirmed by investigating the growth of planktonic PAO1 cultures in the presence of 0.2 mM IPTG in a microtitre plate assay (Figure 5-5).

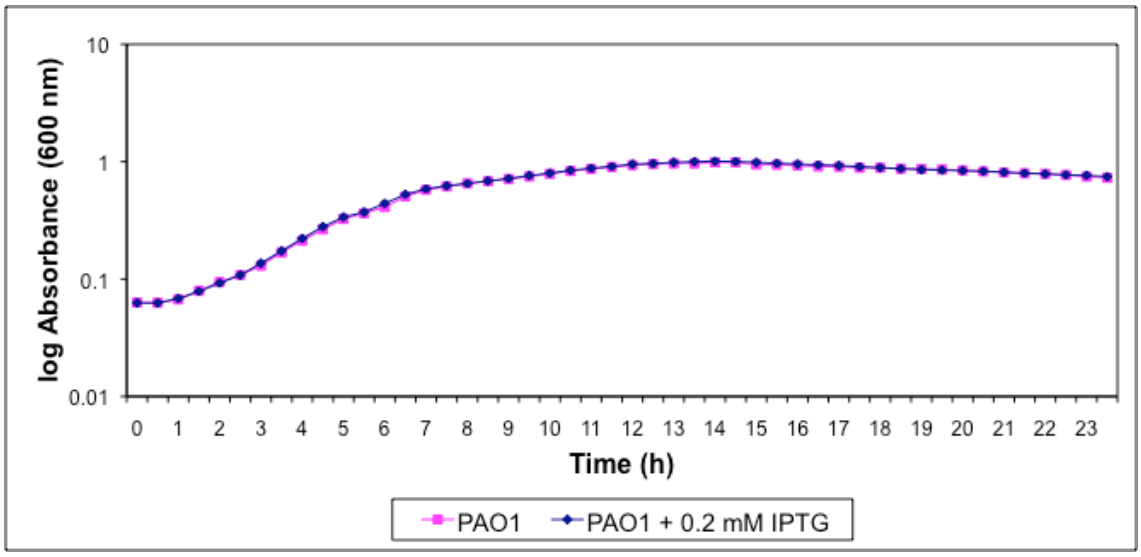


Figure 5-5 Growth of *P. aeruginosa* strain PAO1 in the presence and absence of 0.2 mM IPTG.
Growth of normalised cultures ± IPTG at a final concentration of 0.2 mM over 24 h at 37 °C was performed in a 96 well plate and measured in a spectrophotometer. Mean value ± SEM of 6 independent readings plotted.

The effect of IPTG on biofilm formation by PAO1 and PAO1Δ*lecA* was investigated in the flowchamber biofilm system. The addition of 0.2 mM IPTG to the culture media prior to incubation resulted in the formation of biofilms of similar biomass and thickness by the wild type and mutant strains. Indeed, the previously observed statistically significant differences between PAO1 and PAO1Δ*lecA* were abolished in the presence of IPTG, with all p values being greater than 0.05. As before, no consistent trend for the difference in biofilm heterogeneity was observed. See Table 5-2 and Figure 5-6.

Table 5-2 COMSTAT quantitative analysis of 4-, 5- and 6-day old biofilms formed by PAO1 and PAO1Δ*lecA* in the presence of 0.2 mM IPTG.
Biofilms were grown in media containing 0.2 mM IPTG from the outset. Each strain was inoculated in 4 separate flow channels and 5 image stacks were collected per channel. Mean ± SEM of 3 independent experimental rounds plotted.

Biofilm parameter	Day	Strain		P value
		PAO1	PAO1Δ*lecA*	
Biomass ($\mu m^3/\mu m^2$)	4	13.01 ± 0.76	13.79 ± 0.65	0.44
	5	14.73 ± 0.83	16.27 ± 1.07	0.14
	6	12.89 ± 0.65	12.33 ± 0.75	0.58
Average thickness (µm)	4	12.92 ± 0.93	13.83 ± 0.75	0.45
	5	14.62 ± 0.97	16.95 ± 1.33	0.16
	6	13.37 ± 1.08	13.18 ± 0.92	0.89
Maximum thickness (µm)	4	25.87 ± 1.37	26.77 ± 1.40	0.65
	5	29.87 ± 1.62	33.39 ± 1.94	0.17
	6	26.39 ± 1.83	31.09 ± 1.96	0.085
Roughness coefficient	4	0.37 ± 0.03	0.32 ± 0.01	0.32
	5	0.30 ± 0.02	0.35 ± 0.02	0.098
	6	0.27 ± 0.01	0.39 ± 0.02	0.0004

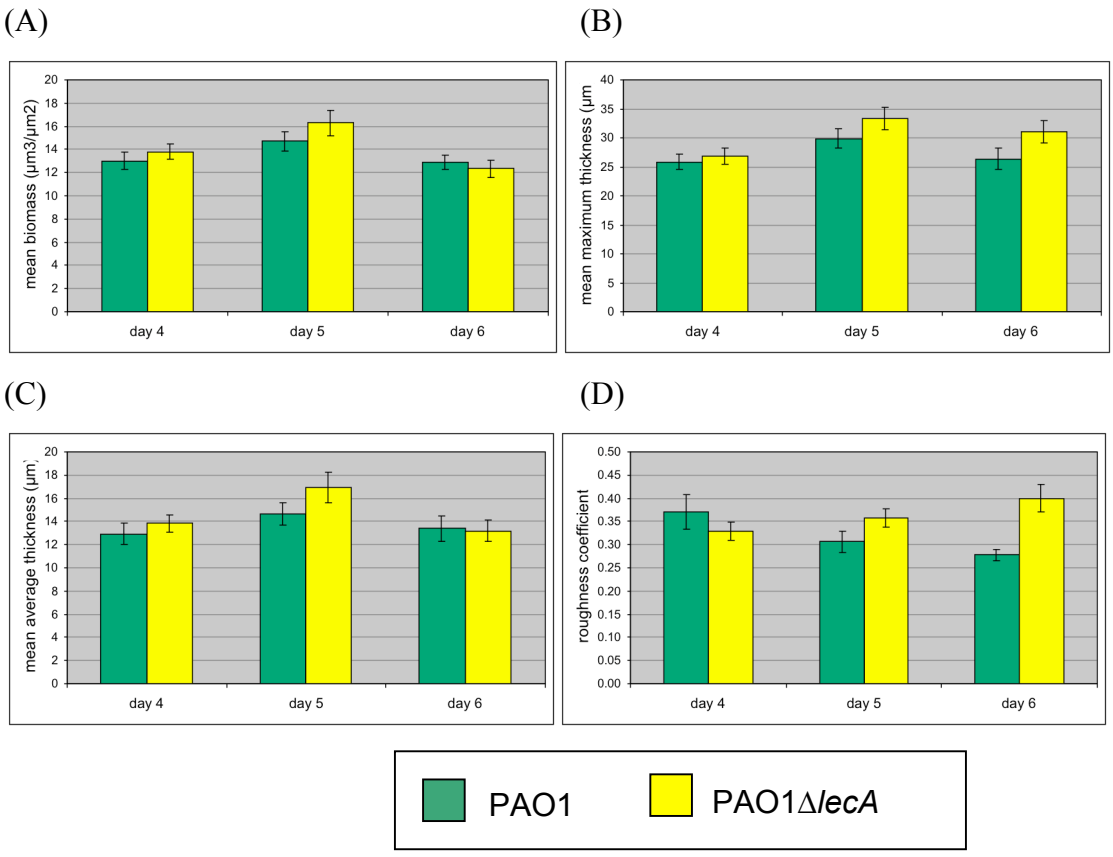


Figure 5-6 Comparison of PAO1 and PAO1Δ*lecA* biofilms grown in the presence of 0.2 mM IPTG.
(A) Biomass (B) Maximum thickness (C) Average thickness (D) Roughness coefficient. Mean values ± SEM are plotted for each biofilm parameter over successive incubation days.

To specifically assess the effect of IPTG on biofilm formation in the wildtype strain, PAO1 flowchamber biofilms were grown in separate channels in the presence and absence of 0.2 mM IPTG in replicate in two experimental rounds. In accordance with the data presented thus far, statistically significant reductions in mean biofilm biomass, average and maximum thickness was seen in the presence of IPTG over days 4 and 5 incubation ($p < 0.05$). No significant difference was seen for biofilm heterogeneity. See Table 5-3 and Figure 5-7.

Table 5-3 COMSTAT Quantitative analysis of 4-, 5- and 6-day old biofilms formed by PAO1 with and without 0.2 mM IPTG.
Biofilms were grown in media ± IPTG at a final concentration of 0.2 mM from the outset. The test strain was inoculated in 2 separate flow channels per test condition and 5 image stacks were collected per channel. Mean ± SEM of 2 independent experimental rounds plotted.

Biofilm parameter	Day	Strain		P value
		PAO1	PAO1 + 0.2 mM IPTG	
Biomass (µm3/µm2)	4	11.96 ± 0.47	9.77 ± 0.63	0.0087
	5	15.45 ± 0.61	10.86 ± 0.95	0.0002
Average thickness (µm)	4	13.36 ± 0.71	10.51 ± 0.99	0.025
	5	16.32 ± 0.63	11.51 ± 1.20	0.0011
Maximum thickness (µm)	4	32.49 ± 1.90	21.28 ± 1.53	< 0.0001
	5	35.84 ± 1.59	23.31 ± 1.73	< 0.0001
Roughness coefficient	4	0.29 ± 0.02	0.30 ± 0.02	0.72
	5	0.30 ± 0.01	0.32 ± 0.04	0.61

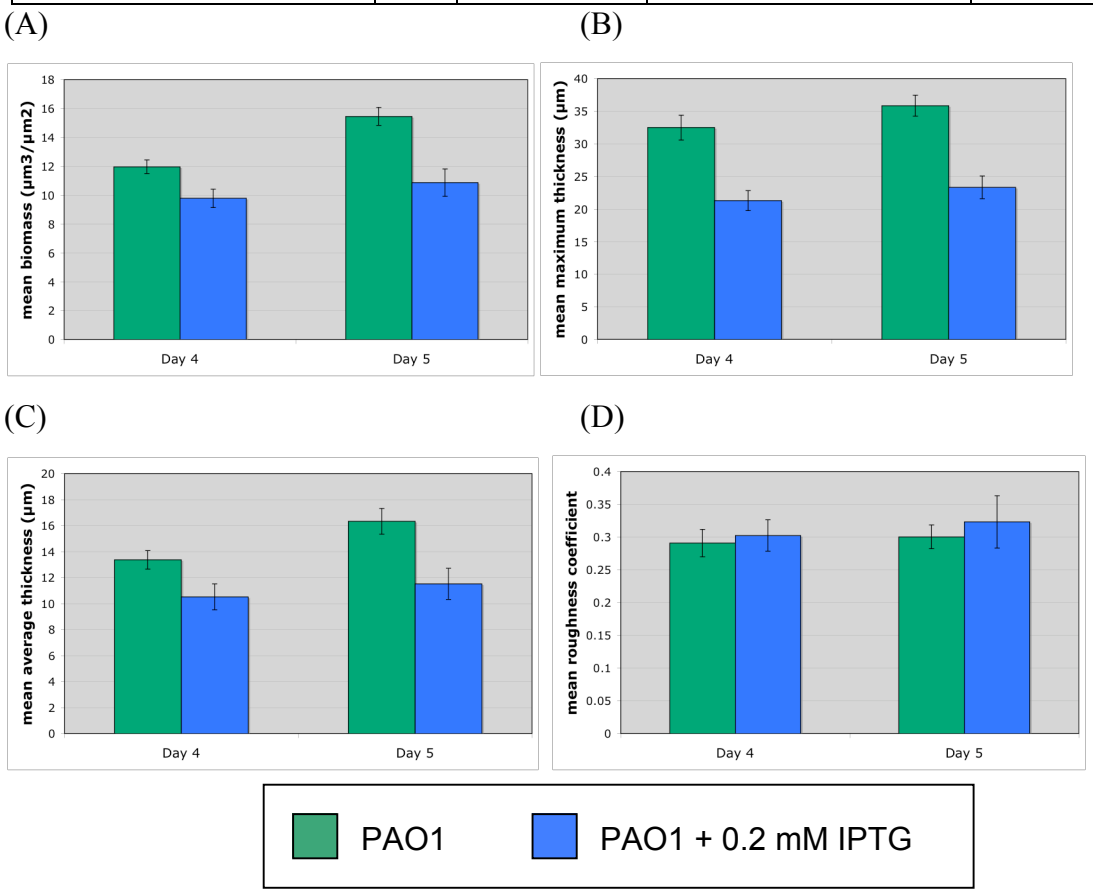


Figure 5-7 Comparison of PAO1 biofilms grown in the presence and absence of 0.2 mM IPTG.
(A) Biomass (B) Maximum thickness (C) Average thickness (D) Roughness coefficient. Mean values ± SEM are plotted for each biofilm parameter over successive incubation days.

5.2.3 Investigation of biofilm development by clinical isolates differing in their LecA status

Given the demonstration of a role for LecA in biofilm development and the ability of the LecA-specific galactoside IPTG to inhibit biofilm formation, three clinical isolates with differing LecA status were identified in Chapter 3 and selected for examination of their biofilm formation in different laboratory biofilm systems in order to determine whether LecA had a role in a clinical context. Table 5-4 details their phenotype and QS profiles. Isolates with sensitivity to carbenicillin were chosen to enable transformation with the plasmid pUCP18::GFPmut3.1. In addition, isolates possessing a full complement of QS signal molecules were chosen so that any differences in biofilm formation could be attributed to differences in lectin status rather than signalling.

Table 5-4 Characteristics of clinical isolates selected for examination in the flowchamber biofilm system

Clinical isolate	Mucoid/ Nonmucoid	Pigment	*lecA*	LecA	C4	C12	HHQ	PQS
A035-051104A	mucoid	Pink	y	y	y	y	y	y
A021-101204A	nonmucoid	Green	y	y++	y	y	y	y
A014-291004	nonmucoid	pink	n	n	y	y	y	y

Isolate A035-051104A was recovered together with a lectin-producing nonmucoid isolate from sputum, whilst A021-101204A was recovered with a nonmucoid 3-oxo-C12-HSL-deficient strain. A014-291004 was isolated on its own.

The examination of the planktonic growth of these clinically derived isolates demonstrated variations in growth rate and the maximum cell density reached, with isolate A035-051104A showing a slower growth rate and A021-101204A reaching a higher maximum cell density compared to the laboratory strain PAO1 (Figure 5-8). These features were of important consideration when interpreting the differences in biofilm formation between the isolates.

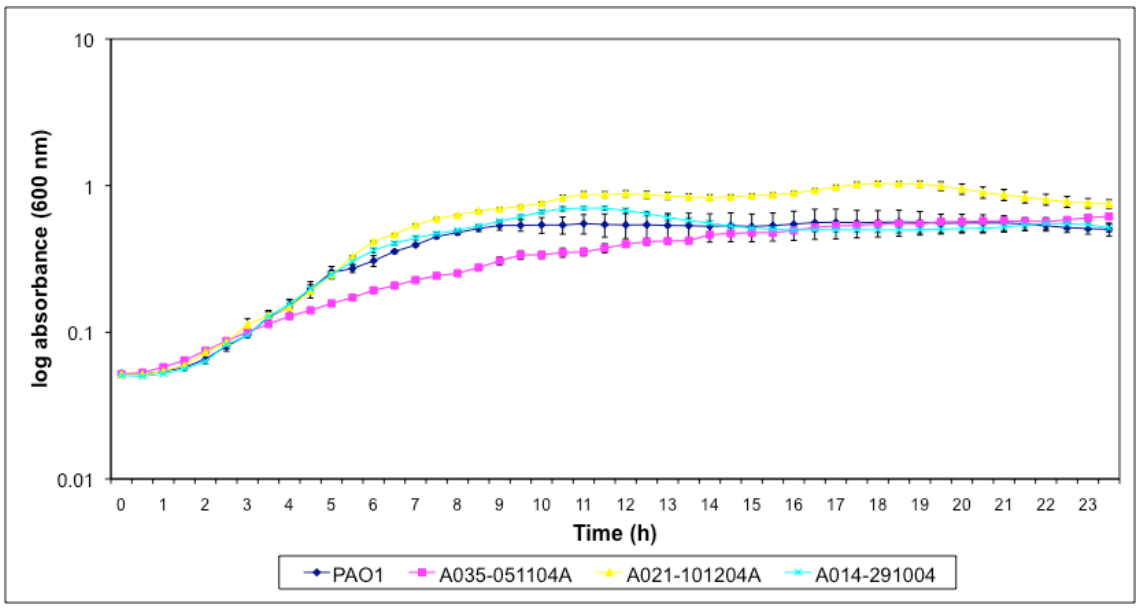
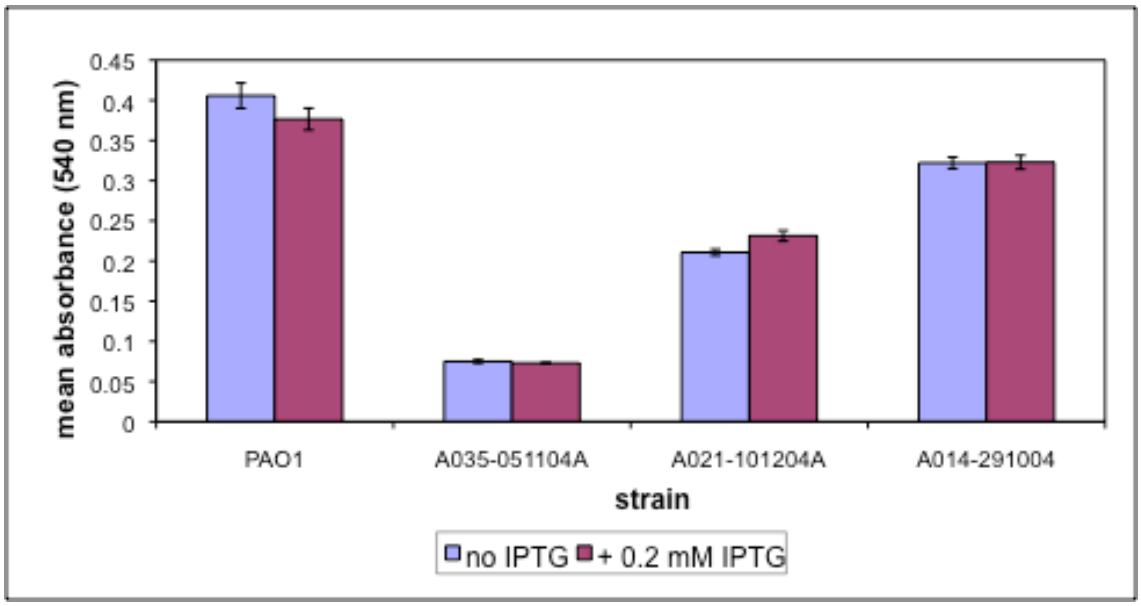


Figure 5-8 Comparison of the planktonic growth of clinical isolates with *P. aeruginosa* strain PAO1.
The growth of normalised cultures over 24 h at 37 °C in a 96 well plate was measured in a spectrophotometer. Mean value ± SEM of 5 independent readings plotted.

The biofilm forming ability of these CF clinical isolates differing in their LecA-status was initially investigated in the microtitre well attachment model (see 2.19.2) and the steel coupon biofilm (2.19.3) assay. Both assays indicated that all three clinical isolates formed biofilms in association with a polystyrene or steel surface. However, the degree of biofilm formation was reduced compared to PAO1 and differed between the isolates, with A035-051104A (LecA +) demonstrating reduced biofilm formation in comparison to A021-101204A (LecA ++) and A014-291004 (LecA -).

There was no significant effect of IPTG on the ability of these isolates to adhere to polystyrene microtitre wells. However, in the steel coupon model, the biofilms formed by PAO1 and A021-101204A were highly significantly reduced in the presence of 0.2 mM IPTG ($p < 0.0001$). The reduction seen for A014-291004 just reached significance with an associated p value of 0.042. See Figure 5-9 and Figure 5-10.

(A)



(B)

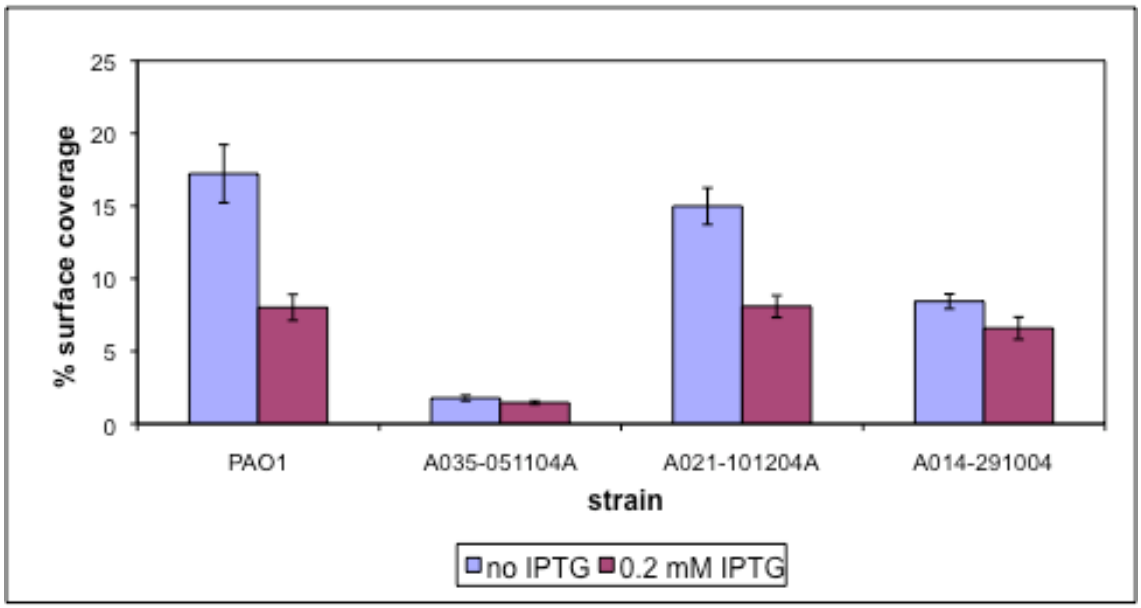


Figure 5-9 Clinical isolate biofilm formation in (A) microtitre well and (B) steel coupon biofilm assays.
(A) Overnight cultures were normalised and grown under static conditions in round bottom wells of a 96 well plate for 24 h at 37 °C. The wells were washed and their contents stained with crystal violet. This was resolubilised and measured in an ELISA plate reader at OD_{540}. Mean value ± SEM of 10 independent readings plotted.
(B) Overnight cultures were normalised and grown in 0.05 % NB ± IPTG at a final concentration of 0.2 mM in Petri dishes containing steel coupons. Following incubation at 37 °C for 40 h with agitation at 60 rpm, the coupons were rinsed and stained with acridine orange. The % surface coverage for each strain was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

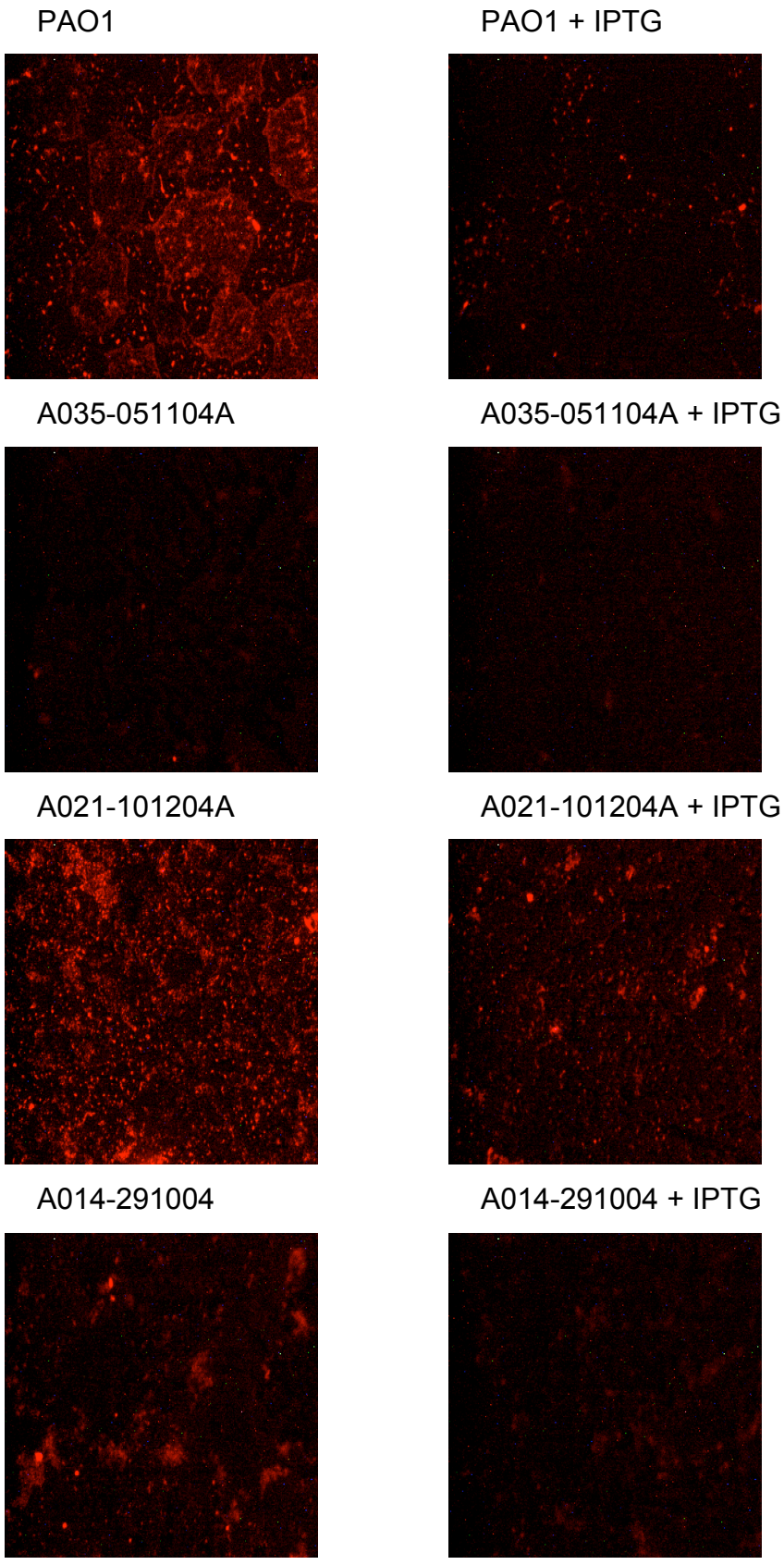
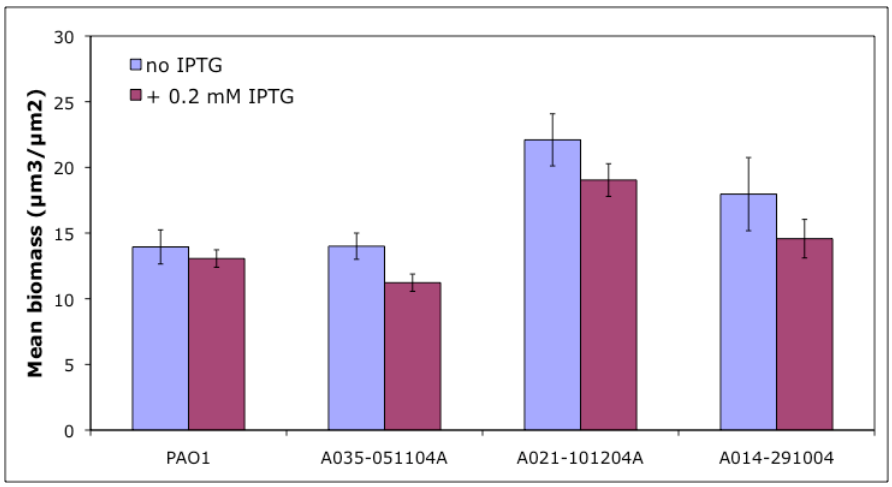


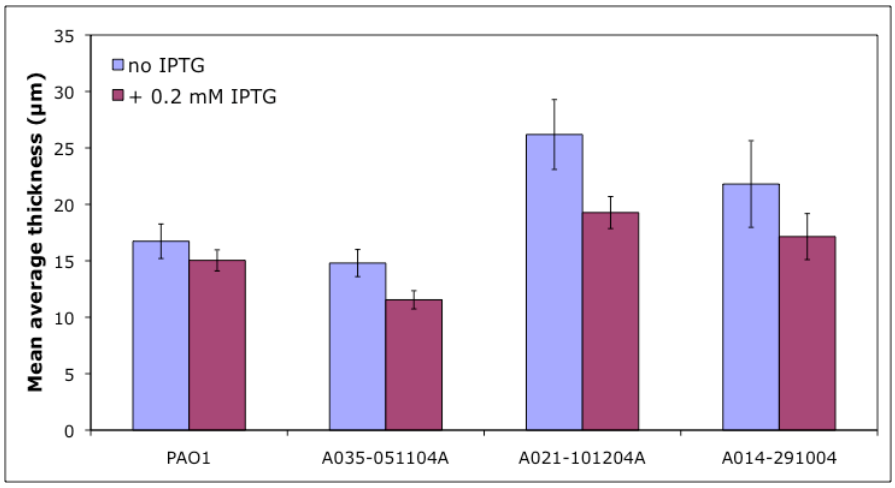
Figure 5-10 Representative images of acridine orange stained biofilms grown in the presence and absence of 0.2 mM IPTG.
Images were captured with the Lucia G/Comet software in conjunction with an inverted fluorescent microscope using the x10 objective lens.

To investigate biofilm formation in the flowchamber system, these clinical isolates were transformed with the plasmid pUCP18::GFPmut3.1 and grown in separate flowchamber channels in the presence or absence of 0.2 mM IPTG from the outset. See Figure 5-11 for day 4 COMSTAT-generated data and Figure 5-12 for IMARIS biofilm reconstructions.

(A)



(B)



(C)

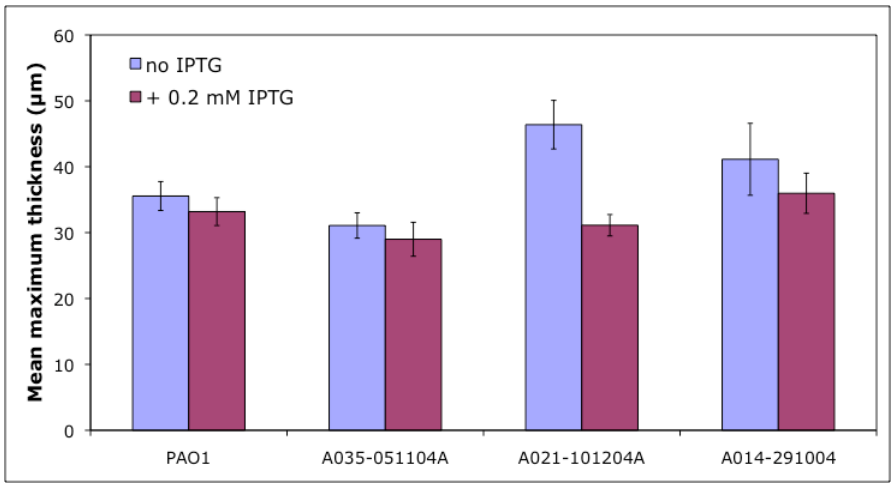


Figure 5-11 Comparison of day 4 flowchamber biofilm formation by fluorescently labelled PAO1 and clinical isolates in the presence and absence of 0.2 mM IPTG (A) Biomass (B) Average thickness (C) Maximum thickness.
The analysis was conducted using the COMSTAT programme. Biofilms were grown in media ± IPTG at a final concentration of 0.2 mM from the outset. Each strain was inoculated in 1 flowchannel per test condition and 9 image stacks were collected per channel. Mean ± SEM of 3 independent experimental rounds plotted.

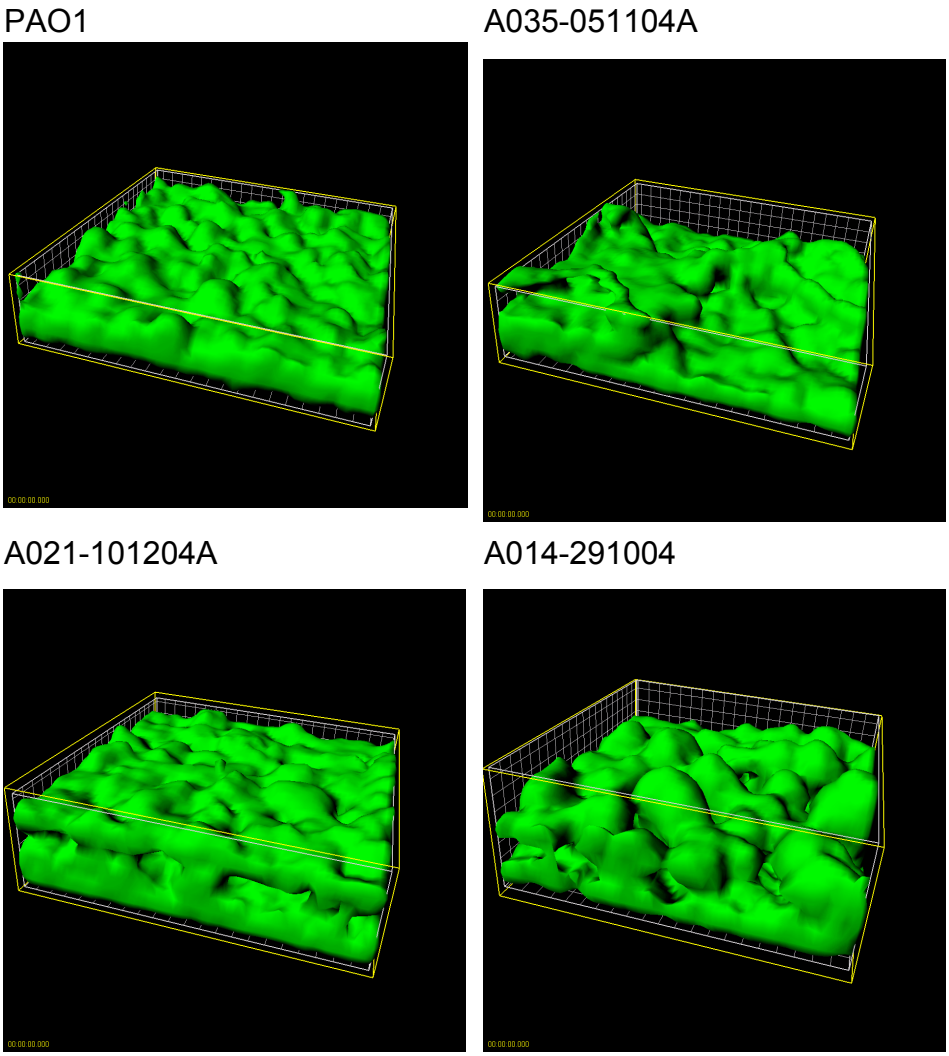


Figure 5-12 Examples of IMARIS rendered images of 4-day old untreated biofilms formed by PAO1 and clinical isolates.
Images depict the 'isosurface' reconstruction, with space-filling and surface smoothing.

In the flowchamber system, the LecA-producing isolate A035-051104A formed comparable biofilms in terms of biomass and thickness to PAO1 and there were some reductions in these parameters in the presence of IPTG. The LecA over-producing isolate A021-101204A formed biofilms with a greater biomass and thickness from the outset in comparison to PAO1 and again, there was an effect of IPTG. The LecA-deficient isolate A014-291004 was able to form structures whose volume and thickness were in keeping with the other strains, yet there was no significant effect of IPTG. Overall, it was not possible to give much weight to the effects of IPTG in this series of

experiments due to the reduced sampling (only one channel sampled per test condition per experimental round compared to 4 on previous occasions).

The IMARIS reconstructions depicted in Figure 5-12 are of particular interest as they suggested that whilst numerically similar, the biofilms formed by the clinical strains differed dramatically in their 3D appearance. In contrast to the familiar regular undulating microcolonies of PAO1, A035-051104A (LecA +) formed structures with an irregular surface morphology. The biofilm of A021-101204A (LecA ++) appeared Swiss-cheese like and dense, whilst that of A014-291004 (LecA -) consisted of large polyp-like structures.

5.2.4 Investigation of the role of Psl as a potential LecA target ligand

The data presented thus far support a role for LecA in *P. aeruginosa* biofilm development by both laboratory strains and clinical isolates, most likely through lectin-carbohydrate interactions. In order to identify the putative carbohydrate target, the ability of LecA to bind bacterial cells was examined in an ELISA plate assay. Purified LecA (PA-IL, Sigma) was immobilised on the surface of test wells, which were then probed with DIG-NHS-labelled bacterial strains. Alongside the three clinical isolates, a *psl*-negative strain (WFPA800) and its parental wildtype PAO1, kindly provided by the Wozniak laboratory, were also investigated to determine whether the Psl carbohydrate was a potential LecA biofilm ligand. The subsequent absorbance readings were an indication of the relative strength of binding of individual strains to the solid-phase LecA target (Figure 5-13). The Psl-producing parent strain showed enhanced binding compared the Psl-deficient mutant. Though the difference in binding was non significant ($p = 0.4$), the trend of the data suggested a possible LecA-Psl interaction. Of interest, the clinical isolates were also able to bind to LecA with A035-051104A (LecA +) behaving like the *psl*-mutant and A021-101204A (LecA ++) and A014-291004 (LecA -) demonstrating levels of binding comparable to the Psl-producing PAO1 strain.

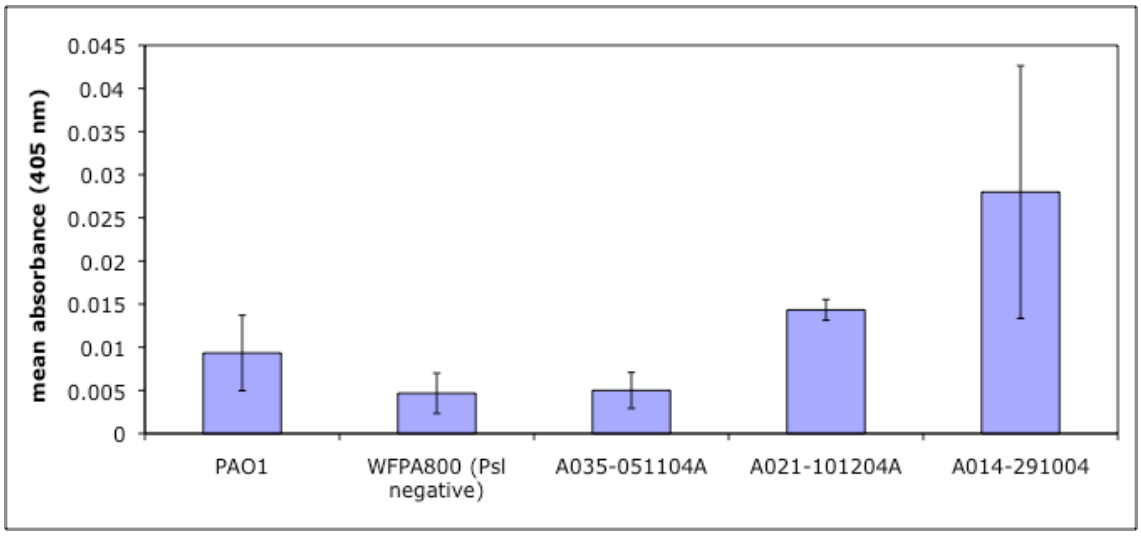


Figure 5-13 ELISA of bacterial cell binding to LecA.
Test wells were covalently labelled with LecA and control wells with BSA. Following overnight incubation with DIG-NHS-labelled bacterial strains, wells were washed and then probed with anti-DIG-NHS antibody. Following the addition of the ABTS enzyme, the resultant colour change was measured with an ELISA plate reader at a wavelength of 405 nm. Mean values minus background BSA binding ± SEM of 3 independent readings plotted.

This putative LecA-Psl interaction was further investigated by examining the effect of IPTG, which was hypothesised to disrupt this binding by competitively blocking the LecA active sites. An ELISA assay was performed as above with the addition of increasing concentrations of IPTG to the test wells. See Figure 5-14. As before, there was increased binding of the Psl-producing PAO1 compared to the Psl-deficient mutant WFPA800. Furthermore, both strains showed a reduction in binding in the presence of both concentrations of IPTG. The sample size was too small for any of these differences to reach significance at $p = 0.05$, hence, these findings were followed up in a steel coupon assay (Figure 5-15). PAO1 was able to form biofilms of significantly greater surface coverage compared to the Psl-deficient strain ($p < 0.0001$) and in addition, there was a significant reduction in the PAO1 surface coverage in the presence of 0.2 mM IPTG ($p < 0.0001$). Overall, this data implied that Psl was a potential candidate carbohydrate target ligand for LecA.

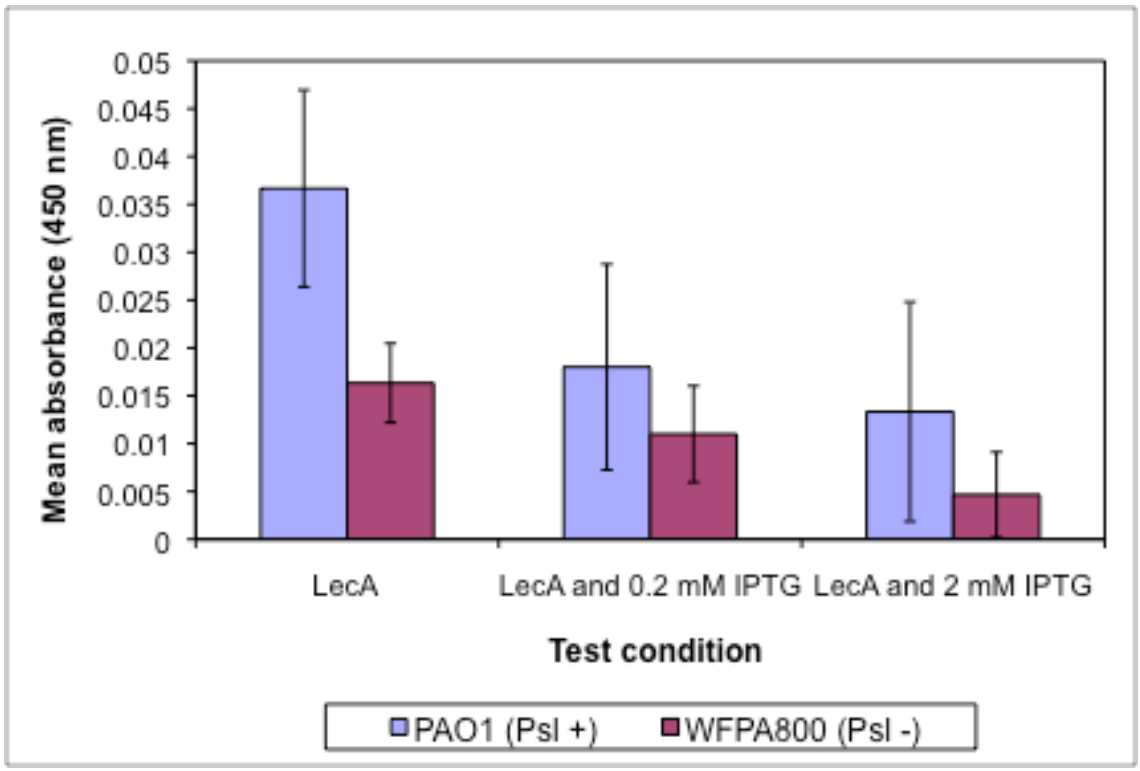
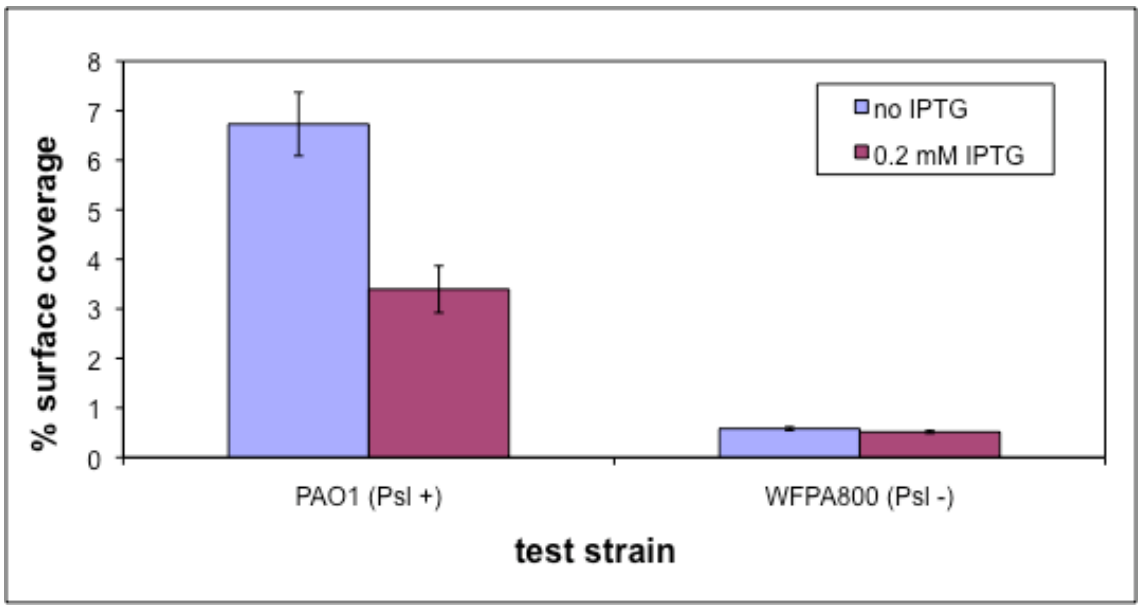


Figure 5-14 ELISA of bacterial cell binding to LecA in the presence of IPTG
Test wells were covalently labelled with LecA and control wells with BSA. Wells were incubated with IPTG at a concentration of 0.2 mM or 2 mM overnight. There then followed overnight incubation with DIG-NHS-labelled bacterial strains in the appropriate concentration of IPTG. Wells were washed and then probed with anti-DIG-NHS antibody. Following the addition of the ABTS enzyme, the resultant colour change was measured with an ELISA plate reader at a wavelength of 405 nm. Mean values minus background BSA binding ± SEM of 3 independent readings plotted.

(A)



(B)

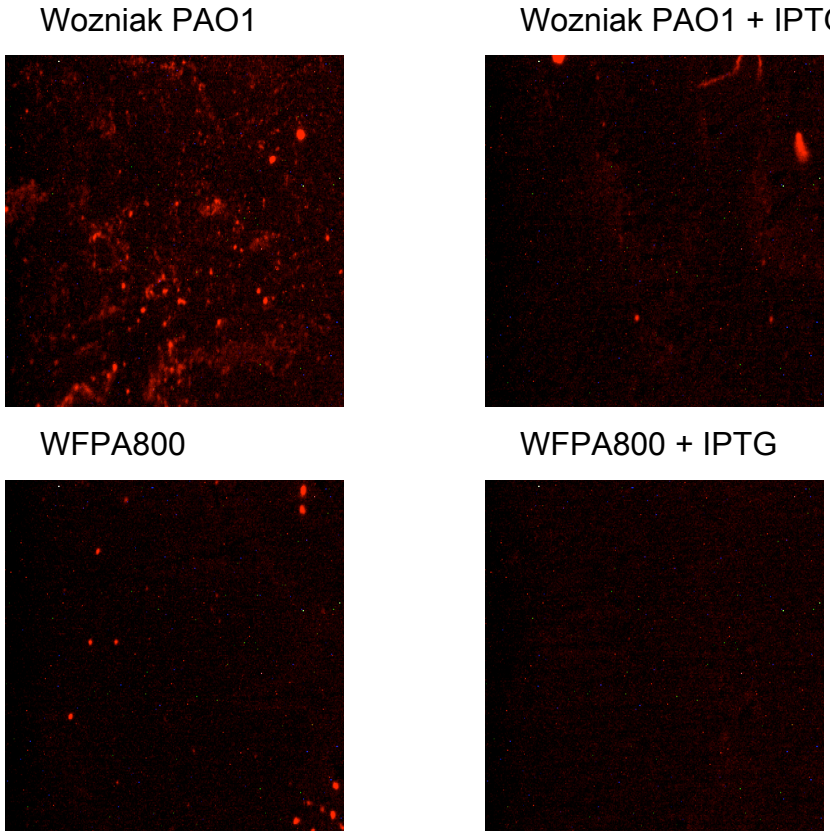


Figure 5-15 The effect of 0.2 mM IPTG on biofilm formation in a stainless steel coupon assay by strains varying in their Psl carbohydrate status.
(A) Steel coupon biofilm % surface coverage (B) Representative acridine orange stained biofilm images. Overnight cultures were normalised and incubated in 0.05 % NB ± IPTG at a final concentration of 0.2 mM in a Petri dish containing 3 steel coupons at 37 °C for 40 h with agitation at 60 rpm. The coupons were rinsed and stained with acridine orange. The % surface coverage for each strain was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

5.2.5 Visualisation of LecA binding within bacterial biofilms

Using chemical composition analyses and lectin staining in conjunction with CLSM and electron microscopy, Ma *et al*. provided evidence that the Psl exopolysaccharide is galactose and mannose-rich (Ma *et al.*, 2007). LecA is a galactose-specific lectin and the data presented above indicated that its putative target may well be the galactose components of Psl. Hence, lectin staining was undertaken to determine whether this indeed was the case.

As a test of the general methodology, the FITC-labelled MOA mushroom lectin specific for galactose moieties on the end of glycan chains, utilised by the Ma study, was obtained and tested for functionality using the *Klebsiella pneumoniae* M10 strain which has a galactose-rich LPS (Tomas *et al.*, 1991). Complete individual cell-associated fluorescent staining was clearly seen under the inverted fluorescent microscope with the appropriate filter set as expected (Figure 5-16). Examination of binding to the Wozniak wildtype and a Psl-inducible strain (WFPA801), grown in the presence of its inducer arabinose, was then performed. This demonstrated that the fluorescent signal was associated with groups of cells only (Figure 5-17). Further high-powered examination using CLSM depicted lectin binding to the material associated with clumps of bacterial cells, presumed to be secreted EPS (Figure 5-18). Luyan Ma from the Wozniak group confirmed that these images were similar to what they have seen (personal communication, January 2008).

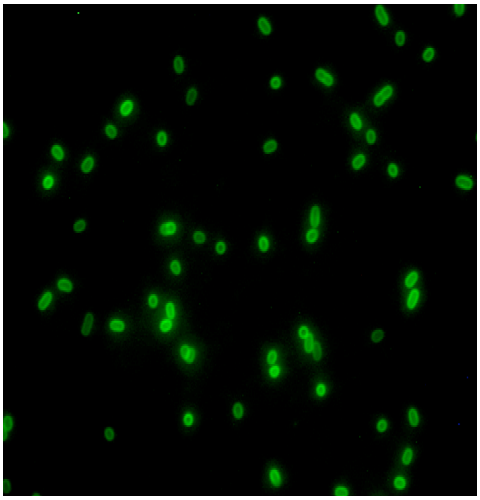


Figure 5-16 FITC-labelled MOA lectin-stained *K. pneumoniae* M10 viewed under the inverted fluorescent microscope.
The x100 oil immersion objective and B_2 filter set were used.

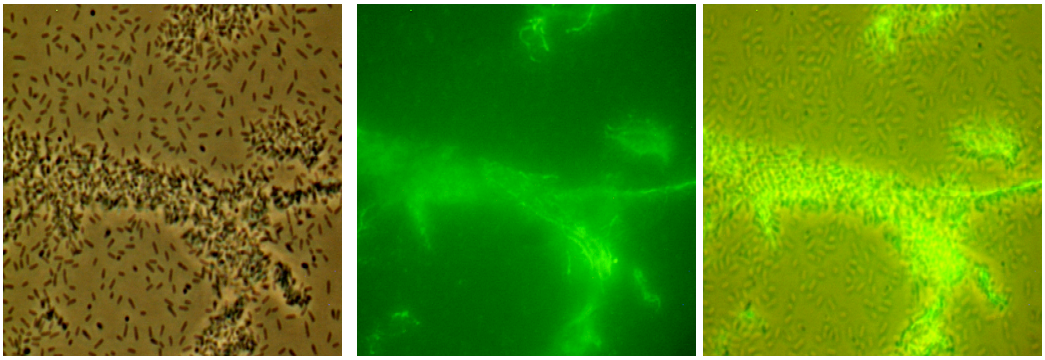
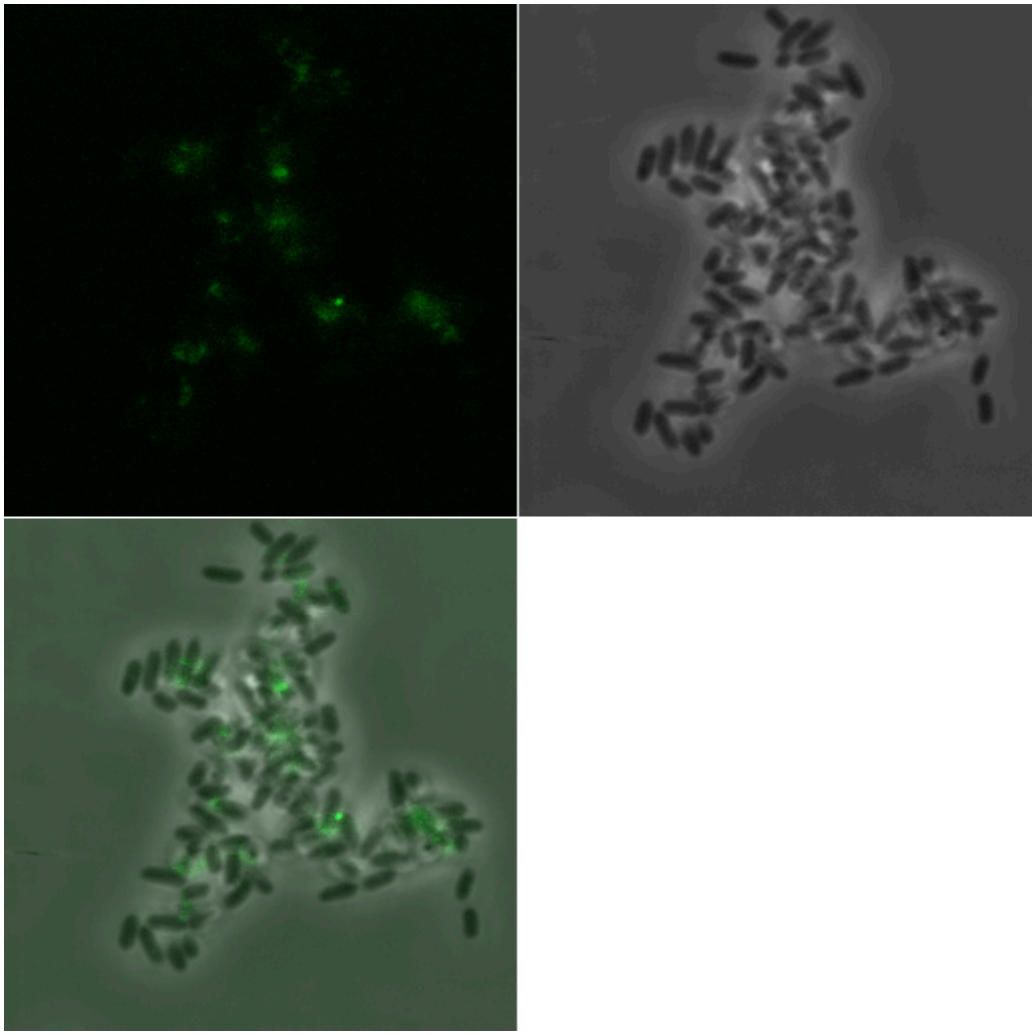


Figure 5-17 FITC-labelled MOA lectin-stained Psl-inducible strain WFPA801 viewed under the inverted fluorescent microscope.
(A) Phase microscopy (B) B_2 filter for capture of fluorescent signal (C) Merged view. All images were obtained using the x100 oil immersion objective.

(A) (B)



(C)

Figure 5-18 CLSM imaging of FITC-labelled MOA lectin-stained Psl-inducible strain WFPA801.
(A) Fluorescent signal captured by CLSM (B) Differential interference contrast (DIC) view (C) Merged view. Microscopy was performed with a Zeiss Plan-NeoFluar 40x/1.3 oil immersion objective. FITC was excited with an Argon laser at 488 nm and emission collected with a LP505 filter.

Hence, to determine whether LecA binding could be similarly visualised and related to the Psl-carbohydrate status of the test strains, purified LecA was fluorescently labelled with a commercially available cyanine dye (Cy5) which fluoresces in the far red end of the spectrum (see section 2.20). Successful staining of *K. pneumoniae* M10 indicated that this lectin-fluorophore conjugate was functional (Figure 5-19), although the intensity of the fluorescent signal and the extent of the individual bacterial cell binding was less than that seen with the FITC-labelled MOA lectin. Indeed, despite repeated experimentation, there was failure to capture any Cy5 fluorescent signal as evidence of

LecA binding to the Psl-producing *P. aeruginosa* strains. A number of technical factors such as choice of fluorophore, optimal ratio of bacterial strain to labelled lectin, length of incubation and nature of the wash steps may have contributed to this. Following unsuccessful attempts to optimise a number of these factors, this avenue of investigation was abandoned. Attention was then turned to the study of the second *P. aeruginosa* lectin LecB and the promising action of anti-lectin compounds on biofilms.

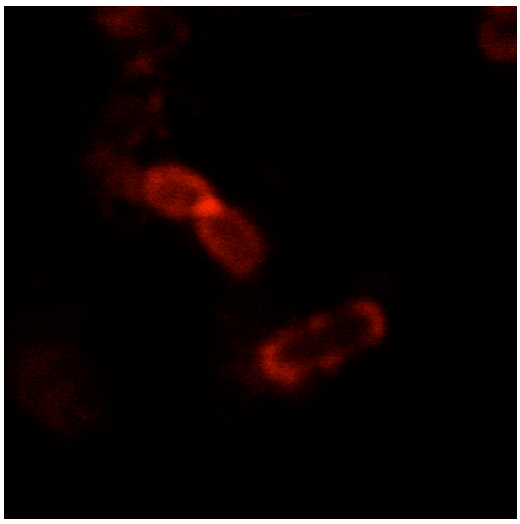


Figure 5-19 CSLM imaging of LecA-Cy5 staining of *K. pneumoniae* M10 strain.
Microscopy was performed with a Zeiss Plan Apochromat 63x/1.4NA oil immersion lens. Cy5 was excited with a HeNe 633 nm laser and emission collected with a LP650 filter.

5.2.6 The contribution of LecB to biofilm development

It is known that biofilm development also involves the second *P. aeruginosa* L-fucose specific lectin LecB (Tielker *et al.*, 2005). Indeed, a preliminary analysis indicated significant inhibition of biofilm surface coverage of stainless steel coupons with the LecB specific monovalent ligand *p*-nitrophenyl-α-L-fucose (NPF) at 0.5 mM ($p = 0.0014$) and an additive effect with 0.2 mM IPTG (Figure 5-20).

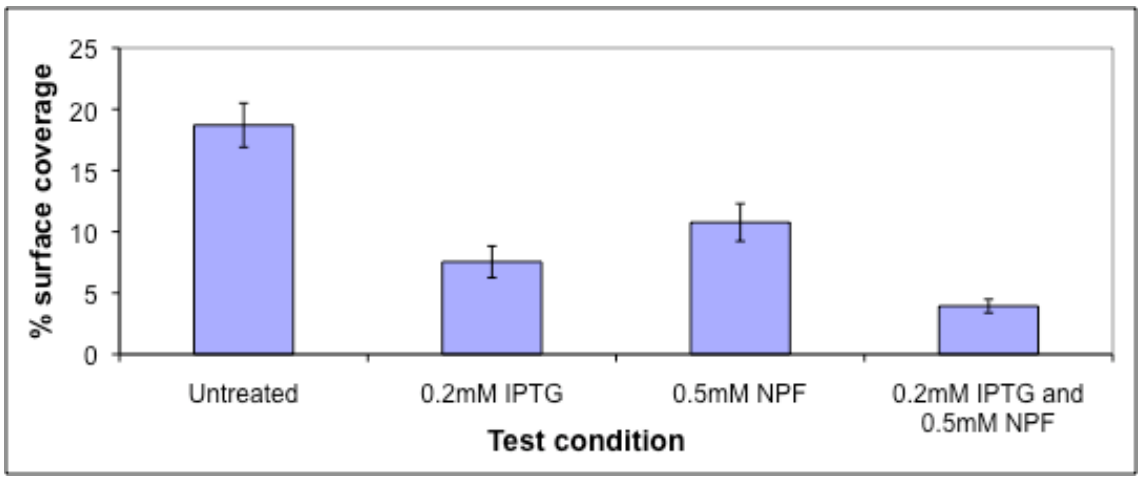


Figure 5-20 The effect of anti-lectin monosaccharide ligands on *P. aeruginosa* PAO1 biofilm formation on steel coupons.
Overnight cultures were normalised and incubated in 0.05 % NB ± the test compound at the appropriate concentration in a Petri dish containing 3 steel coupons at 37 °C for 40 h with agitation at 60 rpm. The coupons were rinsed and stained with acridine orange. The % surface coverage for each strain was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

Hence, in order to further assess the action of anti-lectin compounds on biofilm formation, in addition to the PAO1Δ*lecA* mutant already available, a *lecB*-negative mutant (PAO1Δ*lecB*) was constructed by standard in-frame deletion (see section 2.13) and showed normal growth (Figure 5-21). It was observed that NPF inhibited biofilm formation in wildtype *P. aeruginosa* and in PAO1Δ*lecA* ($P = 0.0001$ and $p = 0.0011$ respectively), but showed no reduction in biofilm formation for PAO1Δ*lecB* ($p = 0.16$). On the other hand, the galactose ligand IPTG inhibited *P. aeruginosa* PAO1 wildtype ($p < 0.0001$) and PAO1Δ*lecB* biofilms ($p < 0.0001$), but not those of PAO1Δ*lecA* ($p = 0.20$). See Figure 5-22.

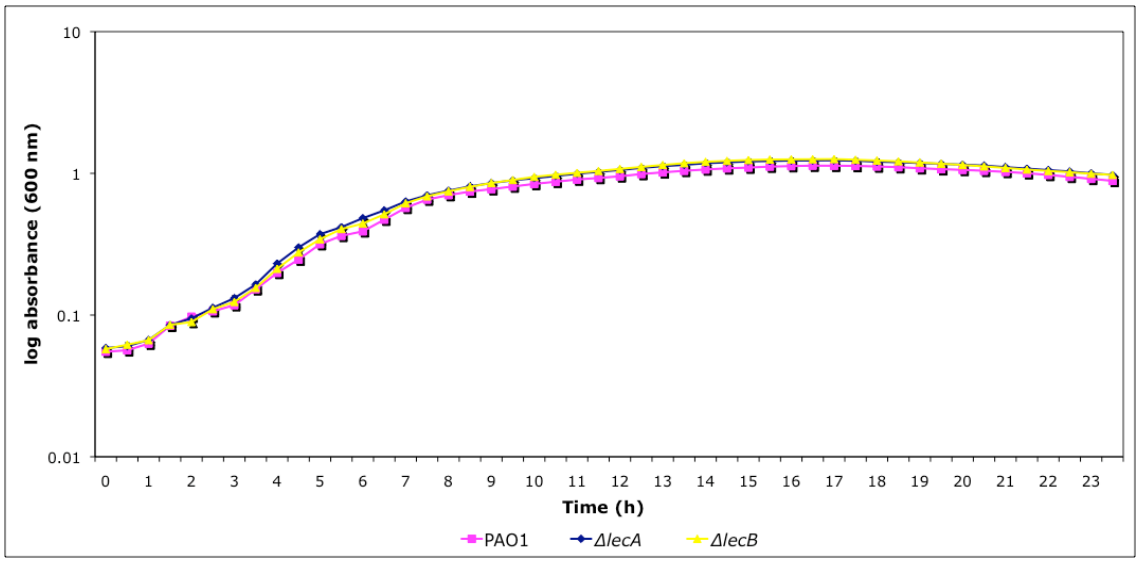


Figure 5-21 Growth curves for lectin mutant strains.
Growth of normalised cultures in a 96 well plate over 24 h at 37 °C was measured in a spectrophotometer. Mean value ± SEM of 10 independent readings plotted.

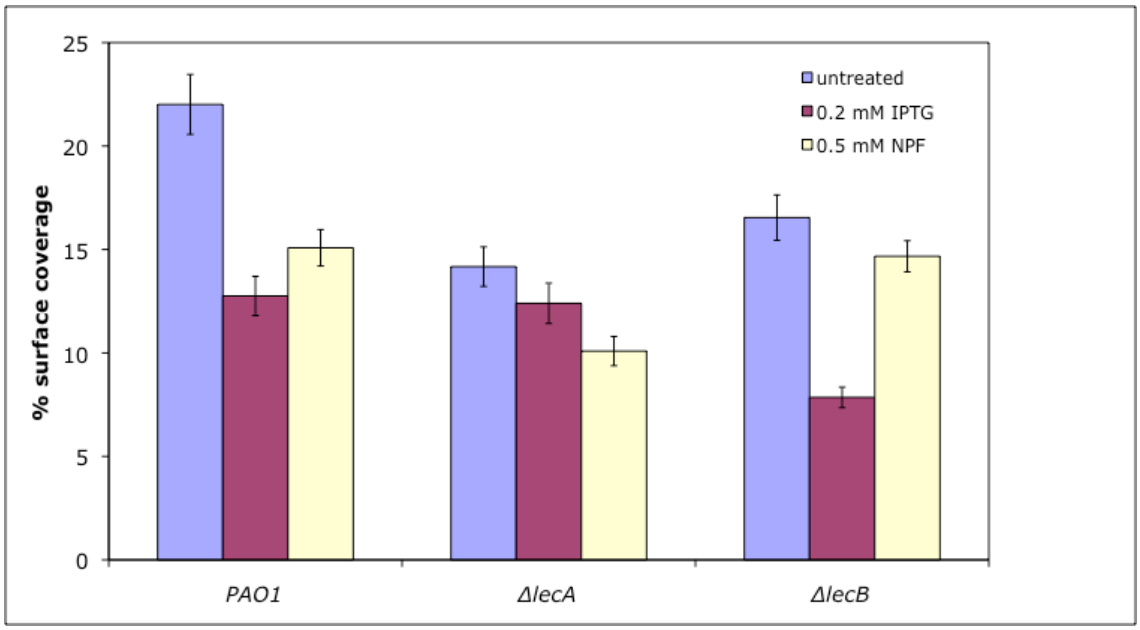


Figure 5-22 The effect of IPTG and NPF on steel coupon biofilm formation by lectin mutants.
Overnight cultures were normalised and incubated in 0.05 % NB ± test compound at the appropriate concentration in a Petri dish containing 3 steel coupons at 37 °C for 40 h with agitation at 60 rpm. The coupons were rinsed and stained with acridine orange. The % surface coverage for each strain was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

5.2.6.1 Inhibition of biofilm formation by targeting LecB with C-fucosyl glycopeptide dendrimers

The recent identification of fucose-based anti-LecB dendrimers (Johansson, 2007) in conjunction with the reduction in biofilm surface coverage by the lectin-deficient strains/lectin-specific ligands prompted the further investigation of these compounds on *P. aeruginosa* biofilm formation. Hence the action of a range of C-fucosyl glycopeptide dendrimers was investigated in collaboration with Emma Johansson, University of Berne, Switzerland, who had previously performed the dendrimer synthesis. The word 'dendrimer' is derived from the Greek 'δενδρον'/*dendron*, meaning 'tree' and is the term for a repeatedly branched compound. Hence, glycopeptide dendrimers are regularly branched structures containing both carbohydrates and peptides. See Figure 5-23 for a depiction of the general structure of a dendrimer.

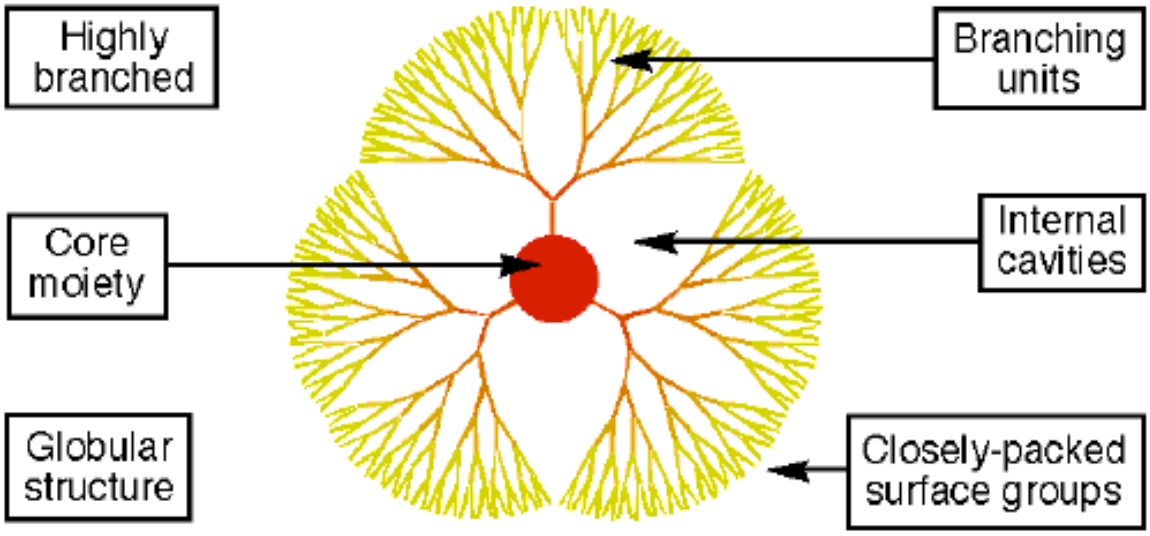


Figure 5-23 General dendrimer structure.
Is treelike with a number of branching units.

Dendrimers, with their globular structure, have a number of biomedical applications. For example, they can be used to mimic enzymes with an active site in the core or the cavities can be loaded with drugs enabling their use as site-specific drug delivery systems. A further application is to functionalise the surface in order to create primary

recognition elements for target ligands. This was achieved for LecB using α-L-fucoside end groups. In this way, a library of dendrimers with variable arm length and multivalency was created (Johansson, 2007). See Figure 5-24.

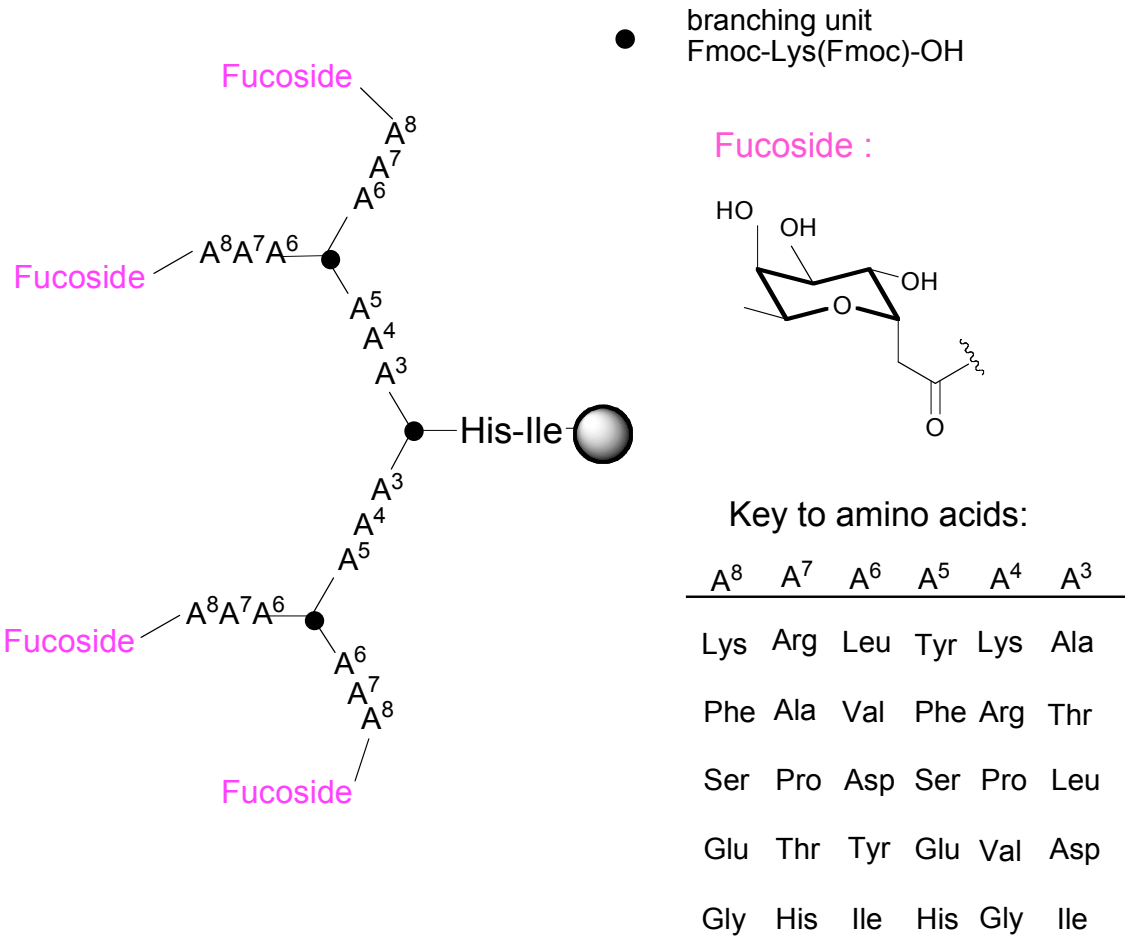


Figure 5-24 General structure of C-fucosyl glycopeptide dendrimers synthesised by Emma Johansson.

Screening of this library identified five dendrimers ("20", "6C", "9Ph", "14Ph" and "17C") with a high affinity for LecB, which were selected for further study. In the first instance, it was established that none of these ligands affected the planktonic growth of the PAO1 wildtype, indicating that any subsequent observed effect was not due to toxicity (Figure 5-25).

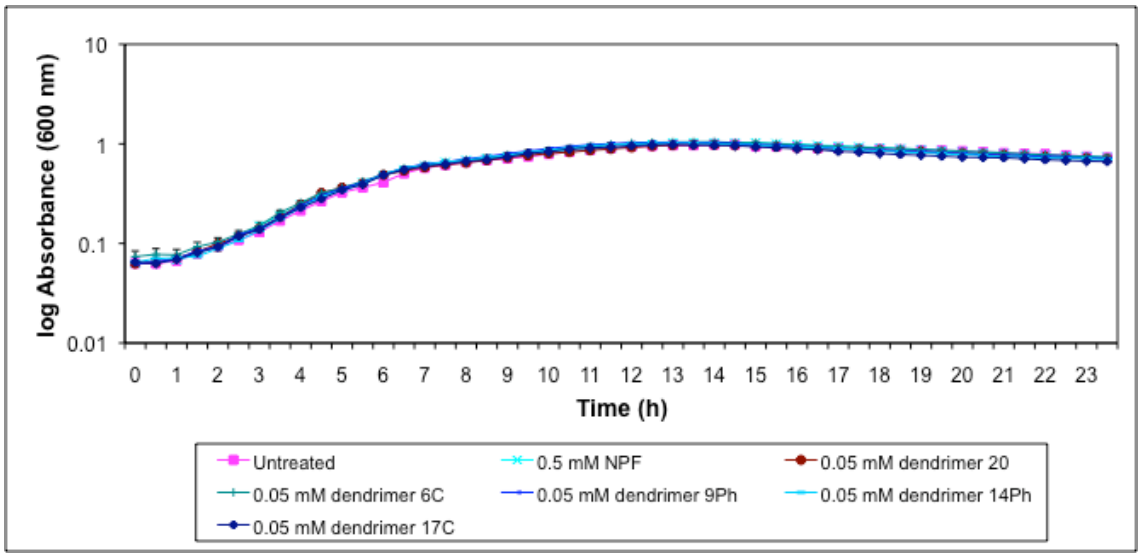


Figure 5-25 Effect of fucose dendrimers on the growth of *P. aeruginosa* PAO1.
Growth of normalised cultures in a 96 well plate with the addition of the test compound at the appropriate concentration over 24 h at 37 °C was measured in a spectrophotometer. Mean value ± SEM of 6 independent readings plotted.

The action of these anti-LecB dendrimers was then investigated in the steel coupon biofilm assay. Indeed, all the dendrimers showed around a 50 % reduction in biofilm surface coverage when added from the outset, with dendrimer 20 showing the most pronounced effect, with almost complete biofilm inhibition ($p < 0.0001$). Furthermore, when added to 40 h pre-formed biofilms, all compounds were able to cause dispersal as indicated by a reduction in surface coverage and an increase in the subsequent viable cell count of released planktonic organisms. The degree of dispersion was similar for all compounds and of the same magnitude as that seen with IPTG (approximately 40 % reduction in surface coverage) with the exception of dendrimer 20 which showed an almost 70 % reduction. See Figure 5-26 and Table 5-5.

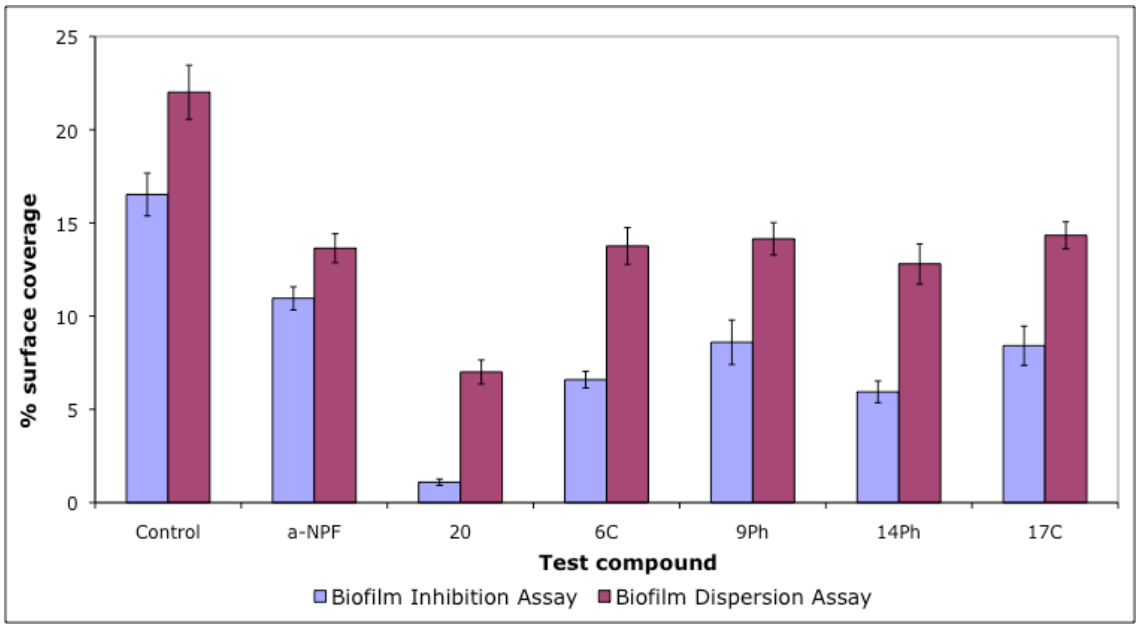


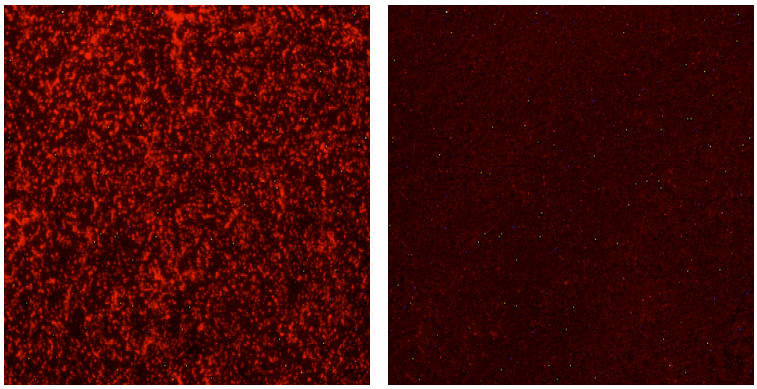
Figure 5-26 The effect of fucose dendrimers on biofilm formation on steel coupons by *P. aeruginosa* PAO1.
Overnight cultures were normalised and incubated in 0.05 % NB in a Petri dish containing 3 steel coupons. Anti-LecB compounds were added to give a final concentration of 0.5 mM for α-NPF and 0.05 mM for the dendrimers. These were either present from the outset (biofilm inhibition assay) or following 40 h incubation (biofilm dispersal assay). The % surface coverage following acridine orange staining was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

Table 5-5 Viable counts of planktonic cells released from 40 h biofilms following exposure to test compounds.

Compound	CFU/plate	Viable count (CFU/ml x 10^7)
Control	123, 120, 95, 83	1.05
IPTG	625, 810, 510	5.13
NPF	1057, 1200, 714	9.90
Dendrimer 20	905, 1100, 875	9.60
Dendrimer 6C	974, 1113, 897	9.94
Dendrimer 9Ph	520, 533, 485	5.13
Dendrimer 14Ph	830, 325, 625	5.93
Dendrimer 17C	1350, 886, 1104	11.1

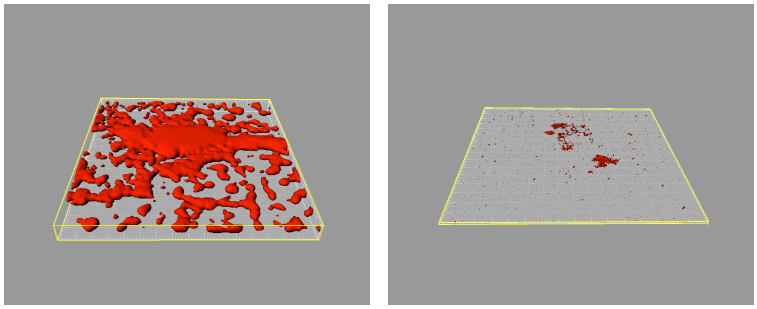
The potency of dendrimer 20 to disperse biofilms formed by the laboratory strain PAO1 was very evident on visual analysis of acridine orange stained steel coupon biofilms viewed under the inverted fluorescent microscope and by CLSM, with virtual clearing of the coupon surface of attached microcolonies (Figure 5-27).

(A)



untreated + dendrimer 20

(B)



untreated + dendrimer 20

Figure 5-27 Images of acridine orange stained PAO1 biofilms with and without treatment with dendrimer 20 after 40 h growth.
(A) Fluorescent microscope view using the x10 objective and green filter, captured with Lucia/G comet software (B) IMARIS reconstructed CLSM stack captured with a Zeiss Plan-NeoFluar 40x/1.3 oil immersion objective. Acridine orange was excited with an Argon laser at 488 nm and emission collected with a LP560 filter.

As a further test of its application, dendrimer 20 was assessed for its ability to inhibit biofilms formed by the clinical isolates A035-051104A, A021-101204A and A014-291004 investigated previously. Highly significant reductions in biofilm surface coverage were seen for all three isolates and PAO1 ($p < 0.0001$) suggesting a generality of the inhibition effect. See Figure 5-28.

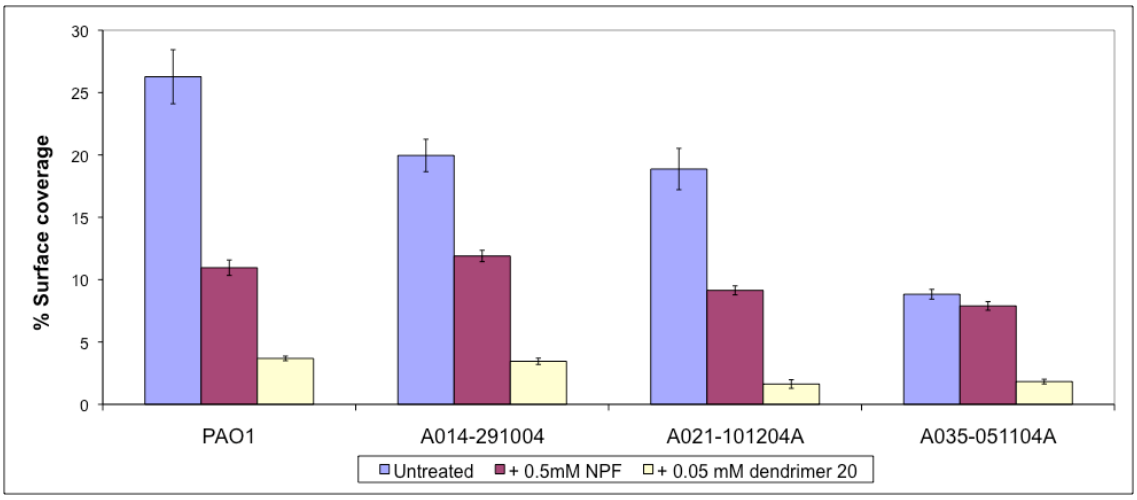


Figure 5-28 The action of dendrimer 20 on steel coupon biofilm formation by clinical isolates.
Overnight cultures were normalised and incubated in 0.05 % NB ± test compound at the appropriate concentration in a Petri dish containing 3 steel coupons at 37 °C for 40 h with agitation at 60 rpm. The coupons were rinsed and stained with acridine orange. The % surface coverage for each strain was measured using the Lucia G/Comet software. Mean value ± SEM of 30 independent readings plotted.

5.2.6.2 Cytotoxicity study of dendrimer 20

The pronounced ability of dendrimer 20 to inhibit biofilm formation by laboratory and clinical isolates of *P. aeruginosa* and in addition, its dispersal effect on preformed PAO1 biofilms, raised the question of its potential therapeutic application. This would only be a possibility if this compound was not harmful to human cells. Hence investigation of dendrimer 20 in a LDH cytotoxicity assay against Human 293T kidney-embryonic cells was performed and indicated that this compound showed no significant increase in LDH production over the untreated cells over a range of concentrations including 0.05 mM (all p values greater than 0.05). See Figure 5-29. This suggests that this dendrimer is not cytotoxic to eukaryotic cells and could potentially be tested in animal models of infection and eventually in humans.

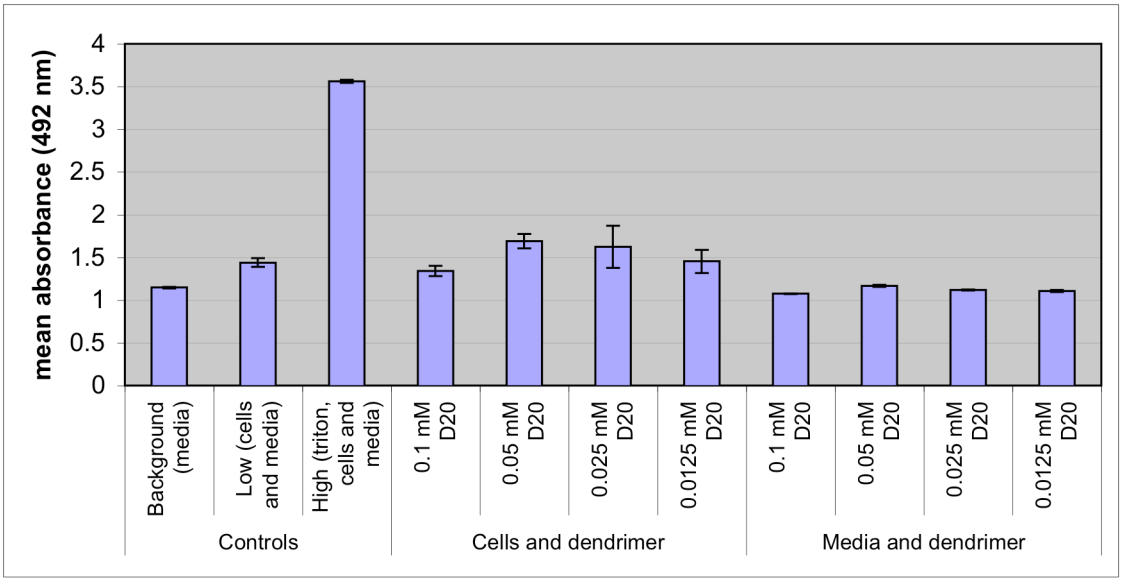


Figure 5-29 LDH release from human 293T kidney-embryonic cells in the presence of dendrimer 20.
Cells were cultured in a 96 well plate and incubated overnight in media containing the appropriate concentration of dendrimer 20. The cell-free supernatant was collected and transferred into the corresponding wells of an optically clear 96-well flat bottom microplate and to these, aliquots of freshly prepared LDH detection Reaction mixture were added. Following incubation for 30 min at room temperature the absorbance of the samples was measured at 492 nm using an ELISA plate reader. Mean values of 3 independent readings ± SEM plotted.

5.3 Discussion

There has been a recent shift in thinking amongst the biofilm community from the view that the EPS is an amorphous physical carbohydrate scaffold, to the realisation that it is "a chemically complex mechanically and structurally adaptive material that has multiple biological functions" (Palmer and Stoodley, 2007). Yet relatively little is known about the regulation, composition and function of this material, which is the hallmark that distinguishes biofilm populations from planktonic cultures.

The report by Diggle *et al*. (2006) highlighted that other adhesins besides alginate may have a larger influence on biofilm structure than was previously supposed. The flowchamber biofilm quantitative and qualitative data comparing the wildtype and *lecA*-negative mutant presented here firmly support the role proposed by Diggle *et al*. (2006b) for LecA in *P. aeruginosa* biofilm development. Specifically, the data demonstrate that whilst the *lecA*-negative mutant can form surface attached biofilms, these were significantly smaller and thinner than those of the wildtype strain. The

IMARIS views implied that these spatial differences reflected a lack of surface differentiation into microcolonies, implying a role for LecA in biofilm maturation. However, a difference in how much the biofilm thickness varied (biofilm heterogeneity), as measured by the roughness coefficient, was expected but not seen. This may be because this particular parameter is not a suitable measure for the variation of surface topology. This also emphasises that whilst the IMARIS biofilm views are visually striking and appealing, they only reflect individual points of the data set. The drier COMSTAT data gives a robust, non-subjective overall description of the structures formed and should generally be more heavily weighted.

IPTG is a hydrophobic galactoside with a high specific affinity for LecA (Garber *et al.*, 1992). The demonstration of its ability to abolish these differences between wildtype and LecA-deficient biofilms by significantly reducing the biomass and thickness of the biofilms formed by the wildtype strain suggested inhibition of LecA-biofilm interactions by specific blockage of its active sites. Whilst it is recognised that LecA is only one of a number of components likely to be important for biofilm development, the fact that *lecA* is the most highly upregulated gene under anaerobic conditions (Marvin Whiteley, personal communication) points to a specific role in the CF lung, where there is increasing evidence for the occurrence of an anaerobic microenvironment (Worlitzsch *et al.*, 2002).

Thinking within the context of the CF lung prompted the important investigation of the behaviour of CF clinical isolates differing in their LecA production. Several interesting points for discussion were raised by this work using three different *in vitro* biofilm assays. The microtitre plate assay is a simple attachment assay and this demonstrated that all three strains, including the LecA-deficient A014-291004, were able to attach to some degree to a polystyrene surface, though less efficiently than the wildtype. This behaviour of A014-291004 and the observation that the inclusion of IPTG in microtitre wells had no effect on the mean absorbance measures for the lectin-producing strains suggested that LecA is not important for attachment to such a surface. Furthermore, it has been reported that LecA is not involved in adhesion to human tracheobronchial mucin (Sonawane *et al*., 2006).

The steel coupon assay is a somewhat more sophisticated system in which measurements of the fluorescence of acridine orange-stained bacterial cells above a certain threshold are made. These reflect the degree of surface coverage of biofilm

microcolonies as can be clearly seen by the microscope images of the untreated WT PAO1. A limitation is that measurements are made in a horizontal plane, yielding no information on biofilm depth. Despite this, this assay provided further information on the behaviour of the clinical strains. A035-051104A showed little microcolony formation, whilst A021-101204A and A014-291004 formed clumps of cells, which though different morphologically to the WT, likely represented differentiated microcolonies. Importantly, the significant inhibition of this microcolony formation in the presence of 0.2 mM IPTG in both the WT and LecA-overproducing strain A021-101204A, further suggested a role for LecA in biofilm maturation that was not restricted to PAO1.

The poor biofilm formation by isolate A035-051104A in these two assays could be a reflection of its reduced planktonic growth compared to the wildtype and other isolates. It was thus very interesting to observe that within the flowcell biofilm system, this isolate formed biofilms with volume and thickness parameters comparable to PAO1, suggesting that the prevailing environmental conditions within this flowing system favoured biofilm formation by this strain. Isolate A035-051104A was known to make LecA and indeed, biofilm inhibition was demonstrated in the presence of IPTG in this system. Interestingly, the LecA-overproducing strain A021-101204A and LecA-deficient strain A014-291004 formed more substantial biofilms compared to PAO1, again suggesting that these isolates were somehow better adapted to forming biofilms in this environment. IPTG inhibition was seen in A021-101204A but not for A014-291004. Whilst the effects of IPTG are in keeping with the known LecA-status of these isolates, some caution is needed in the interpretation of these data as no significant effect of IPTG was seen on the WT strain. This was most likely due to the reduced sampling, with only one channel per strain per test condition, undertaken in this series of experiments.

The IMARIS reconstructions of the flowcell data were particularly interesting, demonstrating strikingly different biofilm morphologies amongst the isolates and from the wildtype laboratory strain. Despite the earlier caution, these observations highlight how informative IMARIS reconstructed biofilm images can be when interpreted in conjunction with the COMSTAT data. From the perspective of understanding *P. aeruginosa* biofilm formation and its implications for pathogenesis in the CF lung, they are in keeping with other reports that CF isolates have a highly variable biofilm

architecture ((Lee *et al.*, 2005) (Kirov *et al.*, 2007), reflecting the great bacterial diversity that occurs within the airways of these patients. These structural differences are certain to represent different adaptive strategies resulting from the effects of the host's genotype (affecting airway physiology and immune response), exposure to antibiotics, cohabiting flora and bacterial interspecies interactions and the length of bacterial colonisation.

Attempts were made to characterise the LecA target ligand by focusing on the recently reported mannose and galactose-rich Psl carbohydrate. The enhanced binding of the Psl-producing Wozniak PAO1 strain to LecA in an ELISA assay and the inhibition of its steel coupon biofilm formation by IPTG were very suggestive of a LecA-Psl interaction. The ability of the clinical isolates to attach to LecA in comparison to the laboratory strains differing in their Psl-status suggested that A035-051104A may be Psl deficient whilst A021-101204A and A014-291004 may be Psl rich. This emphasises that multiple factors are involved in the complex process of biofilm development, particularly in clinical isolates, which have adapted to a unique host environment over a number of years. Thus, the observed differences in the biofilms formed between the clinical isolates cannot simply be attributed to differences in a single component such as their LecA status and will be affected by a range of other factors such as the nature of their EPS.

An assimilation of this data enables the proposition of a hypothesis for the role of LecA in biofilm maturation: in mediating cell–cell and cell–EPS adhesion as well as interactions between inert EPS components themselves (see Figure 5.30). To achieve this, it is known that whilst most of its activity is stored intracellulary, small but significant fractions of LecA are present on the cytoplasmic membrane, on the outer membrane and in the periplasmic space (Glick and Garber, 1983). Hence efforts were made to capture images of fluorescently-labelled LecA binding to *P. aeruginosa* cells. The inability to achieve this demonstrates the great difficulty in undertaking biofilm carbohydrate chemical analyses, due to the diversity of sugar monomers, linkages and unique structures present in the carbohydrate fraction of the EPS matrix material (Flemming *et al.*, 2007). Indeed, replication of the work by Ma *et al*. (Ma *et al.*, 2007) with FITC-labelled MOA lectin was performed with difficulty, likely reflecting that individual-user handling of strains and interpretation of live cell microscopy is variable and subjective.

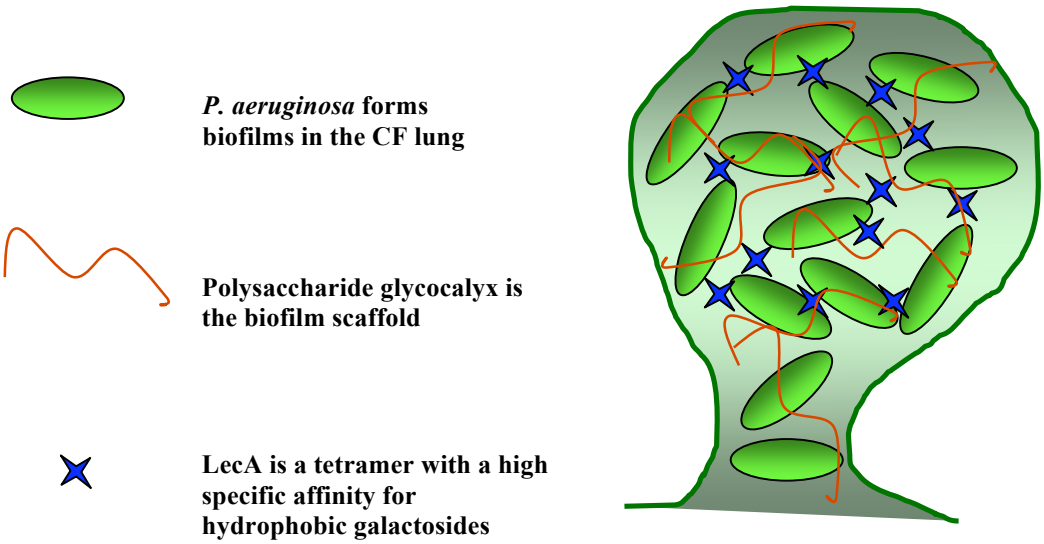


Figure 5-30 Possible role for LecA in biofilm maturation: cross-linking bacterial cells with each other and the polysaccharide matrix.

Whilst there is the need to characterise the nature of biofilm structural interactions, the overall aim of this work was to identify novel therapeutic targets for biofilm disruption. The direction of the study took an exciting turn with the collaborative investigation of the effect of novel anti-LecB synthetic furanoside dendrimers (Johansson, 2007). Previously, Diggle *et al*. (2006) reported no effect of 0.013 mM NPF on biofilm formation. This value, together with the value of 0.2 mM IPTG, were chosen as these concentrations are known to inhibit two haemagglutination units of LecB (Garber, 1987) and LecA (Garber *et al.*, 1992) respectively. This study showed that a much higher concentration of NPF (0.5 mM) had an inhibitory effect on steel coupon biofilm formation and a possible additive effect with 0.2 mM IPTG.

The furanoside dendrimers are many times more potent than NPF and dramatic biofilm inhibitory effects were seen at 0.05 mM concentrations in both the laboratory wildtype and the CF clinical isolates. Importantly, at these test concentrations, there was no inhibitory effect on the planktonic growth of the wildtype. Furthermore, evidence that these biofilm inhibitory effects were mediated by LecB was provided by investigating biofilm inhibition in deletion strains lacking either LecA or LecB. NPF inhibited

biofilm formation in the wildtype and *lecA*-mutant, with no significant effect on the *lecB*-mutant.

Dendrimer 20 showed the most potent biofilm inhibitory effect when added to the incubating media from the outset of growth. Of major significance was its enhanced ability, in comparison to NPF and the other dendrimers, to disperse 40 h preformed *P. aeruginosa* PAO1 biofilms. The associated increase in planktonic viable count was evidence that the mechanism of this action was release of organisms from the biofilm rather than cell death. Furthermore, this dendrimer showed no significant cytotoxic effect against human cells in culture. Taken together, this non-cytotoxic compound, which has the ability to disperse biofilms, has a potentially important clinical application in the treatment of established biofilms, without exerting a selective pressure through a bactericidal action for the emergence of resistance.

These findings will be discussed in the light of the work presented in the previous chapters, together with the indications and implications for future work, in the ensuing final chapter.

Chapter 6: Conclusions and future work

CF is a common incurable inherited disease. *P. aeruginosa* infects and thrives in the CF lung and is the major cause of morbidity and mortality in this patient group. The realisation that within the airways, *P. aeruginosa* adopts sophisticated patterns of behaviour, offers new insights into its disease causing behaviour. Whilst set in the general context of *P. aeruginosa* pathogenesis in the CF lung, this study set out to specifically explore a) the links between QS signal molecule production and disease status in CF patients and b) the role of lectins in biofilm formation.

Crucial to this undertaking was the decision to base the analysis on clinical isolates recovered from sputum samples provided by adult and paediatric patients with CF. The lengthy and tightly regulated process of setting up this clinical study was a worthwhile endeavour for the clinical strain collection established represents a valuable resource, which was utilised for this study and which is already being accessed to investigate other hypotheses relating to the behaviour of *P. aeruginosa* in a clinical context.

The biosensor data presented in Chapter 3 demonstrated that these clinical isolates retained the ability to produce a number of different QS signal molecules *in vitro*, implying a role for QS during *in vivo* pathogenesis. The occurrence of signal-deficient isolates, particularly for 3-oxo-C12-HSL, suggested specific adaptive behaviour within the CF airways.

Given that QS molecules and QS systems are important components of the *in vivo* pathogenesis of *P. aeruginosa*, Chapter 4 assessed the ability to recover these molecules from individual sputum samples by LC-MS. The unique feature of this investigation was that it was performed in conjunction with a review of the patient medical records and the collection of patient clinical information. The analysis revealed that a wide range of QS molecules was detected, including several signalling molecules considered important for virulence gene regulation such as 3-oxo-C12-HSL, C4-HSL and PQS. Furthermore, the data suggested some associations between sputum QS signal molecule level and CF disease status, response to IV antibiotics and the presence of non-cultured *P. aeruginosa*. In particular, it was suggested that a high sputum C4-HSL level could

reflect severe disease progression secondary to chronic biofilm formation and a high 3-oxo-C12-HSL could be due to planktonic organisms leaving the biofilm and causing an acute exacerbation. A fall in signal molecule level after IV antibiotics would be a useful marker of response to treatment and the detection of bacterial signal in the absence of the cultured organism could represent a sensitive early indicator of infection, enabling targeted eradication therapy.

These findings, though limited by the small sample size, clearly indicate the potential for a larger targeted clinical study with prospective collection of clinical data in order to validate the role of QS molecules as biomarkers of lung infection and damage in CF. Future applications might include evaluation of novel antipseudomonal therapies based on QS inhibitors and in other infections caused by *P. aeruginosa*, such as COPD.

The formation of bacterial biofilm communities is of major importance in the pathogenesis of *P. aeruginosa* in the CF lung environment. To enable the study of biofilm formation *in vitro*, a flowchamber biofilm system and allied technology was established and optimised as described in Appendix 4. This very practical undertaking uncovered both the advantages and disadvantages of such a system. It was incredibly exciting to be able to visualise live, fully hydrated biofilms in real-time, with the generation of descriptive quantitative and qualitative data. This was tempered by the realisation that to obtain robust and meaningful measures, simple comparisons with non-subjective sampling and sufficient repeats was essential and this necessitated a lengthy analysis period.

This established flowcell system is a very important analytical tool, which can be further utilised to provide baseline data for examining theoretical questions. It is, however, recognised that there is a need to study biofilm behaviour in a manner that reflects the natural environment. For example, the development of a static tissue culture model system for the study of interactions between *P. aeruginosa* and CF-affected human airway epithelial cells was described by Greg Anderson of Dartmouth College at the 2007 ASM conference on biofilms (Palmer and Stoodley, 2007). Indeed, the potential to incorporate human respiratory epithelium as the biofilm substratum into the flowchamber design is to be actively pursued with Respiratory Medicine colleagues.

Previous findings of a role for LecA in biofilm formation by our laboratory (Diggle *et al.*, 2006b) and the demonstration of the conservation and expression of *lecA* amongst

the clinical isolates examined in Chapter 3 were the basis for the investigation of the contribution of lectins to biofilm formation described in Chapter 6. It was confirmed that LecA contributes to biofilm maturation in both laboratory strains and clinical isolates and hydrophobic galactosides were shown to be able to inhibit biofilm development. The biofilm target ligand for LecA was putatively identified as the Psl exopolysaccharide. Mutants defective in either *lecA* or *lecB* were shown to produce defective biofilms, which could be inhibited and/or dispersed by galactosides or furanosides respectively, including novel synthetic furanoside dendrimers. The latter proved inhibitory to both laboratory and clinical *P. aeruginosa* isolates and constitute a potential novel therapeutic.

The demonstration of this role for lectins in biofilm formation is set in the context of other reports in the literature that these lectins have multiple functions. In addition to those previously discussed, there is the finding that both LecA and LecB immobilise the ciliary beating of *ex-vivo* human nasal mucous membranes by competing for the same binding site(s). This immobilisation of the mucociliary elevator prevents the expulsion of the invading bacteria from the lungs and could be inhibited by lectin-specific sugars (Mewe *et al.*, 2005). Furthermore, it has been reported that LecA binds AHLs (Boteva *et al.*, 2005). The authors hypothesise that this could regulate the free and accessible pool of lactones and prevent destruction of these molecules by maintaining a fraction of them in a protein-bound state. This clearly has important implications within the complex microenvironment of a biofilm and may well offer an explanation for the observation that some of their ascribed functions are secondary effects on other systems rather than direct effects of the lectins themselves (Sonawane *et al.*, 2006).

The implications of the striking and powerful action of anti-LecB dendrimers on biofilm formation are being explored using several lines of investigation. Collaborative work is underway to synthesise and assess dendrimers targeting LecA to determine whether a combination approach targeting both lectins has a more significant impact on the ability to inhibit or disperse *P. aeruginosa* biofilms. The tissue-culture based assay, which indicated that these compounds are unlikely to be toxic to human cells, enables plans to assess their activity in animal models to be developed. Given that these agents both inhibit and disperse biofilms, potential applications include their use to prevent biofilm formation as a prophylactic therapy and their use as a treatment in the case of established biofilm diseases. The finding that biofilm dispersal occurred upon

dendrimer application suggests that any future use will be in combination with other, perhaps traditional, approaches to tackle the potential exacerbation of symptoms caused by the release of viable planktonic bacterial cells.

The widespread occurrence and significant impact of biofilms offers opportunities to exploit the actions of anti-lectin agents in many diverse settings. Beyond CF, *P. aeruginosa* is an important respiratory pathogen in pneumonia and COPD. Elsewhere in the human host, it has the potential to infect chronic wounds such as burns and diabetic foot ulcers. Beyond the clinical setting, *P. aeruginosa* biofilm formation leads to contamination of industrial and commercial waste pipes. The economic implications of these effects are sizeable in terms of costs to patients, their carers, health care providers, industrial and commercial sectors and the state. The clear need for effective anti-biofilm measures needs to go hand in hand with increased recognition and incorporation of biofilm concepts into the routine practices of these settings. For example, in the future, clinical microbiology may embrace biofilm diagnostic technology, which not only involves novel *in vitro* assays, but also includes *in vivo* imaging techniques.

The aim of this thesis was to investigate the molecular mechanisms governing the pathogenesis of *P. aeruginosa* in the CF lung. From the outset, it was recognised that *P. aeruginosa* does not exist as individual bacterial cells in isolation. The approaches used in this study have recognised the extensive individual diversification that occurs within the CF lung and the subsequent impact of the complex interactions, communication and community living of these sophisticated and social microorganisms. This, coupled with their short generation time and genetic agility, enables rapid and successful adaptation to a niche, explaining their success as pathogens. Added complexity occurs in the CF lung with the presence of other bacterial, viral and fungal competitors, further influencing the dynamics and behaviour of population subsets.

Clearly understanding, unravelling, modelling and tackling this complex behaviour is challenging. There is the need to not only consider how pathogenesis occurs in a mechanistic sense, but also consider why such behaviours arise. Hence, the overall conclusion of this body of work is that the important *in vitro* characterisation of discrete pathogenic processes needs to be combined with a consideration of their clinical context, in this instance, the complex ecosystem of the CF lung. This translates as a major shift from the failing single-antibiotic for a single-pathogen traditional approach

to tackling bacterial infection. A more detailed understanding of the ecology of the CF lung together with the identification of alternative therapeutic targets will allow a combination of novel approaches to diagnosis and treatment, with different agents tackling different aspects of the disease process at different stages of its development. It is hoped that this will enable the design of future effective and sustainable strategies to manage chronic lung infections caused by *P. aeruginosa* in CF.

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Appendix 1

STUDY PROTOCOL

Lectin-mediated biofilm maturation, quorum sensing and *Pseudomonas aeruginosa* infections in Cystic Fibrosis

SUMMARY

Chronic respiratory infections with *Pseudomonas aeruginosa* are primarily responsible for much morbidity and mortality in cystic fibrosis (CF) patients. In this Gram-negative pathogen, many virulence determinants including the cytotoxic lectin PA-1L (which also promotes adherence to respiratory epithelial cells) are regulated via cell-to-cell communication (quorum sensing; QS). *P. aeruginosa* forms biofilms in the lungs of CF patients and biofilm formation is also QS-dependent. Preliminary work in The Institute of Infections and Immunity has uncovered a role for PA-1L (the product of the *lecA* gene) in the maturation of *P. aeruginosa* biofilms. The primary aim of this project is to elucidate the molecular basis for the contribution of PA-IL to biofilm development and the persistence of *P. aeruginosa* in the CF lung by investigating (**a**) the conservation of the *lecA* gene in mucoid and non mucoid *P. aeruginosa* CF isolates, (**b**) the expression of *lecA in vitro* and *in vivo* in CF sputum, (**c**) the spatial localization of PA-1L within *P. aeruginosa* biofilms and (**d**) the mechanism by which PA-1L is secreted. Since the identity of the target macromolecule(s) within the biofilm architecture is not known, experiments will be undertaken to determine which exopolymer(s) are cross-linked by this multivalent galactophilic lectin. PA-1L is regulated via *N*-acylhomoserine lactone (AHL)-dependent QS, and this laboratory has shown that AHLs can be sensitively detected in CF sputum using bioluminescence (*lux*)-based biosensors. The potential of *P. aeruginosa* AHLs for predicting the effectiveness of IV antibiotic therapy in pulmonary exacerbations caused by *P. aeruginosa* will therefore be explored. A cohort of CF subjects of differing *P. aeruginosa* sputum status will be studied to determine whether an increase in AHL sputum levels is a useful marker of clinical deterioration. Sputum samples will also be assayed for PA-1L since lectin production is likely to be an indicator of biofilm development and given its cytoxicity, may also prove to be a useful diagnostic indicator of airway damage.

LAY SUMMARY

The bacterium *Pseudomonas aeruginosa* causes chronic chest infections in patients with Cystic Fibrosis. It has many strategies for causing disease which are coordinated by a form of bacterial communication involving small diffusible signal molecules, termed quorum sensing. One such strategy is the formation of slime layers called biofilms. Another is the formation of a protein PA-1L which is toxic to respiratory cells. Interestingly, preliminary work has shown that PA-1L plays an important role in the maturation of biofilms. The project aims to investigate this by determining whether the PA-1L gene is present in *P. aeruginosa* strains isolated from the sputum of Cystic Fibrosis patients, whether the protein is made, how is it transported out of the bacterial cells, where the protein binds in the biofilm, what is binds to, whether this binding be disrupted and if the protein levels in infected sputum and the quorum sensing signal molecules which regulate it are useful markers of disease severity.

DETAILS OF RESEARCH PROJECT

(a) Aims of the project

Chronic infections particularly with *Pseudomonas aeruginosa* (***PA***) are primarily responsible for the decline in lung function and ultimate mortality in cystic fibrosis (CF) patients [**1**]. Preliminary work in this laboratory has uncovered a role for the lectin, PA-1L in the maturation of ***PA*** biofilms. The primary aim of this project is to elucidate the molecular basis for the contribution of the PA-IL lectin to biofilm development and the persistence of ***PA*** in the lungs of individuals with CF. Both lectin expression and biofilm maturation in ***PA*** are dependent on quorum sensing (QS), a cell-to-cell communication mechanism involving the production and sensing of diffusible signal molecules The potential of ***PA*** QS signal molecules which regulate PA-1L expression as diagnostic markers for predicting the effectiveness of IV antibiotic therapy in controlling pulmonary exacerbations caused by ***PA*** will be explored.

(b) Work leading up to the project

In their natural environments, bacteria do not generally exist as isolated, planktonic cells, but grow in organised communities termed biofilms. The attachment process and the formation of an organised bacterial biofilm community are key steps in the chronic establishment of bacterial pathogens on host tissues [**2-4**]. Electron microscope studies indicate that ***PA*** forms biofilms in the lungs of CF patients [**1,4,5**] and as a consequence, can develop unique characteristics which protect the micro-organism from host defences, shear forces and antimicrobials [**6**]. Within the biofilm, bacterial microcolonies are embedded within an exopolysaccharide matrix separated by aqueous channels through which nutrients, waste products, oxygen and quorum sensing signalling molecules can be transported [**3,6**]. In CF, one of the most striking and clinically important features of ***PA*** infections is the conversion of the bacterium to a mucoid phenotype which probably initiates the chronic infection stage of the disease [**1**]. Mucoidy results from the production of an alginate muco-exopolysaccharide which makes an important contribution to the evasion of the host immune response [**1**]. Although the precise contribution of alginate to biofilm architecture is not clear, both mucoid and non-mucoid ***PA*** readily form surface associated biofilms.

In ***PA***, the attachment phenotype is driven by extracellular appendages including flagella and type IV pili [**6**]. During the subsequent stages of biofilm development, other factors are required for biofilm maturation, shape determination and the physical resistance of the biofilm structure. These include exopolysaccharides and the chaperone usher fimbrial pathway genes [**6,7**]. In addition, recent work in this laboratory has uncovered an unexpected function for the so-called "internal" lectin PA-1L, the product of the *lecA* gene [**8**]. PA-1L is a 13 kDa protein which exhibits specificity for hydrophobic galactosides, is cytotoxic for mammalian cells and promotes the attachment of *PA* to respiratory epithelial cells [**9,10**]. In contrast to the parent **PA** strain, a *lecA* mutant was unable to form mature biofilms and mutants defective in MvaT, a negative regulator of *lecA,* overproduce PA-1L and form significantly thicker biofilms which cover a correspondingly greater surface area [**11;unpublished data**]. These observations suggested that biofilm formation in ***PA*** might be prevented and pre-formed biofilms dispersed by the exogenous provision of a non-metabolizable, hydrophobic galactoside. Preliminary experiments suggest that this is indeed the case; inclusion of *p*-nitrophenyl- α-D-galactoside (NPG) abolishes biofilm formation on stainless steel and disperses pre-formed *PA* biofilms [**unpublished data**]. These experiments also indicate that PA-1L is likely to play a major role in stabilizing the biofilm matrix by either promoting bacterial cell-cell interactions, bacterial-host cell interactions and/or by stabilizing the biofilm glycocalyx

through lectin-exopolysaccharide/lipopolysaccharide or lectin-fimbrial interactions. Furthermore, the presence of PA-1L in the cell-free, extracted biofilm matrix confirmed that the lectin must be secreted, even though the *lecA* gene carries no signal sequence.

Expression of the *lecA* gene is directly dependent on QS, a cell-to-cell communication mechanism involving the production and sensing of diffusible signal molecules [**8**]. In *PA*, virulence determinants, secondary metabolites and biofilm maturation are regulated via *N*-acylhomoserine lactone (AHL)-dependent QS systems [**12**]. Work in this and other laboratories has revealed that ***PA*** possesses two AHL-dependent QS circuits termed the *las* and *rhl* systems, comprising of the LuxR/I homologues, LasR/I and RhlR/I. LasI directs the synthesis of primarily *N*-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-HSL) and together with the transcriptional regulator LasR regulates the production of e.g. elastase, alkaline protease and exotoxin A [**12**]. In addition, the *las* system controls genes involved in the regulation of the Type II general protein secretion pathway [**13**] and plays a role in regulating the maturation of ***PA*** biofilms [**14**]. RhlI directs the synthesis of *N*-butanoylhomoserine lactone (C4-HSL) which activates RhlR such that RhlR/C4-HSL induces the production of rhamnolipid, elastase, LasA protease, cyanide, pyocyanin and siderophores as well as the lectins PA-1L and PA-IIL [**8,15**]. The *las* and the *rhl* systems are organised in a hierarchical manner such that the *las* system exerts transcriptional control over *rhlR* and over *rhlI* [**16**].

These data strongly implicate a role for both QS and the PA-1L lectin in the aetiology of *PA* infections in CF patients. However, they were generated using a non-CF, non-mucoid laboratory strain of *PA* and their direct relevance to CF has not been established. This project will therefore combine basic and clinical studies to investigate the conservation and expression of the *lecA* gene in both mucoid and non-mucoid CF clinical isolates and to determine whether the lectin can be detected in infected CF sputum. This work will be complemented by studies of the secretion of PA-1L and its spatial location within biofilms generated *in vitro*.

In addition, the potential of the QS signal molecules and PA-1L as diagnostic markers for predicting the effectiveness of IV antibiotic therapy in controlling pulmonary exacerbations caused by ***PA,*** will be investigated.

(c) Experimental design and methods

Conservation and expression of *lecA* in CF isolates of *PA*.

In addition to the laboratory *PA* strain PAO1, *lecA* is known to be present in several environmental isolates. To determine whether the *lecA* gene is conserved in non-mucoid and mucoid ***PA*** strains isolated from CF sputum will be subjected to PCR and Southern blot analysis using oligonucleotide primers and probes previously synthesized in the laboratory. The expression of *lecA in vitro* will be examined by Northern blot analysis (using as a probe, the cloned *lecA* gene labelled with [α-^{33}P]ATP) and by Western blot analysis using antibodies previously raised against the PA-1L protein [**8**]. To determine whether the *lecA* gene is expressed *in vivo* in the CF lung, infected sputum will be collected, treated with Sputolysin (Calbiochem) and subjected to (a) SDS-PAGE and Western blotting for PA-1L protein detection or (b) real time-RT-PCR (ABI PRISM 7700) after extraction of total RNA to quantify sensitively *lecA* transcript levels. Once these assays are fully established, they will be used to follow the course of *lecA* expression in CF patient sputa during pulmonary exacerbations (**section 4**). To obtain further evidence for

lectin expression in the CF lung, both serum and sputum samples from CF patients colonized with *PA* will be subjected to Western blot analysis and ELISA for the presence of antibodies to PA-1L.

2. Spatial localization and secretion of PA-1L within *PA* biofilms.

Preliminary work in this laboratory has shown that biofilms of the non-mucoid ***PA*** strain PAO1 can be disrupted by hydrophobic galactosides. To determine whether this finding is of relevance in the CF context, biofilms will be formed by non-mucoid and mucoid strains from CF patients and treated with or without NPG. As controls for these experiments, the *lecA* mutation will be introduced into the CF strains using a suicide plasmid as described by Winzer *et al* [**8**] to construct isogenic mutants. Biofilms will be generated statically on glass slides and also using Stovall flow cells (Stovall Life Sciences Inc.; [**17**] which are designed for the direct and non-destructive, on-line microscopic examination of bacterial biofilm development and maturation. Biofilms will be imaged using fluorescence or confocal laser scanning microscopy (CLSM) of acridine orange or green-fluorescent protein (GFP)-tagged bacteria respectively to obtain quantitative information on surface coverage and biofilm depth [**18**]. To gain insights into the spatial localization of the PA-1L lectin in association with the bacterial cells and within the biofilm, *lecA* translational fusions to the GFP will be constructed. Since it is not possible to predict which fusion protein will be most efficiently produced and secreted, both N and C-terminal PA-1L fusions will be engineered. The fusion protein will be introduced on a pUCP-based plasmid vector into ***PA*** by electroporation. Bacterial cells will be fractionated (cyotoplasm, periplasm, outer and inner membranes, extracellular supernatant (planktonic cells) or matrix (biofilm cells) compartments) and the distribution of the fusion examined by Western blotting using antibodies to both PA-1L and GFP to ensure that the lectin-fusion protein is expressed as an intact stable fusion which is exported efficiently. Biofilm development and disruption by hydrophobic galactosides will be followed using the flow cell system and imaged using CLSM. Biofilms will be formed using both parent and isogenic *mvaT* ***PA*** mutants (which over-express the PA-1L and form very thick biofilms; [**11; unpublished data**] transformed with a plasmid carrying the *gfp-lecA* gene an either intact or disrupted chromosomal copy of *lecA*.

Cell-free culture supernatants of ***PA*** contain PA-1L even though the *lecA* gene carries no signal sequence [**8**]. ***PA*** possesses Type I, Type II (two systems - *xcp* and *hxc*) and Type III secretion systems [**19;19a**]. Strains with mutations in each secretion system have been constructed by Dr Alain Filloux (CNRS, Marseille, France) and have been made available to us. Using the cell fractionation techniques described above, Western blot analysis will be used to determine the fraction of PA-1L in each compartment. The accumulation of the protein within the cytoplasm or periplasm for a given secretion mutant will identify the secretion system required. Since, the inability to secrete the PA-1L should correlate with the inability to produce mature biofilms, the nature of the biofilm formed by the relevant secretion mutant will be investigated.

Identification of the PA-1L biofilm target ligand

Since PAO1 biofilms can be disrupted by hydrophobic galactosides and as the corresponding *lecA* mutants form poor biofilms, this suggests that the PA-1L lectin stabilizes biofilms presumably, given its multivalent nature, by cross-linking a bacterially produced polysaccharide (e.g.LPS) or a glycoprotein

(e.g. pilin). Apart from the LPS core oligosaccharide which contains D-galactosamine, there are no obvious galactoside-containing macromolecules although rhamnose, a major component of ***PA*** LPS [**20**] is weakly capable of inhibiting the binding of galactose to PA-1L [**21**]. To determine whether PA-1L is capable of interacting with *PA* LPS O-antigen or core, Western ligand blot analysis will be performed using both glycine and tricine SDS-PAGE [**22**] to separate the purified LPS O-antigen and core components which will be probed with biotin-labelled PA-1L. A similar approach will be used to determine whether PA-1L interacts with any proteins/glycoproteins present in the biofilm extracellular matrix (prepared as described by Wingender *et al* [**23**] or on the bacterial cell surface. These findings will be confirmed using beads coated with PA-1L in agglutination assays with the purified macromolecular target. If PA-1L interacts with any ***PA*** proteins, mass spectrometry will be used to identify the protein; the corresponding gene identified from the genome database and a mutant constructed to evaluate its contribution to biofilm development.

4. **Diagnostic potential of the PA-1L lectin and the QS signal molecules**

Current treatment regimens for controlling ***PA*** infections in CF require aggressive chemotherapy involving prophylactic, nebulised antibiotics and 2 week courses of intravenous antibiotics during infective exacerbations [**1**]. A sensitive marker of airway infection and inflammation would provide a useful tool to guide the initiation and cessation of antibiotic therapy. Previous studies from this group and others have shown that neutrophil elastase/alpha 1 anti-trypsin complex (NE/AAT), lactoferrin and C reactive protein provide an index of the inflammatory process in CF patients and show a reduction following effective anti-pseudomonal treatment in patients colonised with ***PA*** [**24**]. Since PA-1L is regulated via QS and this laboratory has previously shown that AHLs can be detected sensitively in CF sputum using *lux*-based AHL biosensors [**25**], a cohort of CF subjects with differing PA sputum status will be studied to determine whether an increase in sputum AHL levels is a useful marker of clinical deterioration.

In the *PA* colonised CF subjects, we will determine whether the levels of AHLs: i) rise at the time of an exacerbation and ii) fall with antibiotic treatment in line with the clinical response. A group of CF subjects without ***PA*** will serve as a negative control group. However in this group we would expect a proportion of subjects to acquire the organism over the course of the study. By assaying sputum AHL levels in such subjects, we will be able to determine whether AHLs are present in sputum when ***PA*** is acquired which would imply that biofilm formation is an early event or whether AHLs are detectable only when the airways become chronically colonised. The sputum samples obtained will also be assayed for PA-1L (**section 1**) since lectin production is also likely to be an indicator of biofilm formation and also given its cytotoxic properties on respiratory airway cells may also be a useful diagnostic indicator of airway damage. For these clinical investigations, 20 subjects with CF colonised with ***PA***, 20 subjects who have never had ***PA*** isolated and 20 with intermittently positive pseudomonas cultures will be studied. The diagnosis of CF has been established previously in all of these subjects by genetic testing and/or sweat test. The presence of *Burkholderia cepacia* will be an exclusion criteria. There will be no cut off level for FEV_1. To be included in the study subjects must be sputum producers and both adults and children will be studied.

For this clinical study, subjects will be followed longitudinally over 2 years. Subjects will be studied two monthly at clinic visits. Whenever they have an exacerbation they will be studied at the beginning and end of a 2 week course of i.v. antibiotics, one month later then two until their next exacerbation. Measurement of white cell count, C reactive protein, sputum measurement of AHL and spirometry (FEV_1 and FVC) will be made at each visit. Subjects will be treated with a standard antibiotic regime depending on sensitivity testing. This is likely to include an intravenous aminoglycoside (tobramycin/gentamicin) in addition to either Ceftazidime, Meropenem or Aztreonam. Non parametric statistics (Wilcoxon) will be used to determine whether AHL levels are higher during an exacerbation than between exacerbations and whether the levels fall with antibiotic therapy. $P<0.05$ will be regarded as significant.

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Appendix 2

Nottingham City Hospital NHS
NHS Trust

ADULT PATIENT INFORMATION SHEET

LECTIN-MEDIATED BIOFILM MATURATION, QUORUM SENSING AND PSEUDOMONAS AERUGINOSA INFECTIONS IN CYSTIC FIBROSIS

A STUDY OF THE FORMATION OF BACTERIAL COLONIES IN THE LUNGS OF PATIENTS WITH CYSTIC FIBROSIS

PROF A KNOX, DR A SMYTH, DR S CRUSZ

You are being invited to take part in a research study. Before you decide whether you would like to take part it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information (contact details are at the end of this leaflet). Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Pseudomonas aeruginosa is a type of bacteria. It can cause chest infections in many people with Cystic Fibrosis. One way it does this is to form colonies called biofilms in the lungs. Biofilms are made up of bacterial cells and a support structure or matrix. A certain protein made by the bacteria called a lectin is thought to play an important role in this matrix. The aim of this study is to understand this role. To do this we hope to study bacteria from sputum samples collected over a period of two years.

Why have I been chosen?

You have been chosen as you have Cystic Fibrosis and produce sputum. We are studying both people who are known to have Pseudomonas infection and those who are not. We hope to study about sixty patients.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

When you attend the outpatient clinic, we would like to collect a sputum sample. This would be an extra sample in addition to the one that you normally produce. We would also like permission to look at your hospital case notes and test results. We hope to collect a sputum sample each time you attend clinic over a period of two years. We can then study the bacteria in the sputum at times when you are well and during periods when you may be ill.

What are the possible disadvantages of taking part?

There are no specific disadvantages.

What are the possible advantages of taking part?

This study hopes to understand more about how the bacteria Pseudomonas aeruginosa causes lung infections in people with Cystic Fibrosis. This may identify new areas for treatments to be developed. The study will be of no direct benefit to you.

What happens if something goes wrong?

It is unlikely that anything will go wrong as a result of you being in this study. If you are unhappy about anything related to the study, you can mention it to your Cystic Fibrosis doctor or contact me. You are also entitled to contact the complaints department at your hospital.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it. Your CF doctor will know that you are taking part in the study and we will let your GP know if you would like us to.

What will happen to the results of the research study?

We hope to carry out the study over the next two years. We will then publish the findings in a medical research journal. We will send every patient who had participated a summary of the results.

Who is organising and funding the research?

The Cystic Fibrosis team at Nottingham City Hospital is carrying out this study. The Wellcome Trust funds it.

Contact for further information

Dr Shanika Crusz
Wellcome Trust Medical Microbiology Training Fellow
Institute of Infection, Immunity and Inflammation
Centre for Biomolecular Sciences
University of Nottingham
University Park
Nottingham
NG7 2RD

Tel 0115 951 5089

Thank you for taking the time to read this information.

If you wish to take part in this study please keep a copy of this information sheet and a signed copy of the consent form.

Nottingham City Hospital NHS
NHS Trust

PATIENT CONSENT FORM

Title of project	**Lectin-mediated biofilm maturation, quorum sensing and Pseudomonas aeruginosa infections in Cystic Fibrosis**
Site	**Cystic Fibrosis Outpatients' clinics**
Investigators	**Prof A Knox, Dr A Smyth, Dr S Crusz**

The patient should complete the whole of this sheet himself/herself.

Please cross out as necessary

- Have you read & understood the patient information sheet YES/NO
- Have you had opportunity to ask questions & discuss the study YES/NO
- Have all the questions been answered satisfactorily YES/NO
- Have you received enough information about the study YES/NO
- Who have you spoken to Dr/ ……..
- Do you understand that you are free to withdraw from the study
    - at any time YES/NO
    - without having to give a reason YES/NO
    - without affecting your future medical care YES/NO
- Do you agree to take part in the study YES/NO
- Do you agree to your GP being informed YES/NO

Signature (Patient) Date

Name (Block capitals)

I have explained the study to the above patient and he/she has indicated his/her willingness to take part.

Signature (Doctor) Date

Name (In block capitals)

Nottingham City Hospital NHS
NHS Trust

Dear Dr

Your patient,

has given me permission to write to inform you that they have agreed to take part in the study "Lectin-mediated biofilm maturation, quorum sensing and *Pseudomonas aeruginosa* infections in Cystic Fibrosis".

This is a study of bacterial communication (quorum sensing) and the formation of bacterial colonies (biofilms) in the lungs of patients with Cystic Fibrosis. It is funded by The Wellcome Trust and will be conducted by members of the Cystic Fibrosis team, including Prof Alan Knox, Dr Alan Smyth and myself.

We plan to study bacteria from sputum samples collected over a two-year period at Cystic Fibrosis outpatient clinics at Nottingham City Hospital. The aim is to understand more about the pathogenesis of *Pseudomonas aeruginosa* in the Cystic Fibrosis lung and identify novel treatment targets.

The project has been approved by the Director of Research and Development at Nottingham City Hospital and the Nottingham Research Ethics Committee.

Any information collected about your patient will be kept strictly confidential and they have the right to withdraw at any time. At the end of the study, all participants and their GPs will be sent a summary of the findings.

Please do not hesitate to contact me if you would like any further information.

Yours sincerely

Dr Shanika Crusz
Wellcome Trust Medical Microbiology Training Fellow
Institute of Infection, Immunity and Inflammation
Centre for Biomolecular Sciences
University of Nottingham
University Park
Nottingham
NG7 2RD

Appendix 3

Biofilm Study Data Collection Proforma

Patient study no.
Patient sample no.

Current date:					
FEV1	L	FEV1 predicted	L	FEV1	%predicted
FVC	L	FVC predicted	L	FVC	%predicted
Last routine value date:					
FEV1	L	FEV1 predicted	L	FEV1	%predicted
FVC	L	FVC predicted	L	FVC	%predicted

Current Pulse oximetry:

Last available WCC CRP with date:

Antibiotics Dates:
Type:
Duration:
Route:

Clinical features of a pulmonary exacerbation (Rosenfeld *et al*., 2001)

Clinical feature	**A 1 if present 0 if absent**	**B Coefficient**	**AxB**
decreased exercise tolerance		1.8	
increased cough		1.5	
increased sputum / cough congestion		1.5	
absence from school or work		1.6	
increased adventitial sounds on lung examination		1.2	
decreased appetite		1.1	
Total (score of >2.6 = pulmonary exacerbation)			

Decline in FEV1 of >10% in last month Y/N

Microbiology: affix microbiology result overleaf (with identifiers removed)

Appendix 4

Establishing a flowchamber biofilm system

Introduction to the flowchamber biofilm system

The study of the physiological behaviour of bacteria in the laboratory has traditionally been performed on batch-cultures of planktonic cells in suspension. The recognition that most bacterial activity in nature occurs in sessile microbial biofilm communities has prompted the development of technology to enable their study *in vitro*.

Imaging techniques, ranging from simple light microscopy to high resolution electron microscopy, provide visual evidence for the formation of aggregates of bacterial cells, often in association with a surface. This can be applied to a range of ex-*vivo* samples such as sputum and abscess material from the CF lung (Hoiby *et al.*, 2001). The *in vitro* study of these sessile bacterial life forms can be modelled in a number of simple assays of static growth on inert surfaces such as polystyrene microtitre wells (Stepanovic *et al.*, 2000) or stainless steel coupons (Dhir and Dodd, 1995). These approaches, which yield information on bacterial attachment, can be extended to biotic surfaces such as the eukaryotic nematode *Caenorhabditis elegans*. Further animal models of biofilm bacteria can be established in rats and mice by the intratracheal inoculation of bacteria in agar or alginate beads, thus allowing the examination of histopathological changes, the immune response and efficacy of antibiotic therapy (Hoiby *et al.*, 2001).

In nature, biofilms are complex structures consisting of a high density of mixed bacterial populations embedded in a self-produced polymeric matrix, often on submerged surfaces or as flocs in the water column of aquatic environments (Costerton *et al.*, 1999). Within these microbial 'slime cities', internal heterogeneities and structure/function relationships develop which are important to biological activity. It is also known that individual cells and entire communities respond to the prevailing chemical, physical and biological environment. For example, hydrodynamics is known to influence biofilm morphology. Under conditions of low-shear laminar flow, a *P. aeruginosa* PAO1 biofilm was observed to consist of a monolayer of cells with mound-shaped circular microcolonies. However, under high-shear turbulent flow conditions, the biofilm formed filamentous structures (Stoodley *et al.*, 1999). The fluid dynamics within a flowing system assists the dispersal of cells, the arrival of bacteria at

the substratum as well as the concentration of nutrients, signal molecules, waste products and antibiotics.

Whilst a number of laboratory devices such as the rototorque bioreactor and Robbins device have been developed, the flowchamber biofilm system is widely favoured due to its relatively simple design and its applicability to direct on-line microscopic examination of the biofilms. This system utilises a number of important molecular and imaging tools. Biofilms are grown in a defined nutrient and temperature stable environment with a fixed rate laminar flow. Live, fluorescently-tagged bacterial biofilms can be periodically and non-destructively examined with confocal laser scanning microscopy (CLSM). Here, digital images are acquired by photomultiplier tube detection of fluorescence excited by a laser light source. This technology overcomes the problem faced by traditional light microscopy of a loss of resolution as the thickness of the specimen increases due to the contribution from unfocused parts of the viewing field. Instead, by collecting fluorescent light from only the thinnest focal plane afforded by the objective lens and by scanning several planes interspersed by short distances, it is possible to reconstruct virtual three-dimensional (3D) images of the biofilm structures tens of microns thick.

This high quality reconstruction of biofilm images can be achieved by the powerful Unix-based software package IMARIS (Bitplane, Switzerland). In addition, this digital data can be analysed using the COMSTAT image processing software (Heydorn *et al.*, 2000b) to yield a number of biofilm parameters. Together, this qualitative and quantitative approach has greatly assisted in the application of laboratory biofilm study to natural, clinical and industrial settings.

Within this Appendix, the processes undertaken to establish a local flowchamber biofilm system and allied technology are described in detail.

Construction of flowchamber

Biofilms were cultivated in flowchambers kindly provided by Dr Thomas Bjarnsholt of the Center of Biomedical Microbiology (CBM), Danish Technical University (DTU), Copenhagen. A flowchamber was composed of three parallel channels machine-cut in perspex (poly[methyl methacrylate]), covered with a no. 1 24 x 50 mm glass coverslip (SLS Ltd.) which served as the biofilm substratum. Each channel had a dimension

(length x width x height) of 40 x 4 x 4 mm and was wiped down with 70 % (v/v) industrial methylated spirit (IMS) prior to use. To assemble the flowchamber, a thin continuous layer of clear silicone (3M, U.S.A.) was applied sausage-like between the channels with a 2 ml syringe with a cut 200 µl pipette inserted as a nozzle. The glass cover slip was placed on top of the silicone and carefully pushed down until the silicone covered the whole area of perspex between the channels, without actually entering the channels themselves. The silicone was allowed to dry overnight before use. Silicone tubing (Versilic, France) with dimensions 1 mm inner diameter (ID), 3 mm outer diameter (OD), was connected to each end of the flow channel. See Figure A-1.

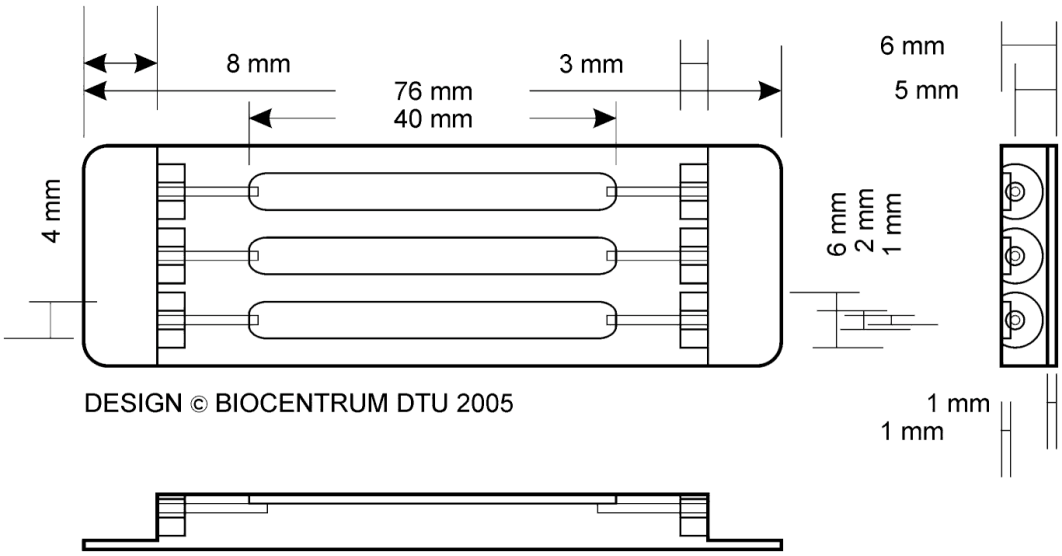


Figure A-1 A 3-channel flowchamber.
A glass cover slip was attached with silicone glue to a perspex base containing three machine-drilled flow channels with dimensions 40 x 4 x 4 mm. Silcone tubing (ID 1 mm and OD 3 mm) was connected to the inlet and outlet of each channel.

Assembly of flow system

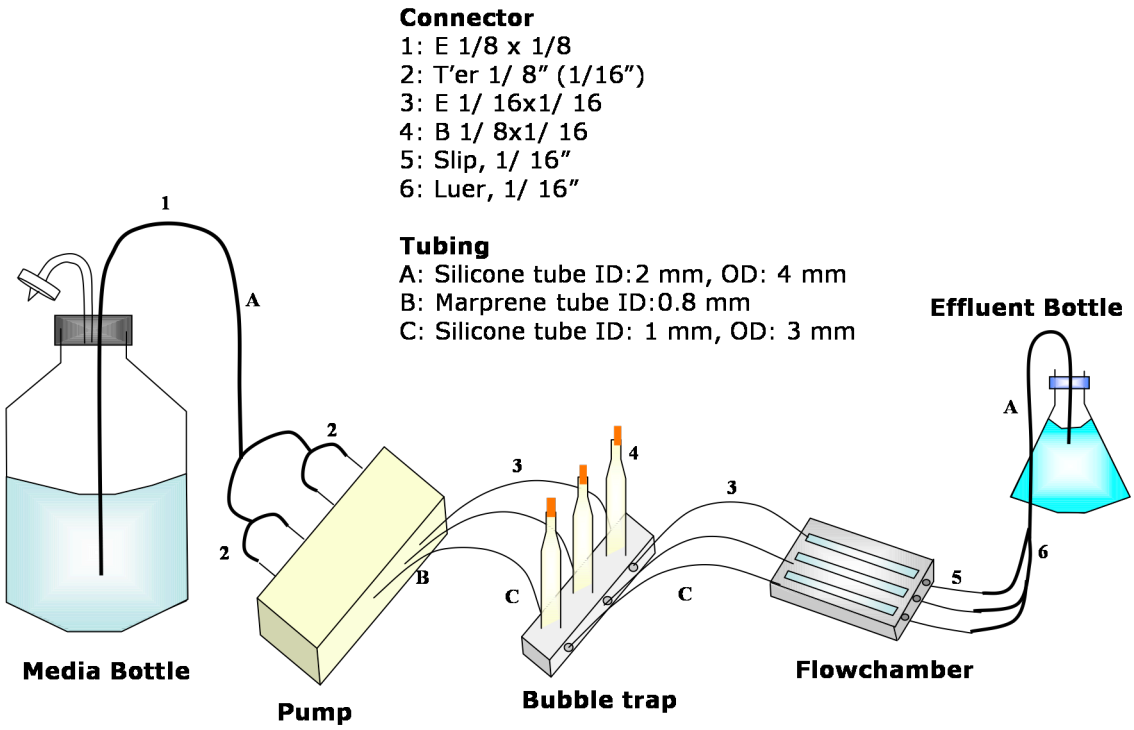
To assemble the flow system, the protocol developed by the CBM, DTU (Haagensen *et al.*, 2006) was followed. Except for the tubes passing through the pump and those attached to the flow channels, the flow system was assembled using 2 mm ID and 4 mm OD silicone tubing. For the pump, the much stronger Marprene tubing, ID 0.88 mm (Watson Marlow Ltd., Cornwall, England), was used. The media bottle (Nalgene

Company, U.S.A.), bubble traps and flowchambers were connected with silicone tubing and plastic connectors as shown in Figure A-2. Midistart 2000 0.20 µl pore venting filters (Sartorius, Germany) were fitted to the media and effluent containers. The connectors between the silicone and inlet Marprene tubing were covered with several layers of parafilm to prevent drag once the pump was operational.

The bubble traps were 5 ml syringes mounted on a perspex base (DTU) with inlet and outlet within the bubble trap. The inlet holes were situated higher than the outlet holes to allow bubbles to rise and hence not enter the onward flowing media. The syringes were closed at the top with stoppers and the bubble traps were placed in petri dish lids to collect spills.

The effluent media was collected in a 10 L vessel which contained 200 ml Trigene surface disinfectant (Medichem International, UK) to give a final working strength of 1:50.

(A)



(B)

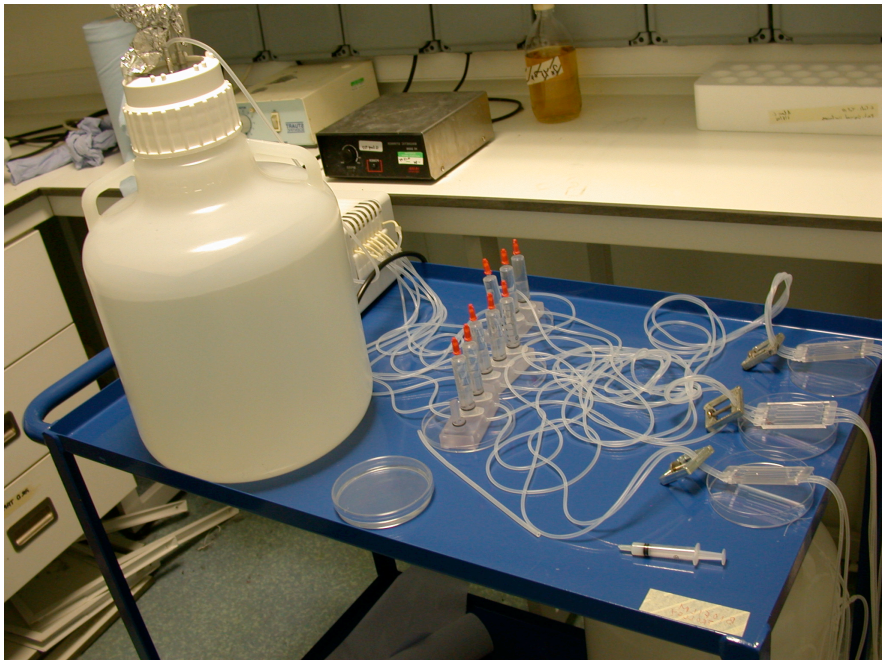


Figure A-2 Original biofilm flowchamber experimental setup.
(A) Schematic representation of the closed, continuous flow system which was constructed by connecting the inlet media vessel, flowchambers and effluent vessel by a series of tubes fitted with connectors. Media was circulated by means of an 8-channel peristaltic pump. Bubble traps were incorporated in to the design upstream of the flow chambers (diagram not to scale). (B) Photograph of the fully assembled system.

Modifications to the design of the flowchamber biofilm system

When running the original flowchamber system at 37 °C, persistent problems of air bubble formation within the tubing and flowchambers were encountered. In order to dislodge bubbles, the flowchambers were gently tapped at their base, however this mechanical force was likely to disrupt the developing biofilms and necessitated constant vigilance.

To overcome this problem, advice was sought from the Renal Dialysis Technicians, Mr John West and Mr Paul Harbuz, Nottingham City Hospital Campus Renal Unit. Together with this and further experimentation, a number of modifications were made to the system design. Firstly, inlet media vessels were rendered airtight with silicone sealant and fitted with an internal noncollapsable tube connected to a venting air filter. These vessels were then inverted and suspended above the level of the flowchambers on clamp-stands, allowing the flow of media by gravity, thus reducing the work of the peristaltic pump. This also reduced the negative pressure created within the tubing by the pulling action of the pump, which itself had encouraged air bubbles to come out of solution.

Prior to running the system, the media outlet port was clamped off and using a 50 ml syringe, air within the inlet vessel above the media was drawn off via the air filter in order to create a negative pressure within the vessel. This allowed any dissolved gases within the media to be drawn out of solution. The system was then allowed to equilibrate prior to running.

It was observed that the 8 channel peristaltic pump itself introduced bubbles into the system, which were able to enter and lodge within the flow channels despite the presence of bubbletraps. Hence, the pump was moved downstream of the flowcells and the bubbletraps were removed from the design. This modification had the added advantage of allowing the pump to gently pull non-attached free-floating biofilm material distally out of the channel so that it would not interfere with the microscopy. See Figure A-3 for a depiction of the unique technological modifications made to the design of the flowchamber system.

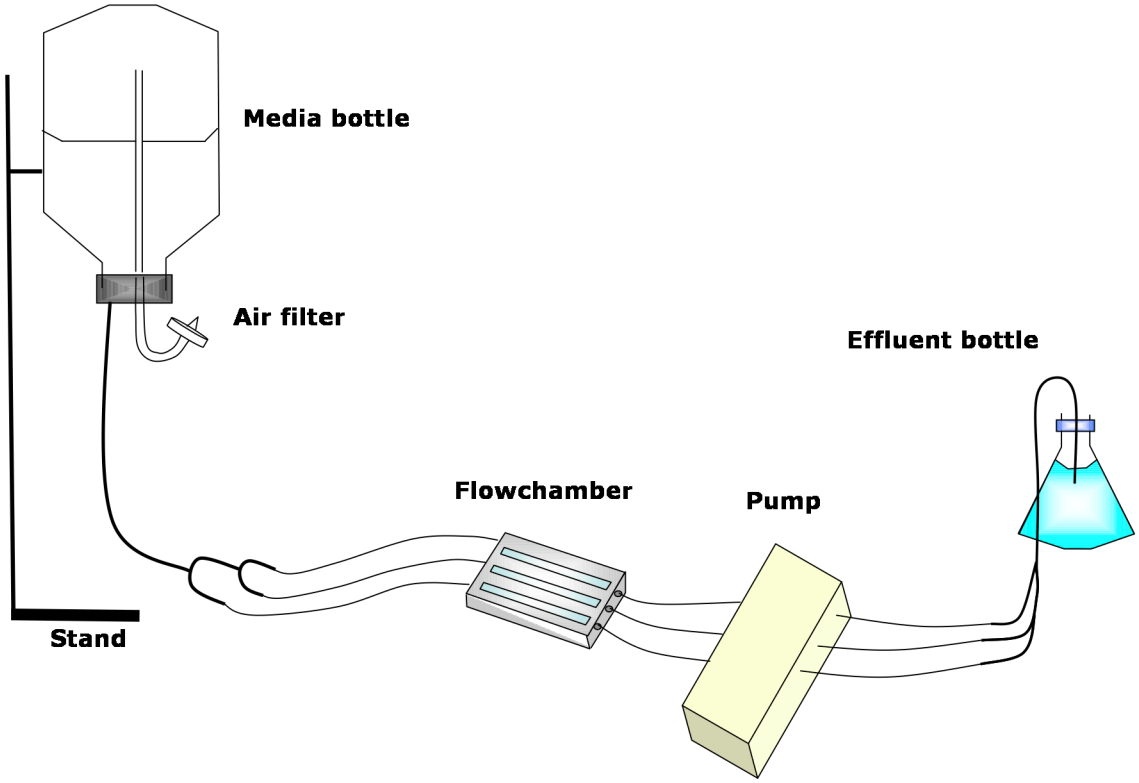


Figure A-3 Modified flowchamber experimental set-up.
The media inlet bottle was inverted and suspended above the height of the flowchamber. Air could be drawn out of the vessel via the air filter connected to a rigid tube in order to create a negative pressure within the chamber, forcing dissolved gases to rise out of solution. The peristaltic pump was positioned downstream of the flowchamber and the bubbletraps were removed from the design (diagram not to scale).

Sterilisation and saturation of flow system

Sterilisation of the flow system was performed by pumping 1 L 0.5 % (v/v) sodium hypochlorite bleach through the system over 3 hours at a pump setting of 0.450 ml/min. The flow system was then washed to remove the hypochlorite by filling and emptying the system two to three times with 1.5 L sterile dH_2O. The flow system was then filled with media pre warmed to 37 °C and the pump was set to 48.3 µl/min which gave a flowrate of 3.3 ml/hr/channel when the system was calibrated. The system was allowed to run overnight at 37 °C to saturate the silicone tubing before inoculation.

Inoculation of flow channels

Before inoculation of the flow channels, the flow was stopped and the tubing between the flow channel and bubble trap clamped off. The effluent container was placed at a

level higher then the flowchambers to prevent air being drawn in to the system when the tubing was breached. The tubing at the flow channel inlet was sterilised with 96 % (v/v) EtOH. A 1 ml insulin syringe (Sterilin) was filled with prepared culture and air bubbles expelled from the syringe. The syringe needle was inserted in to the tubing as near as possible to the flow channel inlet and the cells carefully injected in to the channel. After inoculation, the tubing was sterilised with 96 % (v/v) EtOH and the injection hole sealed by applying a thin layer of silicone. To allow the cells to establish on the glass coverslip substratum, the flowchamber was inverted with the glass surface facing downwards and incubated at 37 °C for 1 h. Following this, the tubing was unclamped, the media flow resumed and the chambers were suspended vertically to ensure that any air bubbles that did enter rose to the distal end of the channel and passed out of it.

Incubation of biofilm flowchamber system

Flowchambers were incubated at 37 °C for up to 6 days. Any backgrowth that formed in the flow channel inlet tubing was cut out on a daily basis to prevent the influence of upstream biomass on the flow chamber biofilms. This was performed by clamping off the tubing downstream of the flowchamber and sterilising the tubing upstream with 96 % (v/v) EtOH. This upstream tubing was then cut with a sterile scalpel immediately above the backgrowth and the tubing containing the backgrowth removed. The upstream tubing was then reconnected aseptically to the flowchamber ensuring that no bubbles were introduced into the channel. The clamps were removed and the flow resumed.

Live / dead staining with propidium iodide

As for inoculation of the flow channels, the flow was stopped and the tubing between the flowchamber and bubble trap clamped off. A bolus of 250 µl 0.2 mM propidium iodide (PI) solution in DMSO was injected into the channel via the downward facing surface of the inlet tubing and the injection site was quickly sealed with silicone. The flowchamber was covered in tin foil to avoid degradation of PI by light and the cells were allowed to incubate for 10 min before two channel CLSM was undertaken.

CLSM image collection

- **Specimen transportation**

The flow system was periodically transported on a two-tier trolley to the microscope facility in order to undertake live confocal microscopic examination of the biofilms on days 4, 5 and 6 incubation. This was generally for a 3 h period, during which unavoidable temperature fluctuations were encountered. This was addressed by covering the inlet media vessel with a plastic insulating jacket to reduce cooling. The flow chambers were examined on a heated microscope stage maintained at 37 °C. The pattern of microscopic examination and hence temperature shift was kept constant for all experimental rounds as far as was practically possible.

- **Microscope settings**

Biofilm images were captured with a Zeiss LSM 510 UV META Kombi confocal system on an inverted Zeiss Axiovert 100M microscope (Carl Zeiss, Germany). Scanning was performed with the Argon ion and HeNe 1 laser units to enable excitation of GFP and PI respectively. The Plan-Neofluar 40x/1.3 oil phase 3 working objective lens was chosen to enable good biofilm depth penetration in addition to visualising reasonable size areas of the field of interest.

The fluorescent filters and tracking were configured to enable the detection of one or more fluorescent proteins. For GFP detection, single-track imaging was performed using the Argon 488 nm laser to excite the specimen and the long pass (LP) 505 emission filter to capture the signal. For simultaneous detection of GFP and PI, multi-track imaging with sequential excitation and capture was performed to reduce bleed through of signal. The 488 nm laser coupled with the 505-530 band pass (BP) filter and the 543 nm laser coupled with the LP560 filter were used for GFP and PI detection respectively.

The scanning parameters were set to capture a frame size of 512 x 512 pixels, with a scan speed of 9 in one direction only. To improve the signal to noise ratio, mean line averaging was performed 4 times. The data depth was set as 8 bit and the pinhole size adjusted to equal 1 airy unit.

- **Specimen placement**

An existing microscope stage with a central 2 x 2.5 cm viewing aperture was utilised which could hold an individual flowchamber. As the microscope was an inverted system, the flowchamber was mounted onto the stage with the coverslip facing downwards. Scanning was undertaken with the pump running at 48.3 µl/min.

Any excess silicone present on the upper surface of the flowchamber prevented it lying flat when inverted and positioned on the microscope stage. This consequently led to distorted and tilted images. Equally, any silicone applied to seal the inlet tubing would also prevent the flowchamber lying flat, hence all inoculations to the channels were performed via the dorsal aspect of the tubing. In addition, imaging of certain peripheral areas of the flowchamber led to tilting by the conical microscope objective, restricted by the fixed viewing aperture of the microscope stage. Furthermore, at these peripheral areas, oil could seep under the stage and its surface tension led to movement of the chamber as well as difficulties with fine focusing. The use of lead weights to secure the flowchamber was necessary to overcome this mobility and in addition, exerted a downward pressure to level the chamber.

When turned coverslip-side down, any non-adherent free-floating biomass within the flowchamber channels settled on top of the biofilms growing on the glass substratum (Figure A-4). This material reduced the quality of the images obtained by causing movement artefact and gave a false impression of the size of the biofilm, necessitating the collection of larger stacks over longer time periods (Figure A-5). This was overcome by gently tapping each channel to dislodge loosely- or non-attached debris and then standing vertically inlet-end up for 15 min prior to imaging to allow this matter to settle and be drawn out of the distal end of the channel (Figure A-6). In addition, subsequent COMSTAT image analysis was performed with the default option of connected volume filtration, which removes biomass that is not in some way connected to the substratum.

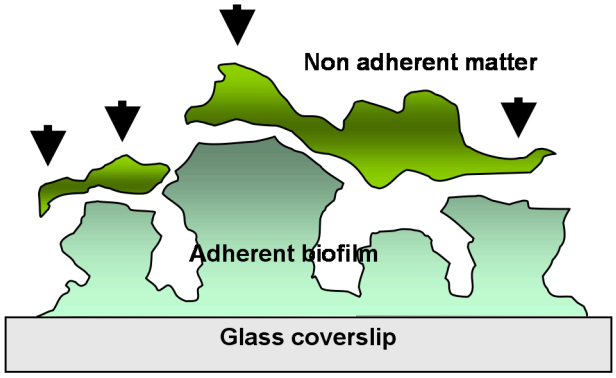


Figure A-4 Non-attached cellular debris settles on top of the glass substratum-attached biofilms when flowchambers are positioned on an inverted microscope system.

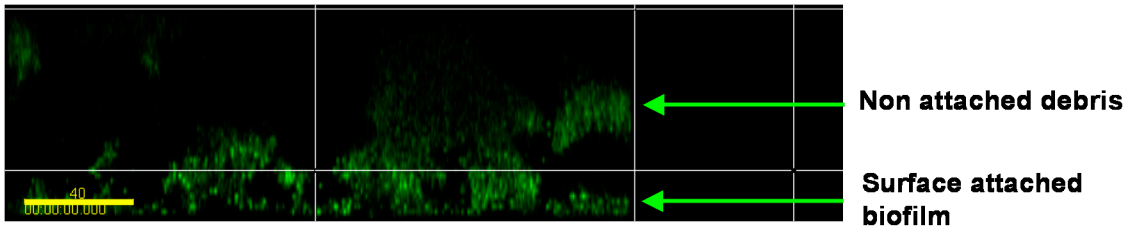


Figure A-5 Side-profile (z view) CLSM image of 3-day old PAO1 biofilm forming on glass coverslip.
Free-floating unattached biomass settles on top of the glass substratum-attached biofilms, giving a false representation of the size and morphology of the structures formed. Micron bar = 40 μm.

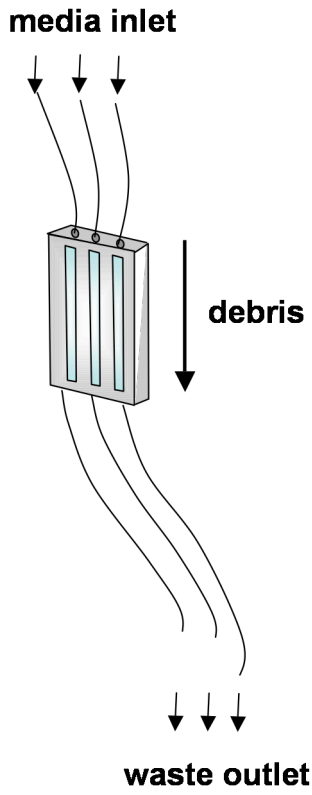


Figure A-6 Inversion of the flowchamber prior to imaging.
Allows unattached debris to be drawn out of the channels by gravity and the action of the downstream pump.

- **Image collection**

With the microscope in visual mode, the bacterial cells were located with phase contrast microscopy at the centre of the channel 5 mm in from the inlet. The microscope was then placed in LSM mode and the first image acquired with the detector sensitivity automatically pre-adjusted. Using continuous fast scanning, the fine focus was adjusted to find the area of maximum brightness (and hence thickness). At this position, the detector gain and amplifier offset were adjusted in order to reduce over (red pixels) and undersaturation (blue pixels) of the image.

A z-series was collected by selecting the first image at the level of the coverslip at the point when the cells first appeared. The focus was then moved to the top of the biofilm and the last image of the z series was set. The interval between slices was adjusted to between 1-2 μm and typically 10 to 40 slices were collected per stack. Four further z-stacks through the biofilm from within an area of 1 mm^2 in each flow channel were

captured randomly by moving the fine position dial a set number of turns in x and y. Each image depicted an area of 230.3 µm x 230.3 µm. See Figure A-7. In total, the 5 captured stacks represented an area of 2.651 x 10^5 μm^2 at the centre of the flowchannel.

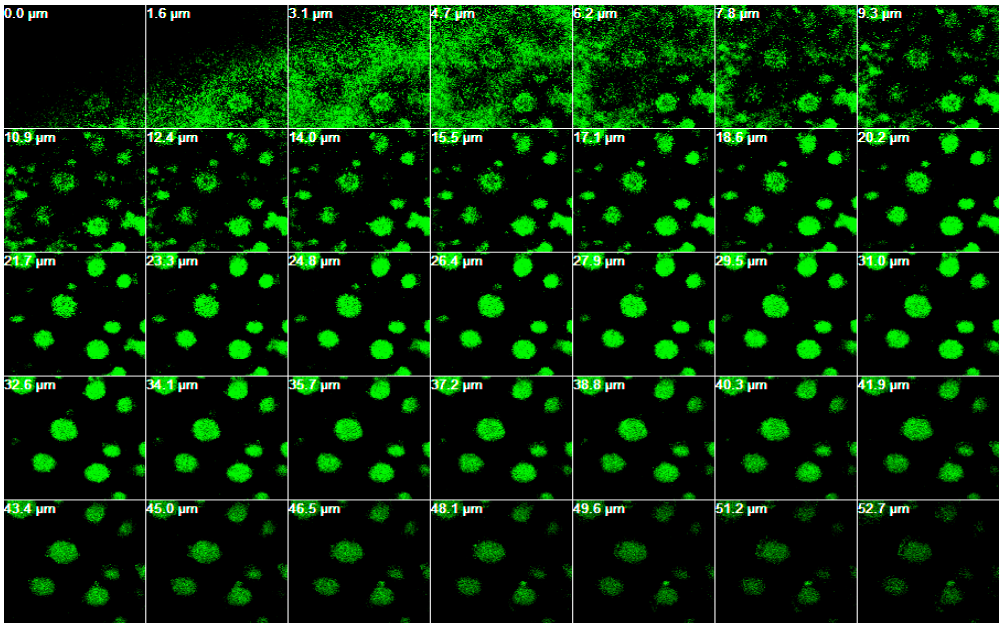


Figure A-7 Series of xy slices collected through z from 4 day PAO1 biofilm.
Slices were separated by an interval of 1.6 µm and each slice represented 230.3 µm x 230.3 µm. A tilted coverslip lead to non-flat initial images.

COMSTAT image analysis

Biofilm structure was quantified from the confocal stacks using the image analysis software package COMSTAT (Heydorn *et al.*, 2000b). The program was written as a script in MATLAB 5.1 (The MathsWorks Inc., Natick, Massachusetts), equipped with the Image Processing Toolbox and was originally designed to analyse images from the Leica TCS4D confocal microscope. Hence to enable COMSTAT analysis of the Zeiss LSM 510 data, the '.lsm' images were converted to '.tif' files using the batch export facility. A user written MATLAB function 'tiffread' (Dr François Nédélec, Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, Heidelberg, Germany) was used to extract the necessary information from the '.tif' files to produce an '.info' file. This contained details of the number of slices in each stack and their

pixel size in x, y and z. A MATLAB script was subsequently written in-house to automate generation of '.info' files for image stacks.

Prior to running COMSTAT, the CONVERT000 program was used to name the image stacks in the appropriate format. The CHECKALL program ensured that all the image stacks were intact. Threshold values were determined with the aid of the LOOK and LOOKTIF programs. Thresholding an image stack resulted in a 3D matrix with a value of ONE in positions where the pixel values in the original image were above or equal to the threshold value and ZERO where the pixel values were below the threshold value. The value ONE represented biomass, while ZERO represented background. To determine the threshold, a random selection of images was chosen. These were assigned different thresholds and the most representative threshold value was determined subjectively by simultaneously scrutinising the LSM acquired images. This value was then applied to all images undertaken within that session.

The COMSTAT program was capable of generating up to 11 different statistical parameters for the purpose of quantifying biofilm 3D structure (see Table A-1). For the present study, three parameters were employed to quantify the differences between biofilms formed by different strains or under different experimental conditions. These parameters were biomass (option 1), average biofilm thickness (option 3) and maximum biofilm thickness (option 11). See Table A-2 for a description of these select image analysis features (Heydorn *et al.*, 2000b).

In addition, the option to correct for a non-flat first image was selected as the calculations performed by COMSTAT depended on the first image of the stack showing exactly the layer of biomass at the substratum. As described earlier, by default, connected volume filtration was performed on all images to remove biomass that was not connected to the substratum. This was deemed to be reasonable for when working with a continuous-flow system, biomass that was not connected to the substratum would be expected to be washed away. However, when analysing biofilms additionally stained with PI, this default was overridden in order to determine the relative biomass of the live and dead cells.

Table A-1 COMSTAT image analysis tools.

COMSTAT option	Description of image analysis tool
1	Biomass
2	Area occupied by bacteria in each layer (also executes 3)
3	Thickness distribution & average thickness
4	Identification and area distribution of microcolonies at the substratum
5	Volumes of microcolonies identified at the substratum (also executes 4)
6	Fractal dimension (Minkowski sausage) of each microcolony identified at the substratum (also executes 4)
7	Fractal dimension (slope of the cross correlation function) in the x and y direction of each image in the stack
8	Dimensionless roughness coefficient (also executes 3)
9	Distribution of diffusion distances and maximum diffusion distance
10	Surface area and surface to volume ratio (also executes 1)
11	Maximum thickness of the biofilm

Table A-2 Description of the COMSTAT image analysis features used in this study.

COMSTAT feature	Unit of meassurement	Description
Biomass	$\mu m^3/\mu m^2$	The biomass was calculated by normalising the volume of the biofilm by the surface area of the field of view, which gives the biomass parameter as biofilm volume per unit surface area. The biomass parameter represents the volume of the biofilm cells present in a given confocal image. stack.
Average thickness	µm	The average thickness was the average biofilm height taken over the entire field of view. Hence average biofilm thickness was a measure of the spatial size of the biofilm.
Maximum thickness	µm	The maximum biofilm thickness was the maximum distance from the substratum that the biofilm colony reaches.
Roughness coefficient	none	Biofilm roughness provided a measure of how much the thickness of the biofilm varied and was an indicator of biofilm heterogeneity.

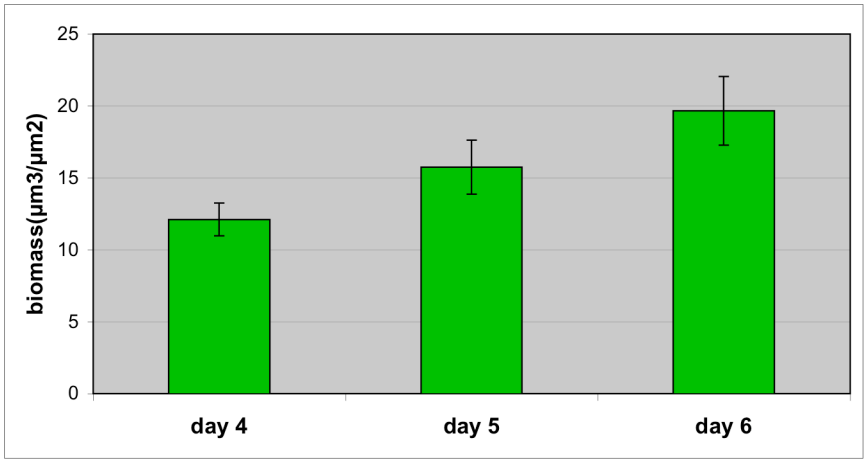
Statistical analysis

Mean and standard error of the mean (SEM) values were based on the average of each parameter calculated by COMSTAT from 5 confocal image stacks per channel per experiment. To illustrate this, see Table A-3 and Figure A-8 for COMSTAT generated data on mean biomass, average thickness and maximum thickness for *P. aeruginosa* PAO1 biofilms grown in four channels from a single experimental round.

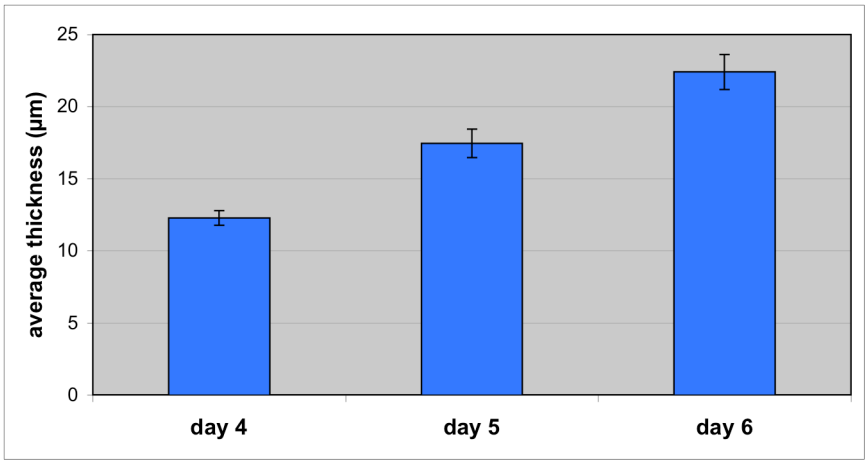
Table A-3 Biomass, average thickness and maximum thickness of 4-, 5- and 6-day old biofilms formed by *P. aeruginosa* PAO1 pUCP18::GFPmut3.1.
Values are means ± SEM of data from 20 image stacks (5 image stacks from 4 channels).

Biofilm parameter	**Day**	***P. aeruginosa* PAO1**
Biomass ($\mu m^3/\mu m^2$)	4	12.10 ± 0.57
	5	15.74 ± 0.94
	6	19.67 ± 1.19
Average thickness (μm)	4	12.27 ± 0.50
	5	17.44 ± 0.98
	6	22.40 ± 1.21
Maximum thickness (μm)	4	28.34 ± 0.93
	5	36.25 ± 1.86
	6	44.72 ± 2.56

(A)



(B)



(C)

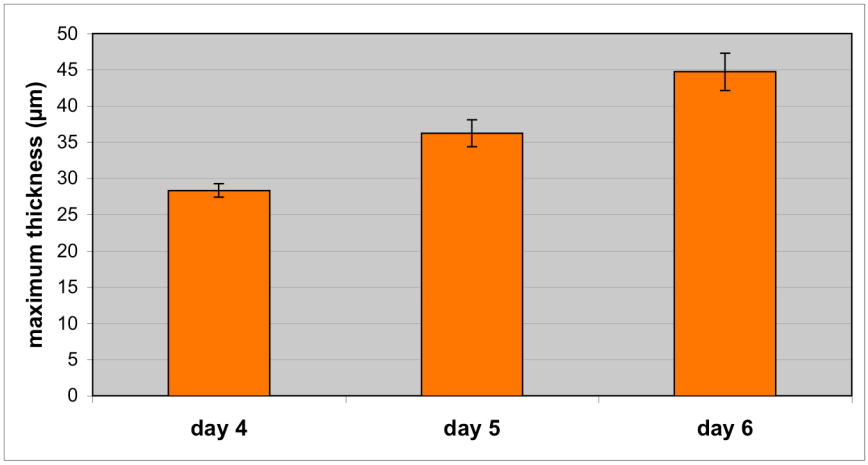


Figure A-8 (A) Biomass, (B) average thickness and (C) maximum thickness of 4-, 5- and 6-day old biofilms formed by *P. aeruginosa* PAO1.
Values are means ± SEM of data from 20 image stacks (5 image stacks from 4 channels).

When investigating the difference between test conditions or test strains, usually three independent experimental rounds were performed with analysis of the pooled data. Using SPSS for windows, interexperimental variation and interchannel variation were assigned as random effects and were incorporated into an analysis of variance (ANOVA) using a general linear model (GLM). This indicated that whilst both channel and experiment were having an effect, this could be disregarded. Subsequently, therefore, the analysis applied the student's t test to determine whether the difference between the means of two data sets was significant at $P < 0.05$ (http://www.physics.csbsju.edu/stats/t-test.html).

IMARIS image processing and rendering

Simulated 3D images were obtained using the IMARIS package (BITplane, Switzerland). Raw z-stack data sets were imported as confocal '.lsm' files and opened within the software. Image processing functions were applied to enable 3D reconstruction with the option to render the surface morphology solid. This enabled a visual representation of the biofilms captured which were saved using the 'snapshot' function as '.tif' files. See Figure A-9 for examples of *P. aeruginosa* PAO1 pUCP::GFPmut3.1 four-day old biofilms.

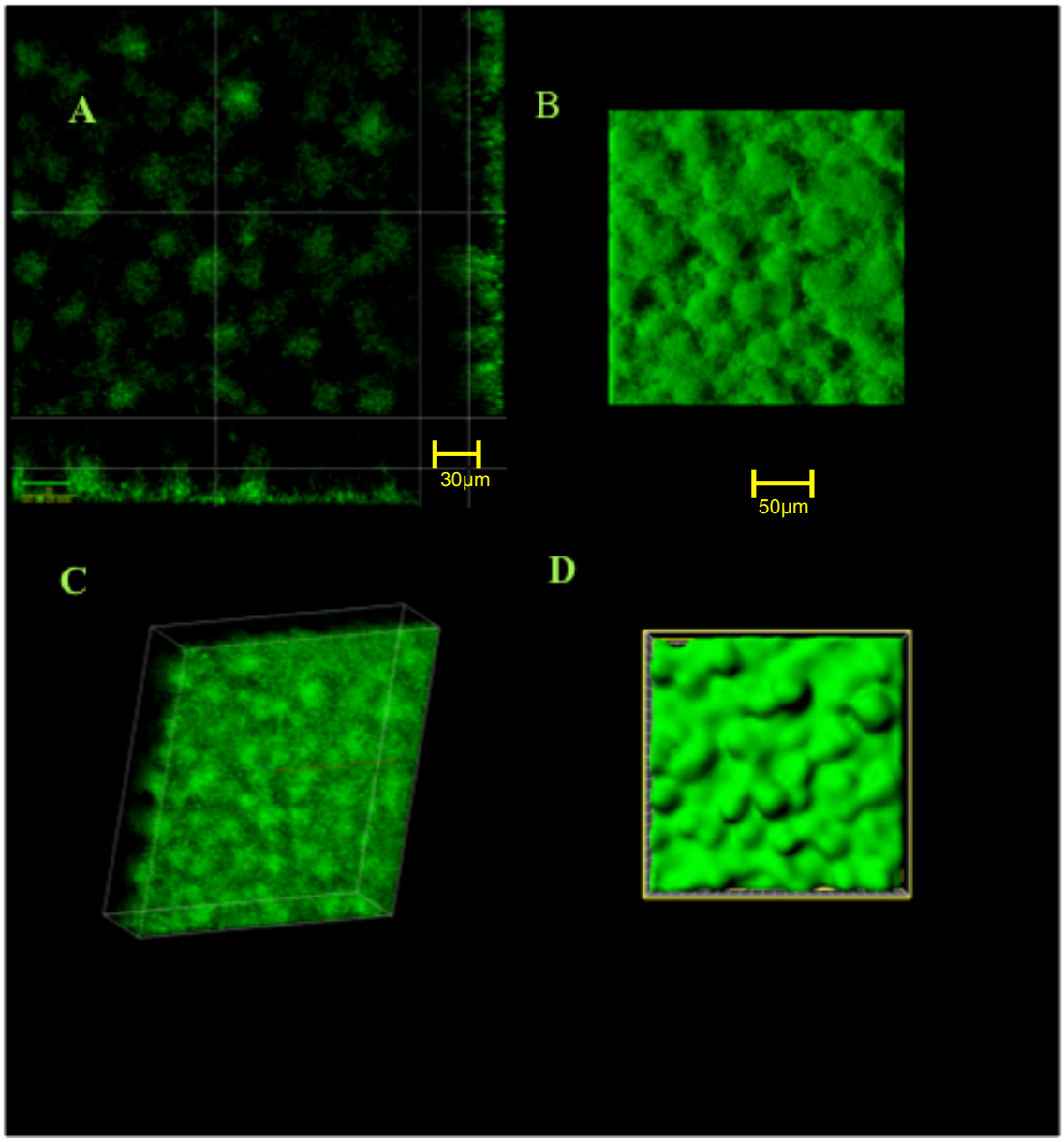


Figure A-9 IMARIS reconstruction of 4-day old PAO1 biofilm image stack.
(A) xy section through biofilm with side panels depicting microcolony profiles through z.
(B) 3D rendered image viewed from above.
(C) Full 3D rendered image
(D) 'Isosurface' image with space-filling and surface smoothing

Live / dead staining

With the flowchamber system established and optimised and the technology in place to undertake the image analysis, it was possible to increase the sophistication of the methodology to capture two separate fluorophores. Thus far, live cells expressing a plasmid-based GFP (pUCP::GFPmut3.1) were captured. This 26.9 kDa fluorescent protein was originally isolated from the jelly fish *Aequorea victoria* and has a major excitation peak at a wavelength of 395 nm and an emission peak at 509 nm, which is in the lower green portion of the visible spectrum. Extensive engineering of the original protein has led to many mutants with different spectral characteristics, which have a wide range of applications.

Propidium iodide is a fluorescent molecule and intercalating agent, which binds both DNA and RNA. PI is membrane impermeant and is therefore generally excluded from viable cells. This, together with its broad emission spectrum peaking in the orange at 620 nm, allows it to be used to identify dead cells in a population and as a counterstain in multicolour fluorescent techniques.

The potential to use GFP in conjunction with PI staining to distinguish live and dead biomass was investigated in the flowchamber system. Five-day old *P. aeruginosa* PAO1 pUCP::GFPmut3.1-labelled biofilms were treated with the aminoglycoside antibiotic tobramycin, which is frequently used in the treatment of CF lung disease. Confocal imaging, with the simultaneous use of the Argon ion and HeNe 1 laser units, was undertaken on day 6 following incubation with locally applied propidium iodide. COMSTAT calculation of biomass was performed on the separate green (live) and red (dead) signals (Figure A-10). The IMARIS reconstructions of these dual labelled images gave a striking view of the action of this antibiotic on the biofilm (Figure A-11).

Figure A-10 Effect of tobramycin on day 6 PAO1 biofilm biomass viability.
The analysis was conducted using the COMSTAT programme to quantify biofilm biomass. Day 5 biofilms growing in 2 separate channels were incubated with 340 µg/ml tobramycin for 20 h. Multi-track CLSM was undertaken on day 6 following PI staining, with 5 image stacks collected per channel. Mean ± SEM of 2 independent experimental rounds (10 image stacks per experiment) plotted.

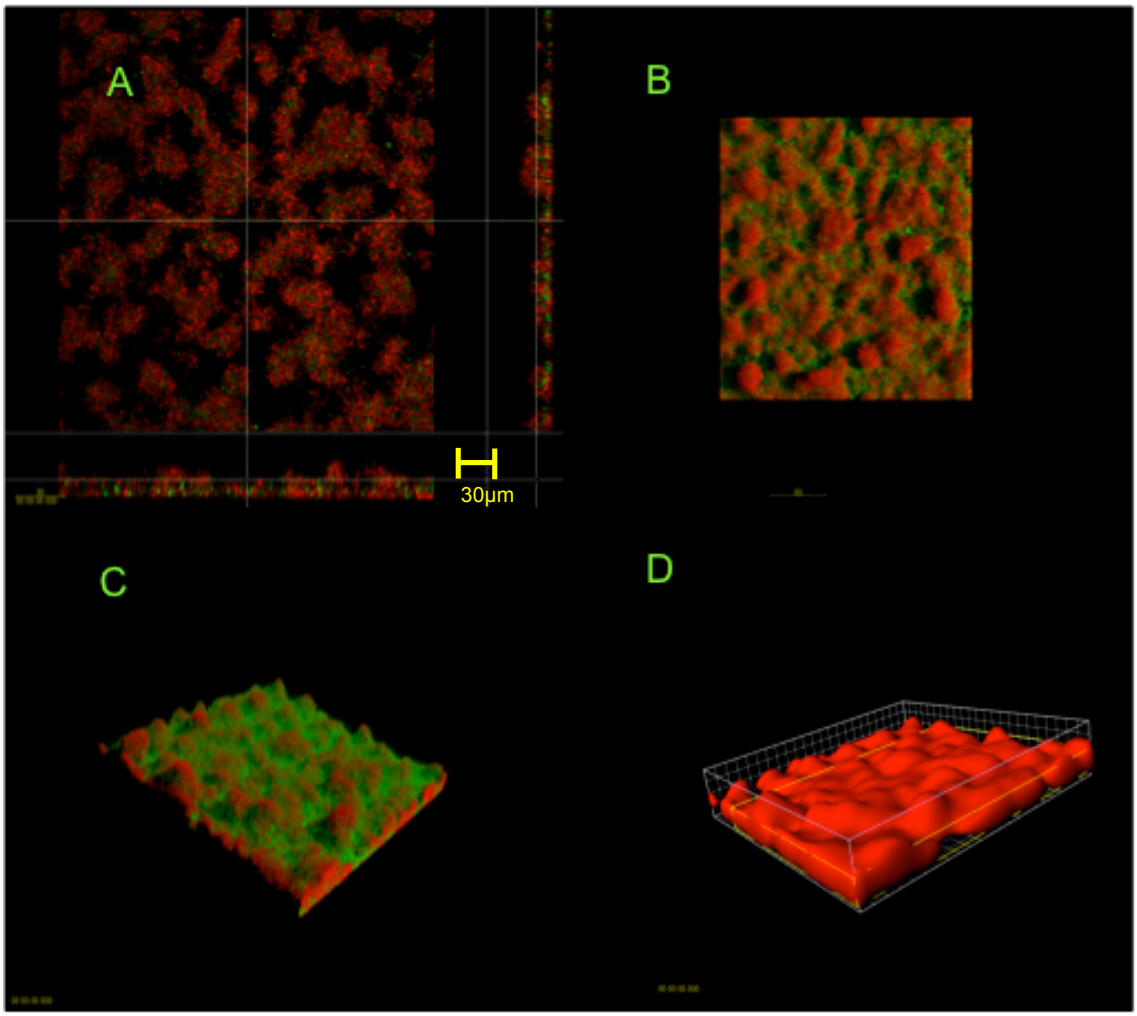


Figure A-11 IMARIS reconstruction of two-channel CSLM imaging of 6-day old PAO1 biofilm following 20 h exposure to 340 µg/ml tobramycin.
Bacterial viability was assessed using PI staining: live cells appear green; dead cells are red.
(A) XY section through biofilm with side panels depicting microcolony profiles through Z
(B) 3D rendered image viewed from above
(C) Full 3D rendered image
(D) 'Isosurface' image, with space-filling and surface smoothing

General comments on the establishment and operation of a flowchamber biofilm system

The construction and operation of a flowchamber biofilm system posed a number of technical and practical considerations such as access to dedicated equipment (peristaltic pump, media vessels, flowchambers etc.) and appropriate autoclave, incubation and imaging facilities. The general design was modified to overcome the issue of bubble formation in the system and the method of image capture adapted to the particular specification of the confocal microscope. Furthermore, the analysis of the digital data captured required the application of the specialist software packages COMSTAT and IMARIS.

Whilst a popular model system, the flowchamber biofilm method does have some disadvantages. Contamination of the large volumes of liquid media can occur unless adequate autoclaving is performed. Chemical sterilisation with hypochlorite bleach requires careful attention to aseptic technique as do subsequent manipulations to the tubing in order to inoculate strains and remove backgrowth. Any enclosed system is subject to wall effects with aberrations in flow in the corners of the chamber (Palmer, 1999). Finally, in cases of high biomass within the flowchamber, it is possible that a gradient of nutrients and waste products could be established over its length.

These latter issues expose one of the fundamental challenges to any study of biofilm formation in that it is to a certain extent a stochastic process (Heydorn *et al.*, 2000a). To address this and in order to achieve robust biofilm descriptive data, guidance from Heydorn *et al*. (2000) was followed in an effort to ensure that all allied conditions were kept constant so that any variations observed could be attributed to differences in the test condition.

Specifically, in order to minimise variation between successive experimental rounds, the main factors that influence biofilm formation were kept constant. Hence, flow rate was fixed at 3.3 ml/hr/channel, biofilms were routinely grown at 37 °C and any temperature shifts were reproduced. The nutrient composition of the media was not varied and cultures with the same history were used for all experiments. In addition, the same operator performed all experimental rounds and specifically fixed the microscope and COMSTAT thresholds (which is a subjective process).

To minimise variation between channels containing the same strain, machine-made flow channels were used so that biofilm structure variation caused by small differences in channel shape and size could be minimised. Attention was paid to large macrocolonies growing at the inlet of the channel which could exhaust the carbon source, leaving the downstream growth as essentially a single layer of cells. Additionally, backgrowth forming in the upstream tubing was removed daily for the same reasons.

Within each channel, spatial heterogeneity in biofilm structure was observed. For example, the biofilm near the inlet was typically thicker than the biofilm near the outlet and the biofilms in the regions near the sides of the channel often displayed differences in structure to those forming in the centre. Hence, the issue of representative sampling was considered. The nature of the microscope stage itself limited the area of the flow channel that could be imaged as previously discussed. The decision to capture images from 5 random positions from an area 1 mm^2 at the centre of the channel 5 mm from the inlet balanced practical considerations with the satisfaction that an adequate area had been investigated in order to obtain representative data (Korber, 1993).

The impact of these practices was assessed statistically using both sophisticated (ANOVA using a GLM within SPSS for windows) and simple (student's t test via a web-based link) approaches. The effect of doing the more sophisticated analysis incorporating experimental round and different channel would be to explain some of the variation as variation between experimental round and channel and leave some as random error. By performing the simple t-test, the assumption was made that all the variation was random error and this was in fact the more conservative approach. Simply stated, if a significant difference was observed with a t-test then the difference would also be significant in the GLM. The added complexity of performing the GLM would be useful in the situation when the t-test was not significant and further work could be performed to find evidence for a difference with the GLM.

Full implementation of this locally developed system enabled the successful growth, imaging and analysis of biofilms of *P. aeruginosa* PAO1 labelled with the fluorescent tag pUCP18::GFPmut3.1. By day 4, established biofilms around 12 μm deep had formed with a characteristic surface topology made up of microcolonies. Additional growth of around 5 μm in thickness was observed each day thereafter. This data represented the benchmark for the behaviour of the wildtype PAO1 strain in this particular system. In addition, the ability to undertake more sophisticated analyses was

confirmed using live / dead staining following exposure of the biofilm to tobramycin. These successes firmly established this locally-implemented flowchamber system as a robust model with many potential applications.

Appendix 5

The QS signal molecule profile and *lecA* status of the cohort of clinical isolates

LecA produced								
Strain	**Mucoid/ nonmucoid**	**Pigment**	***lecA***	**LecA**	**C4**	**C12**	**HHQ**	**PQS**
A001-200804	nm	none	*y*	y	y	y	y	y
A002-051104	m	none	*y*	y	y	y	y	y
A003-280504	nm	green	*y*	y	y	y low	y	y
A004-130804	m	pink	*y*	y	y	y	y	y
A005-100904	nm	green	*y*	y	y	y low	y	y
A007-110604	nm	green	*y*	y	y	y	y	y
A009-110604	nm	green	*y*	y	y	y low	y	y
A012-180604A	nm	green	*y*	y	y	**n**	y	y
A012-180604B	m	none	*y*	y low	**n**	**n**	y	y
A017-081004	m	green	*y*	y	y	**n**	y	y
A019-040205	nm	green	y	y	y	**n**	y	y
A021-101204A	nm	green	y	y	y	y	y	y
A021-101204B	nm	green	y	y	y	**n**	y	y
A023-200804	nm	green	y	y	y	y	y	y
A024-270804	nm	green	y	y	y	**n**	y	y
A026-130804	nm	green	y	y	y	**n**	y	y
A029-110305	nm	green	y	y	y	**n**	y	y
A032-200804	nm	green	y	y	y	**n**	y	y
A033-200804	nm	green	y	y	y	y	y	y
A035-051104A	m	pink	y	y	y	y	y	y
A035-051104B	nm	green	y	y	y	y	y	y
A037-230205A	m	pink	y	y	y	y	y	y
A037-230205B	nm	green	y	y	y	y	y	y
P003-170804	nm	green	y	y	y low	**n**	y	**n**
P004-010205	nm	green	y	y	y	y low	y	y
P006-170804	nm	green	y	y	y low	**n**	y	**n**
P007-280904	nm	green	y	y	y	**n**	y	y
P009-280904	nm	green	y	y	y	**n**	y	y
P010-211204	m	green	y	y	y	**n**	y	y
P013-101204A	m	none	y	y	y	**n**	y	y
P013-101204B	nm	green	y	y	y	**n**	y	y
P015-170804A	m	none	y	y	y	**n**	y	y
P015-170804B	nm	green	y	y	y	**n**	y	y
P016-280904	nm	none	y	y	y	**n**	y	y
P018-161104	nm	green	y	y	y	**n**	y	y
P024-070904	m	green	y	y	y	y	y	y

no LecA								
Strain	**Mucoid/ nonmucoid**	**Pigment**	***lecA***	**LecA**	**C4**	**C12**	**HHQ**	**PQS**
A014-291004	nm	pink	**n**	**n**	y	y	y	y
A018-151004	nm	green	**n**	**n**	y	y	y	y
A025-221004	m	pink	**n**	**n**	y	y	y	y
A031-030904	nm	green	**n**	**n**	y	**n**	y	y
P010-191004	nm	green	**n**	**n**	y	y	y	y
P020-191004	m	none	**n**	**n**	y	y	y	y
P021-211204	nm	green	y	**n**	y	**n**	y	y