

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Spatial and temporal localisation of exopolysaccharide  
gene expression in mucoid and non-mucoid  
*Pseudomonas aeruginosa* biofilms

a thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Microbiology

Massey University, Manawatu,

New Zealand

Stacey Ann Kashel Holbrook

2014

## Abstract

The biofilm, or surface-associated microbial community, is the preferred method of growth for most bacteria. *Pseudomonas aeruginosa* is an ubiquitous, opportunistic pathogen capable of biofilm formation in a wide range of natural and clinical environments. In particular, biofilms formed by *P. aeruginosa* in the lungs of people with cystic fibrosis (CF) are responsible for a significant decline in the health and prognosis of these patients. Once established, *P. aeruginosa* biofilms begin to excrete an exopolysaccharide (EPS) called alginate which protects the bacterial microcolonies from antimicrobial molecules and confers a mucoid phenotype. Once this phenotypic switch has occurred, the biofilm becomes impossible to eradicate and ultimately leads to the death of the patient. Here, fluorescent signalling systems and confocal laser scanning microscopy (CLSM) have been used to spatially and temporally resolve the expression of three EPSs produced by *P. aeruginosa*; the pellicle-forming EPS (Pel), the EPS encoded by the polysaccharide synthesis locus (Psl) and alginate. In order to observe the effect (if any) of EPS production on spatial localisation of the cells within the biofilm, the biofilm-associated characteristics of three *P. aeruginosa* double-knockout mutants, each able to produce only one EPS has been observed. In analysing these biofilm structures, it was found that Pel has a role in facilitating an increased surface area of the biofilm, while Psl-producing mutants form a biofilm structure with a significantly increased biomass. By visualising fluorescent signals throughout a biofilm consisting of a mixture of the three mutants, the spatial localisation of EPS-producing bacterial populations has been observed. Here, Pel-producing mutants tended to aggregate at the attachment surface, suggesting a role in adhesion of the biofilm structure. Spatial and temporal localisation of EPS promoter activity was achieved by transforming the prototypic *P. aeruginosa* PAO1 strain with one of three plasmids encoding unstable *gfp* expression under the control of each EPS's promoter sequence. Overall, this study has demonstrated the applications and limitations of fluorescence-based localisation of bacterial gene expression throughout *P. aeruginosa* biofilm development. Collectively, this information can help to guide future investigations into the expression and regulation of the genes associated with a biofilm phenotype, with the aim of identifying a target for effective therapy against this important pathogen.

## Acknowledgements

To everyone who

believed in me when I didn't believe in myself,

helped me find my way when I was lost

and didn't give up on me.

You know who you are.

Thank you.

## Abbreviations

3-D	3-dimensional
Ap	ampicillin
Ap <sup>R</sup>	ampicillin resistance
bfp	blue fluorescent protein
bp	base pair(s)
c-di-GMP	cyclic diguanylate monophosphate
Cb	carbencillin
Cb <sup>R</sup>	carbencillin resistance
CF	cystic fibrosis
cfp	cyan fluorescent protein
CLSM	confocal laser-scanning microscopy
Δ	delta (deletion of)
DGC	diguanylate cyclase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
g	gram(s)
GDP	guanosine diphosphate
<i>gfp</i>	gene encoding green fluorescent protein
gfp	green fluorescent protein
Gm	gentamicin
Gm <sup>R</sup>	gentamicin resistance
h	hour(s)
HcRed	<i>Heteractis crispata</i> red fluorescent protein
HSL	homoserine lactone

IFN	interferon
L	litre(s)
LB	Luria-Bertani
m	milli-
M	moles per litre
μ	micro-
Milli-Q	ultrapure, Type 1 filtered and deionised water (Millipore™)
min	minute(s)
MOPS	3-propanesulfonic acid
Mm	millimetre
nm	nanometre(s)
OD <sub>600</sub>	optical density at 600 nm
<i>pel</i>	gene encoding Pel (pellicle-forming) exopolysaccharide
Pel	exopolysaccharide encoded by <i>pel</i> gene
PDE	phosphodiesterases
<i>psl</i>	polysaccharide synthesis locus
Psl	exopolysaccharide encoded by <i>psl</i> gene
QS	quorum sensing
RNA	ribonucleic acid
RSCV	rugose small-colony variant
s	second(s)
SDS	sodium dodecyl sulfate
TBE	tris/borate/EDTA
TCS	two-component signalling
U	unit(s)
V	volt(s)
w/w	weight by weight
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

## Table of contents

Abstract.....	ii
Acknowledgements.....	iii
Abbreviations.....	iv
Table of contents.....	vi
Figure legend.....	viii
Table legend.....	x
1.0 Introduction.....	1
1.1 Biofilms.....	1
1.2 <i>Pseudomonas aeruginosa</i> .....	2
1.3 <i>P. aeruginosa</i> biofilms in cystic fibrosis patients.....	3
1.4 Discovery of EPSs in <i>P. aeruginosa</i> .....	3
1.4 EPSs in biofilm development.....	5
1.5 EPS biosynthesis and regulation.....	7
1.6 EPSs as potential targets for biofilm therapies.....	9
1.7 <i>In vitro</i> biofilm studies.....	10
1.8 Current understanding.....	11
1.9 Hypothesis and design.....	12
1.10 Aims and objectives.....	13
2.0 Materials and methods.....	15
2.1 Strains, plasmids and oligonucleotides.....	15
2.2 Media.....	18
2.3 DNA manipulation.....	21
2.4 Continuous-culture biofilm system.....	29
2.5 Microscopy techniques.....	32

3.0	Results.....	34
3.1	Introduction.....	34
3.2	Fluorescent protein labelling of PDO300 double-knockout mutants .....	34
3.3	Effect of EPS production on biofilm structure .....	36
3.4	Spatial localisation of PDO300 strains in mature, mucoid biofilms. ....	38
3.5	Temporal localisation of EPS gene expression in PAO1 biofilms.....	41
3.6	Spatial localisation of EPS gene expression in PAO1 biofilms.....	46
3.7	Attempted generation of blue and red unstable fluorescent proteins.....	51
4.0	Discussion.....	53
5.0	Conclusions.....	56
6.0	Limitations and future directions.....	57
7.0	Publication .....	58
8.0	References .....	59

## Figure legend

Figure 1.1. Schematic representation of the stages involved in polymicrobial biofilm formation (Phillips, <i>et al.</i> , 2009). .....	2
Figure 2.1. The continuous-culture biofilm system (Jakobsen, <i>et al.</i> , 2011). .....	29
Figure 2.2. The dimensions of the bubble trap apparatus (Nielsen, <i>et al.</i> , 2011). .....	30
Figure 2.3. The dimensions of the flowcell chamber (Nielsen, <i>et al.</i> , 2011). .....	30
Figure 3.1. Confirmation of the fluorescence of the double-knockout, single-EPS-producing <i>P. aeruginosa</i> PDO300 strains. ....	35
Figure 3.2. Biofilm structure produced by <i>P. aeruginosa</i> PDO300 double-knockout mutants. ....	37
Figure 3.3. The mature biofilm structure formed by wild-type <i>P. aeruginosa</i> PDO300 compared with the mixed mutant biofilm. ....	39
Fig. 3.4. Spatial localisation of the fluorescently-labelled <i>P. aeruginosa</i> PDO300 double-knockout mutants within the mixed mutant biofilm. ....	40
Fig. 3.5. EPS promoter activity demonstrated with unstable <i>gfp</i> expression in 24-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	42
Fig. 3.6. EPS promoter activity demonstrated with unstable <i>gfp</i> expression in 48-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	43
Fig. 3.7. EPS promoter activity demonstrated with unstable <i>gfp</i> expression in 72-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	44
Fig. 3.8. EPS promoter activity demonstrated with unstable <i>gfp</i> expression in 96-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	45
Fig. 3.9. EPS promoter activity demonstrated with stable <i>gfp</i> expression in 24-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	47
Fig. 3.10. EPS promoter activity demonstrated with stable <i>gfp</i> expression in 48-hour-old <i>P. aeruginosa</i> PAO1 biofilms. ....	48

Fig. 3.11. EPS promoter activity demonstrated with stable *gfp* expression in 72-hour-old *P. aeruginosa* PAO1 biofilms. .... 49

Fig. 3.12. EPS promoter activity demonstrated with stable *gfp* expression in 96-hour-old *P. aeruginosa* PAO1 biofilms. .... 50

Fig. 3.13 Synthesised blue fluorescent protein gene sequence (blue) with AAV tag (red)... 52

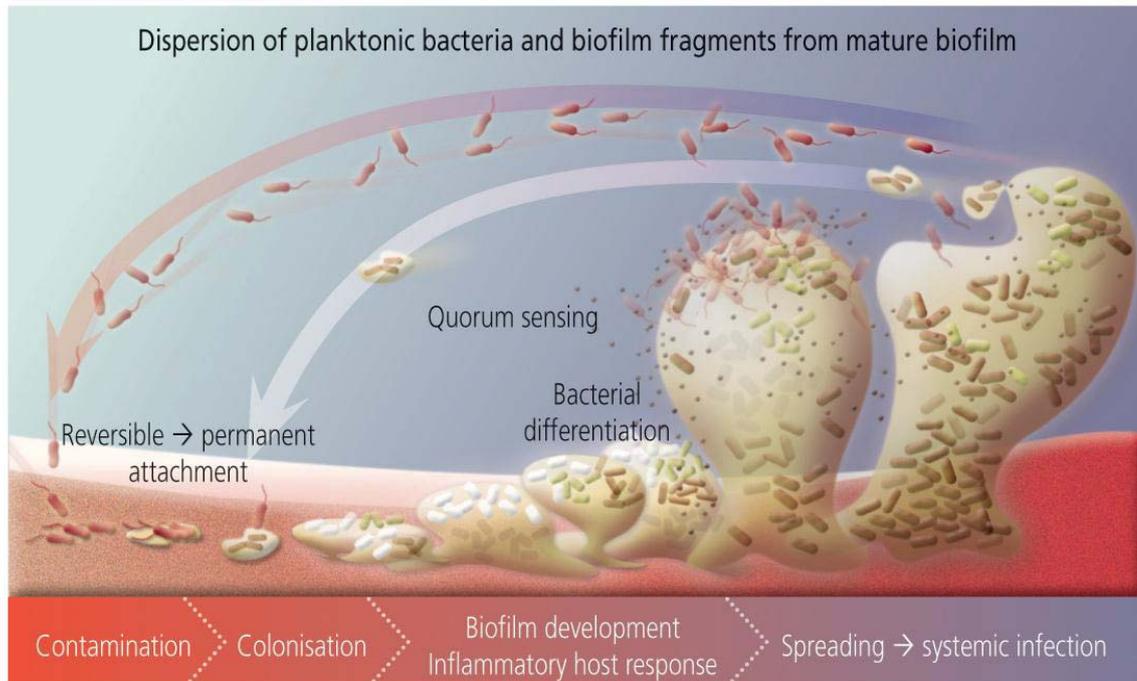
## Table legend

Table 1.1. Established roles of EPSs in <i>P. aeruginosa</i> biofilms (Wei & Ma, 2013).....	4
Table 2.1. Bacterial strains used in this study.....	15
Table 2.2. Plasmids used in this study.....	16
Table 2.3. Oligonucleotides used in this study.....	17
Table 2.4. Antibiotic concentrations used in this study.....	20
Table 2.5. <i>Pfx</i> DNA polymerase PCR reaction mixture.....	23
Table 2.6. <i>Taq</i> DNA Polymerase PCR reaction mixture.....	23
Table 2.7. A-tailing reaction mixture.....	24
Table 2.8. Tn7 transposon delivery plasmids and strains used to label <i>P. aeruginosa</i> PDO300 double-knockout mutants.....	25
Table 2.9. Restriction endonuclease reaction mixture.....	26
Table 2.10. Fluorescence parameters for CLSM.....	32

## 1.0 Introduction

### 1.1 Biofilms

A biofilm is a community of microorganisms embedded within an extracellular matrix which is immobilised at a surface. It has been estimated that over 99% of bacteria in the natural and pathogenic environment exist within a metabolically integrated biofilm, with the majority of these biofilms consisting of numerous bacterial species which undergo mutually beneficial interactions (Costerton, 2007). Biofilm-associated bacteria express a physiologically distinct phenotype when compared with their planktonic counterparts, and are generally found in aqueous environments. Bacterial biofilms were first described in 1935, through an accidental observation that bacterial aggregates would attach to glass slides left submerged in aquatic ecosystems (Henrici & Johnson, 1935). Today, these bacterial communities account for a large proportion of chronic infections seen in hospitalised and immunocompromised patients (Mulcahy, *et al.*, 2013). Biofilms pose a significant threat to hospitalised patients, with over 50% of nosocomial infections being attributable to bacterial biofilms associated with the surface of endoprostheses. (Camarrota & Sant'Anna, 1998). Because the interactions within multispecies biofilms are extremely complex and difficult to study, this complicates their analysis and eradication via traditional diagnostic and treatment methods. *Pseudomonas aeruginosa* is a significant opportunistic pathogen which has been extensively utilised as a model organism for biofilm studies (Mulcahy, *et al.*, 2013). Through *in vitro* investigations, the stages which are involved in the formation of a mature biofilm structure have been clearly established (Figure 1.1). Briefly, planktonic bacteria settle on a surface and first attach reversibly before becoming permanently attached and non-motile. They then form microscopic colonies or microcolonies; essentially an aggregation of bacterial cells which begin to signal to each other and work together to form the biofilm matrix. The protein expression profile of these microcolony-associated bacteria is significantly different to their planktonic counterparts, allowing the production of compounds and signalling molecules which facilitate co-operative growth (Sauer, *et al.*, 2002).



**Figure 1.1. Schematic representation of the stages involved in polymicrobial biofilm formation (Phillips, *et al.*, 2009).**

Biofilm development can be categorised into the following distinct stages; reversible attachment, permanent attachment, microcolony formation, biofilm maturation and dispersion of planktonic bacteria.

## 1.2 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an adaptable and ubiquitous bacterium with a relatively large genome and non-fastidious nutritional requirements. These characteristics have allowed it to become established as an important opportunistic pathogen, particularly in nosocomial infections such as wounds, burns and infections of prostheses such as breathing tubes and catheters. The inherent resistance of *P. aeruginosa* biofilms to exogenous stresses allow it to flourish and persist in clinical infections at a significant rate (Mulcahy, *et al.*, 2013). An important characteristic of *P. aeruginosa* biofilms is their ability to produce a viscous exopolysaccharide (EPS) called alginate which confers a mucoid phenotype to the biofilm-associated cells. The antiphagocytic properties of the alginate polymers which coat mucoid *P. aeruginosa* biofilms make this pathogen almost impossible to eradicate from the body once a bacterial community has been established (Govan & Deretic, 1996). The production of alginate is also a significant contributing factor to the high level of morbidity and

mortality associated with pulmonary colonisation of mucoid *P. aeruginosa* amongst cystic fibrosis (CF) patients. (Pedersen, *et al.*, 1992).

### 1.3 *P. aeruginosa* biofilms in cystic fibrosis patients

Understanding the pathogenesis of biofilm-associated *P. aeruginosa* can be, in part, contributed to the use of a CF disease model. *P. aeruginosa* rugose small-colony variants (RSCVs) were found to have up-regulated EPS loci which cause increased adherence and reduced motility, similar to the non-mucoid phenotype isolated from CF patients (Kirisits, *et al.*, 2005). The presence of a non-mucoid phenotype in the sputa of CF patients precedes the isolation of a mucoid strain, and has a similar clinical presentation to that of patients not harbouring any *P. aeruginosa* isolates in their lungs (Govan & Deretic, 1996). Conversely, the isolation of mucoid *P. aeruginosa* is closely associated with a marked decline in the clinical condition of the patient (Pedersen, *et al.*, 1992). It has been demonstrated that alginate-overproducing *P. aeruginosa* form a biofilm which is loosely attached to surfaces and has less densely aggregated microcolonies (Hay, *et al.*, 2009). The initial colonisation of the CF lung with non-mucoid *P. aeruginosa* is therefore a pathogenic strategy, whereby the cells form a firmly-attached and closely-aggregated biofilm before the genes encoding a protective, mucoid phenotype are expressed (Stapper, *et al.*, 2004). Once a mucoid biofilm has been established, subsequent antibiotic therapy and immune responses are unable to clear the infection (Pedersen, *et al.*, 1992). Alginate encapsulation allows the biofilm to evade the immune response by a number of mechanisms including interference of opsonisation, inhibition of phagocytosis and suppression of immune cell function (Govan & Deretic, 1996). Overproduction of alginate also confers increased antibiotic resistance to biofilm-associated *P. aeruginosa in vitro* (Hentzer, *et al.*, 2001). In understanding these EPSs and their role in biofilm formation, it is necessary to review the way in which they were discovered and the current understanding of their contribution to biofilm development.

### 1.4 Discovery of EPSs in *P. aeruginosa*

*P. aeruginosa* is currently known to produce three EPSs which contribute to its biofilm-associated virulence; alginate, Psl and Pel. Alginate produced by bacteria was first described in 1966, when it was isolated from the slime coating mucoid *Pseudomonas* species from the sputa of CF patients. This compound is made up of a variable proportion of  $\beta$ -1-4-linked

D-mannuronic and L-guluronic acids, with structural and physiochemical properties similar to the alginic acids produced by some species of seaweed (Linker & Jones, 1966). Although it was the first to be discovered, it was later demonstrated that alginate production is not necessary for biofilm formation, nor is it the predominant EPS in *P. aeruginosa* biofilms (Wozniak, *et al.*, 2003). The major carbohydrate constituents of the biofilm matrix were found to be mannose and glucose, which were later identified as primary residues in the Psl and Pel EPSs respectively (Friedman & Kolter, 2004). The gene cluster encoding Psl was concurrently discovered by three independent research groups; two of whom discovered the polysaccharide synthesis loci (*psl*) through sequence homology of the operon to the EPS biosynthesis loci of other biofilm-forming bacteria, while the third isolated this loci as a necessary requirement for the formation of a phenotypic RSCV (Friedman & Kolter, 2004, Jackson, *et al.*, 2004, Matsukawa & Greenberg, 2004). The *pel* operon was discovered in screening *P. aeruginosa* mutants for genes involved in production of RSCVs, as well as the ability to form surface-associated and liquid-air interface-associated or pellicle biofilms (Friedman & Kolter, 2004). These three EPSs have since been extensively studied and the current understanding of their relative roles in biofilm development is summarised in Table 1.1.

EPS	Locus *	Roles
Psl	PA2231– PA2245	Initial attachment and adhesion
		Primary biofilm scaffold
		Proinflammatory signaling
		Antibiotics resistance
		Avoidance of host defence mechanisms
		Signaling molecule to stimulate biofilm formation
		Resistance to biofilm inhibitor Polysorbate 80
		Guide of exploration and microcolony formation
Pel	PA3058– PA3064	Pellicle formation and solid surface-associated biofilm formation
		Aggregating of bacterial cells
		Aminoglycosides antibiotic resistance
		Initial attachment in the absence of type IV pili
Alginate	PA3540– PA3548	Persistence and immune evasion
		Resistance to antibiotics as well as opsonophagocytosis
		ROS scavenge
		Leading to mucoid
		Water and nutrient retention

**Table 1.1. Established roles of EPSs in *P. aeruginosa* biofilms (Wei & Ma, 2013).**

A summary of the current understanding of the roles each EPS have in the formation and maintenance of *P. aeruginosa* biofilms.

## 1.4 EPSs in biofilm development

EPSs produced by bacteria make up the majority of the polymeric matrix which surrounds the surface-associated community, with an essential role in the following aspects of biofilm development:

### 1.4.1 Surface attachment

Surface-induced expression of EPSs has been implicated in overcoming the electrostatic repulsion observed when bacterial cells come into close proximity with solid surfaces and enhancing the polymeric interactions required for surface attachment (Tsuneda, *et al.*, 2003). The Psl EPS has been identified as an essential factor in the attachment, formation and maintenance of biofilm structures (Ma, *et al.*, 2006). *P. aeruginosa* deficient in Psl production were found to be significantly impaired in initial attachment (Overhage, *et al.*, 2005), with this effect being more pronounced during biofilm formation in high-flow systems (Jackson, *et al.*, 2004). This implies that Psl has an important role in overcoming attachment difficulties associated with turbulent environments which may provide an attractive habitat. It has been shown that the production of alginate can be induced by surface-associated activation of *algC* expression in *P. aeruginosa* (Davies, *et al.*, 1993). As alginate-overproducing *P. aeruginosa* have been shown to form a biofilm which is only loosely attached to the surface, this is not necessarily beneficial to the developing biofilm (Hay, *et al.*, 2009). The transcription of certain genes within the *pel* operon was observed to significantly increase after establishment of the biofilm phenotype, with some expression only detectable during the biofilm growth mode (Colvin, *et al.*, 2011). In contrast, the *psl* operon is constitutively expressed and the Psl EPS is localised on the surface of planktonic cells, possibly as a pre-emptive mechanism for attachment when an appropriate environmental surface is encountered (Overhage, *et al.*, 2005). The Pel EPS has also been implicated in the attachment of cells to a solid surface, however there appears to be an adaptive response whereby type IV pili can rapidly compensate for the loss of *pel* expression. Type IV pili have been shown to facilitate irreversible surface attachment (Vasseur, *et al.*, 2005) and microcolony formation in *P. aeruginosa* biofilms (Klausen, *et al.*, 2003), making the role of *pel* in surface attachment somewhat redundant.

### 1.4.2 Microcolony formation and maturation

Pel is a fundamental component of cell-cell interactions within the surface-associated microcolony, as demonstrated by studies which show that *pel*-deficient mutants form

biofilms which are smaller in mass and less tightly aggregated than the wild-type strains (Colvin, *et al.*, 2011). The predominant residue in the matrix of biofilms produced under nutrient-limiting conditions is glucose, suggesting that the Pel EPS plays an important role in biofilm maturation during times of stress (Myszka & Czaczyk, 2009). Constitutive *psl* expression is also required for development and maintenance of a stable biofilm structure by facilitating intercellular reactions (Ma, *et al.*, 2006). Although the production of alginate is not necessary for initiation of biofilm development, it does have a significant impact on the structure and integrity of *P. aeruginosa* biofilms. Microcolonies formed by alginate-overproducing strains such as PDO300 are thicker and exhibited the characteristic mushroom-like architecture when compared to the wild-type PAO1 strain (Hentzer, *et al.*, 2001). The degree to which alginate is O-acetylated determines its viscosity and subsequently how resistant it is to degradation by alginate lyases. The deacetylation of alginate *in vitro* leads to inhibition of surface attachment and biofilm maturation, suggesting that O-acetylation is a necessary factor when alginate is produced (Nivens, *et al.*, 2001). Once cells begin to mature within the microcolony, there are conflicting views as to where Psl production is localised. Overhage *et al.* (2005) localised *psl* expression to the centre of *P. aeruginosa* microcolonies with a proposed role in cell differentiation during maturation of the biofilm (Overhage, *et al.*, 2005). In contrast, Ma *et al.* (2009) propose that distinctly higher levels of Psl production are found in the periphery of mature microcolonies. This finding is linked to the necessity of cavity formation for planktonic cell dispersal in order for bacterial biofilms to establish new microcolonies (Ma, *et al.*, 2009). This is one area of conflict within current literature which this study will attempt to resolve.

### 1.4.3 Cell dispersal

Seeding dispersal of free-living cells from a bacterial biofilm is an essential step in maintaining a viable population in nutrient-deficient environments. Selection within the mature biofilm during antibiotic treatment facilitates the presence of a resistant phenotype, which is then passed on to daughter cells during the dispersal process (Drenkard & Ausubel, 2002). Experimental evidence suggests that this process involves reversion of a subset of the population to a planktonic phenotype and subsequent controlled cell lysis to form a cavity and release mechanism for this free-swimming population (Webb, *et al.*, 2003). The production of Psl and extracellular DNA is co-ordinated with this process to provide elevated nutrient levels for the pre-dispersal population (Ma, *et al.*, 2009). Alginate lyase determines the length of the alginate polymer by cleavage of glycosidic bonds, effectively

decreasing the viscosity of the compound. It has been hypothesised that an increase in expression of *algL* may have an essential role in the dispersal of *Pseudomonas aeruginosa* biofilms, although this exact mechanism is not yet understood (Boyd & Chakrabarty, 1994).

## 1.5 EPS biosynthesis and regulation

Relatively little is known about the regulatory mechanisms by which bacteria switch to a biofilm phenotype, due to the complexity of bacterial messenger systems and the relatively recent discovery of the *pel* and *psl* operons (Merritt, *et al.*, 2010). *P. aeruginosa* invests a large proportion of its energy into the production of transcriptional regulators, having a relatively large number of two-component signalling (TCS) pathways involved in the phenotypic switching required for effective biofilm development. The regulator of EPS and Type III secretion (RetS) is a sensor histidine kinase responsible for the expression and repression of numerous TCS pathways which are essential for the biofilm-associated phenotype of *P. aeruginosa*. Repression of *RetS* results in overexpression of *pel* and *psl* expression through complex interactions with numerous other signalling molecules, essentially signalling for the switch from a planktonic to a biofilm-associated phenotype (Goodman, *et al.*, 2004). *Psl* transcription is also regulated by the stationary-phase  $\sigma$  factor RpoS, which reciprocally increases Psl production in response to a reduction in growth rate (Ventre, *et al.*, 2006). This may be a survival mechanism, whereby the biofilm phenotype is selectively expressed in an environment with limited nutrients or external stressors. *Psl* is also post-transcriptionally repressed by the RNA binding protein RsmA, although this mechanism is not well understood (Irie, *et al.*, 2010). Alginate biosynthesis in *P. aeruginosa* requires activation of the *algD* promoter, *PalgD*, by numerous transcriptional and environmental factors. AlgD is a GDP-mannose dehydrogenase responsible for the conversion of fructose-6-phosphate to GDP-mannuronic acid, an activated precursor of the alginate polymer (Deretic, *et al.*, 1987). In nonmucoid isolates, the *algD* operon is not expressed due to a functional *mucA* gene. MucA is involved in the sequestration of the alternative sigma factor,  $\sigma^{22}$  or AlgT/U, and subsequent down-regulation of alginate production. A mutation within *mucA* is the most commonly observed genotype of mucoid *P. aeruginosa* isolates from CF patients (Stapper, *et al.*, 2004). The production of alginate can be induced by cell wall stressors which activate proteases such as AlgW to degrade MucA and allow  $\sigma^{22}$  to activate *PalgD* expression (Ohman, 2009). *P. aeruginosa* can also regulate alginate production in response to reactive oxygen species such as hydrogen peroxide produced by

polymorphonuclear leukocytes during acute inflammatory reactions (Mathee, *et al.*, 1999). Expression of the ampicillin resistance promoter,  $P_{ampR}$ , is positively regulated by  $\sigma^{22}$ , while the presence of AmpR has the opposite effect on the expression of *algT/U*. This system provides a negative feedback loop to limit alginate production while facilitating antibiotic resistance of *P. aeruginosa* in chronic infections. Interestingly, both AmpR and  $\sigma^{22}$  negatively affect the transcription of *lasA*, an essential element of quorum sensing pathways in *P. aeruginosa* (Balasubramanian, *et al.*, 2011).

### 1.5.1 Quorum sensing

Quorum sensing (QS) is a system whereby bacteria collectively optimise gene expression for a particular environment by regulating transcription in response to population density (De Kievit, *et al.*, 2001). QS provides a mechanism for the ‘switch’ from rapid replication of planktonic cells to high-level production of EPS, the production of which is inversely proportional to the volume of cells in the biofilm matrix (Frederick, *et al.*, 2011). There are two main regulatory systems which utilise homoserine lactone (HSL) signalling molecules to regulate phenotypic switching and biofilm formation in *P. aeruginosa*; these are controlled by the *lasI* and *rhlI* loci (Davies, *et al.*, 1998). The Las-QS system was found to specifically upregulate *pel* transcription, under the influence of the *rhlI* loci (Sakuragi & Kolter, 2007). Knockout-*lasI* mutants were unable to produce detectable levels of EPS (Shih & Huang, 2002), with their biofilm structure being relatively undifferentiated and significantly more susceptible to detachment and dispersal when exposed to sodium dodecyl sulphate (SDS) treatment (Davies, *et al.*, 1998). Sauer *et al.* (2002) found that EPSs are more closely associated with the interstitial spaces between bacterial cells in wild-type *P. aeruginosa*, however the *lasI* mutants were found to form biofilms with more closely packed cells and a closer cell-EPS association (Sauer, *et al.*, 2002). The intercellular arrangement of the EPS matrix is therefore a crucial factor for biofilm stability, the production of which is reliant on an efficient QS system.

### 1.5.2 Cyclic diguanylate monophosphate

The production of EPS in *P. aeruginosa* is regulated by various pathways requiring cyclic diguanylate monophosphate (c-di-GMP) (Merritt, *et al.*, 2010). C-di-GMP is a nucleotide which acts as a second messenger involved in the phenotypic switching of a wide range of

bacteria (D'Argenio & Miller, 2004). The post-transcriptional binding of c-di-GMP to specific gene products is essential for Pel and alginate production in *P. aeruginosa* (Lee, *et al.*, 2007, Lory, *et al.*, 2009). Intracellular c-di-GMP levels are controlled by a number of regulatory mechanisms which modulate the reciprocal actions of synthesis by diguanylate cyclases (DGCs) and degradation by phosphodiesterases (PDEs) (Tamayo, *et al.*, 2007). While some studies have indicated a link between intracellular c-di-GMP levels and the biofilm phenotype, recent research suggests a spatial element has a significant role. It was demonstrated that c-di-GMP production is localised to the area of the inner membrane associated with specific DGCs (Merritt, *et al.*, 2010). More specifically, expression of the membrane-associated complex *mucR* directly upregulates alginate synthesis by increasing the level of this signalling molecule production near the c-di-GMP binding region of Alg44 (Hay, *et al.*, 2009). In addition to the controlled locality of production, there may be potential differences in the microstructure of c-di-GMP molecules which modulate the biochemical pathways with which they can interact and therefore the control of expression of EPS genes. If these systems can be better understood they could form the basis of effective therapies targeted at inhibiting EPS production.

## 1.6 EPSs as potential targets for biofilm therapies

It has been demonstrated that the adhesion and accumulation of an established biofilm was severely diminished when a blocking agent of EPS biosynthesis, in this case 2,4-dinitrophenol, was introduced into the milieu (Cammarota & Sant'Anna, 1998). Bismuth dimercaprol (BisBAL) treatment was also found to significantly reduce EPS production in biofilm-associated *P. aeruginosa* (Huang & Stewart, 1999). Psl production has been shown to have a significant effect on antibiotic resistance of *P. aeruginosa* and mixed species biofilms with *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. Biofilms in which the wild-type *P. aeruginosa* PAO1 was unable to produce Psl were significantly more susceptible to a range of antibiotic treatments, while overproduction of Psl conferred increased resistance (Billings, *et al.*, 2013). This finding infers that substances directed at degradation or inhibition of these polymers could significantly improve therapies aimed at the eradication of mixed species biofilms in clinical disease. Treatment utilising the potent leukocyte activator interferon-gamma (IFN- $\gamma$ ) has been shown to aid in clearance of chronic *P. aeruginosa* infection within the CF lung. The presence of IFN- $\gamma$  significantly enhanced phagocytosis of alginate-negative biofilm bacteria, however resistance was restored with the

addition of exogenous alginate suggesting this potential therapy would only be effective for non-mucoid biofilms (Leid, *et al.*, 2005). Compounds which block the formation or enhance degradation of c-di-GMP or QS molecules could also potentially be used to chemically attenuate the intercellular and intracellular signals required for EPS production in biofilm development (Hentzer, *et al.*, 2002).

## 1.7 *In vitro* biofilm studies

Numerous studies have investigated the role of extracellular polymeric substances in the development, maintenance and persistence of biofilms. The dynamic and complex nature of bacterial biofilms means the control of environmental conditions during biofilm studies is essential in interpreting and applying data obtained *in vitro* (Billings, *et al.*, 2013). Previous studies have used isogenic strains with various mutations to identify genes and conditions affecting EPS production in *P. aeruginosa* biofilms. The commonly used laboratory strains, PAO1 and PA14, do not produce significant amounts of alginate when they are grown as a biofilm *in vivo* (Wozniak, *et al.*, 2003). PDO300 is a mucoid variant of the prototypic PAO1 strain with a knockout mutation in the regulatory pathway of alginate, allowing sufficient quantities to be produced for biofilm analysis (Mathee, *et al.*, 1999). In terms of visualisation of biofilms, light microscopy techniques offer limited information about thickness, while the fixation step required for electron microscopy radically alters the architecture being investigated (Lawrence, *et al.*, 1991). As opposed to electron microscopy techniques which require dehydration of the biofilm, confocal laser scanning microscopy (CLSM) has been applied to effectively visualise fluorescence in a viable bacterial biofilm structure (Caldwell, *et al.*, 1992). With the increasing application of CLSM in biofilm studies, technological advances in analytical software means that high-resolution image analysis can be obtained with quantification of physical parameters in a viable biofilm structure. Using the image stacks acquired through CLSM, textural parameters can be applied to demonstrate the heterogeneity of biofilms while volumetric parameters describe the size and morphology of the biomass (Beyenal, *et al.*, 2004). When this technology is coupled with a continuous flow culture system and fluorescent reporter constructs, the localisation of gene expression within viable biofilm-associated bacteria can be observed in real time and applied in understanding the regulatory systems which facilitate the pathogenesis of *P. aeruginosa* biofilms *in vivo* (Caldwell, *et al.*, 1992).

## 1.8 Current understanding

*P. aeruginosa* is an incredibly efficient opportunistic pathogen, with the ability to form persistent, surface-associated biofilms in a wide range of habitats. Current understanding of EPS gene expression and regulation in biofilm formation by *P. aeruginosa* is limited, probably due to the relatively recent discovery of the *pel* and *psl* operons. As it has with alginate, the use of infectious models may help to further elucidate the mechanisms through which *pel* and *psl* expression is regulated by environmental factors to contribute to the pathogenesis of this organism. *P. aeruginosa* is able to inhabit a wide variety of ecological niches due to highly regulated gene expression systems with a significant level of redundancy within the biochemical pathways. This is observed by the ability of various mutants to achieve the same goal through utilisation of a different set of genes, significantly increasing the complexity of any biofilm investigation. The human immune response is incredibly efficient at opsonisation and phagocytosis of planktonic cells during the acute infectious stage, however responses against sessile bacterial communities are often ineffective and damaging to host tissues (Costerton, 2007). Further investigation is required to spatially and temporally resolve the location and regulation of EPS production in *P. aeruginosa* biofilms before the functional relationship of these secreted polymers within the biofilm development model can be explained. An increased understanding of the localisation of EPS biosynthesis will benefit both therapeutic and commercial endeavours involving biofilm formation by *P. aeruginosa*.

## 1.9 Hypothesis and design

The expression of alginate, Psl and Pel exopolysaccharides will be distinctly located throughout the development of *Pseudomonas aeruginosa* biofilms.

This study will utilise two different strains to localise EPS gene expression in *P. aeruginosa* biofilms. In order to be able to investigate the effect of alginate production on bacterial cell localisation, a mucoid variant of the prototypic *P. aeruginosa* PAO1 strain which produces a significant amount of this EPS will be used. *P. aeruginosa* PDO300 has a knockout mutation in the alginate regulation gene, *MucA*. This isolate has been subject to three different combinations of double-knockout mutations to produce three bacterial strains, each capable of producing only one of the three EPSs being investigated. To address the first aim of this study, these strains will be grown as individual biofilms to elucidate the effect that the production of each EPS has on the structure of *P. aeruginosa* biofilms. These three strains will then be mixed in an equivalent ratio determined by their cell density, and each EPS-producing population will be visualised within a mature, mixed mutant biofilm to observe the effect of EPS production on bacterial localisation in mucoid biofilms. This part of the study will address the second aim of this paper, to observe whether the production of a particular EPS influences the localisation of *P. aeruginosa* within the biofilm matrix.

Localisation of EPS gene expression throughout biofilm development will be achieved by using the wild-type *P. aeruginosa* PAO1 strain. To observe the spatial localisation of cells expressing each EPS gene, this strain will be transformed with a plasmid encoding the expression of the stable green fluorescent protein (gfp) under the control of one of the three EPS operon promoters and grown into biofilms by methods which have been previously described (Nielsen, *et al.*, 2011). Therefore, when the corresponding EPS promoter is activated within the cell, transcription of the stable fluorescent protein encoded by this plasmid will occur. Temporal localisation of EPS gene expression will be achieved by transforming the prototypic *P. aeruginosa* PAO1 strain with a plasmid encoding expression of an unstable *gfp* gene under control of each of the three EPS promoters. As this protein has a shorter half-life, it will be degraded by the cell if promoter activity ceases. This part of the study will allow identification of temporal patterns in EPS gene expression by visualising unstable fluorescent signals over the course of biofilm development. The fluorescent signal emitted by cells with active EPS promoters will then be detected using CLSM and visualised using images generated with IMARIS software.

## 1.10 Aims and objectives

The following aims and objectives were addressed by this study.

1. Assess the effect of EPS production on the architecture of *P. aeruginosa* PDO300 biofilms by:
  - growing individual biofilms of the three single-EPS-producing *P. aeruginosa* PDO300 strains.
  - visualising fluorescence of bacterial cells using CLSM after 96 hours and generating images with IMARIS software.
  
2. Assess the effect of EPS production on localisation of *P. aeruginosa* PDO300 bacterial cells within biofilms by:
  - using the Tn7 mini transposon system to chromosomally label three single-EPS-producing *P. aeruginosa* strains with the following fluorescent protein-encoding genes to generate three strains;
    - PDO300 $\Delta$ *pslA* $\Delta$ *pelF*::*gfp* - Green fluorescent, alginate producing strain.
    - PDO300 $\Delta$ *pelF* $\Delta$ *alg8*::*HcRed* - Red fluorescent, Psl producing strain.
    - PDO300 $\Delta$ *pslA* $\Delta$ *alg8*::*cfp* - Cyan fluorescent, Pel producing strain.
  - growing a mixed biofilm consisting of an equivalent number of each of the labelled double-knockout mutant strains under the same conditions as a biofilm of the parent strain.
  - visualising the structure and fluorescence of bacterial cells within the biofilms using time-lapse CLSM and generating images with IMARIS software after 24, 48, 72 and 96 hours.

3. Visualise temporal localisation of alginate, Psl and Pel EPS promoter activity in *P. aeruginosa* cells within a viable biofilm matrix by;
  - transforming prototypic PAO1 strains with a reporter plasmid construct expressing unstable *gfp* under the control of three EPS promoters.
  - growing individual biofilms of each confirmed strain under the same conditions.
  - visualising promoter activity within the biofilm using time-lapse CLSM data collected after 24, 48, 72 and 96 hours, and images generated with IMARIS software.
  
4. Visualise spatial localisation of alginate, Psl and Pel EPS promoter activity in *P. aeruginosa* cells within a viable biofilm matrix by;
  - transforming prototypic PAO1 strains with a reporter plasmid construct expressing stable *gfp* under the control of three EPS promoters.
  - growing individual biofilms of each confirmed strain under the same conditions.
  - visualising promoter activity within the biofilm using time-lapse CLSM data collected after 24, 48, 72 and 96 hours, and images generated with IMARIS software.
  
5. Concurrently visualise promoter activity of all three EPS genes by generating a multi-promoter reporter plasmid with three genes encoding labile fluorescent proteins under the control of each EPS promoter by;
  - adding an [AAV] tail to the DNA sequences for red and blue-fluorescent protein genes (HcRed and *bfp*) by PCR and sequence confirmation.
  - confirming the lability of these unstable protein by ligating into an inducible expression vector.\*

\* *This aim was not completed because the synthesised proteins did not exhibit any fluorescence.*

## 2.0 Materials and methods

### 2.1 Strains, plasmids and oligonucleotides

Table 2.1. Bacterial strains used in this study

Strain	Relevant phenotype or genotype	Reference
<i>Escherichia coli</i> TOP10	F- <i>mcrA</i> $\Delta(mrr-bsdRMS-mcrBC)$ $\phi 80lacZ\Delta M15 \Delta lacX74 recA1 araD139$ $\Delta(aralen) 7697 galU galK rpsL (StrR)$ <i>endA1 nupG</i>	Invitrogen*
<i>E. coli</i> SM10	Used for replication of Tn7 elements and pUX-BF13	(Lambertsen, <i>et al.</i> , 2004)
<i>P. aeruginosa</i> PAO1	Wild-type	(Holloway, <i>et al.</i> , 1986)
<i>P. aeruginosa</i> PDO300	$\Delta mucA22$ variant of PAO1, mucoid phenotype	(Mathee, <i>et al.</i> , 1999)
<i>P. aeruginosa</i> PDO300 $\Delta pelF\Delta alg8$	Isogenic deletion mutant derived from PDO300; Psl-producer	(Ghafoor, <i>et al.</i> , 2011)
<i>P. aeruginosa</i> PDO300 $\Delta pslA\Delta alg8$	Isogenic deletion mutant derived from PDO300; Pel-producer	(Ghafoor, <i>et al.</i> , 2011)
<i>P. aeruginosa</i> PDO300 $\Delta pslA\Delta pelF$	Isogenic deletion mutant derived from PDO300; Alginate-producer	(Ghafoor, <i>et al.</i> , 2011)
<i>P. aeruginosa</i> PDO300 $\Delta pelF\Delta alg8:HcRed$	Psl-producer, chromosomally labelled with stable red-fluorescent protein	This study
<i>P. aeruginosa</i> PDO300 $\Delta pslA\Delta alg8:cfp$	Pel-producer, chromosomally labelled with stable cyano-fluorescent protein	This study
<i>P. aeruginosa</i> PDO300 $\Delta pslA\Delta pelF:gfp$	Alginate-producer, chromosomally labelled with stable green-fluorescent protein	This study

\* Invitrogen Corporation, San Diego, USA

**Table 2.2. Plasmids used in this study**

Plasmid name	Relevant phenotype or genotype	Reference
pUX-BF13	R6K replicon-based helper plasmid; transposase genes <i>tns-ABCDE</i> , Ap <sup>R</sup> , mob <sup>+</sup>	(Lambertsen, <i>et al.</i> , 2004)
miniTn7(Gm)P <sub>A1/04/03</sub> <i>gfp</i>	Tn7 element encoding green-fluorescent protein	(Lambertsen, <i>et al.</i> , 2004)
miniTn7(Gm)P <sub>A1/04/03</sub> <i>ecfp</i>	Tn7 element encoding cyan-fluorescent protein	(Lambertsen, <i>et al.</i> , 2004)
miniTn7(Gm)P <sub>A1/04/03</sub> <i>HcRed</i>	Tn7 element encoding red-fluorescent protein	(Lambertsen, <i>et al.</i> , 2004)
pProbe'-gfp[AAV]:: Gm-P <sub>psl</sub>	Unstable gfp expressed under Psl promoter with constitutive gentamicin resistance	(Overhage, <i>et al.</i> , 2005)
pProbe'-gfp[AAV]:: Gm-P <sub>palg</sub>	Unstable gfp expressed under Alg promoter with constitutive gentamicin resistance	Aamir Ghafoor†
pProbe'-gfp[AAV]:: Gm-P <sub>pel</sub>	Unstable gfp expressed under Pel promoter with constitutive gentamicin resistance	Aamir Ghafoor†
pProbeAT'::P <sub>psl</sub>	Stable gfp expressed under Psl promoter with constitutive ampicillin (carbencillin) resistance	(Miller, <i>et al.</i> , 2000)
pProbeAT'::P <sub>pel</sub>	Stable gfp expressed under Psl promoter with constitutive ampicillin (carbencillin) resistance	(Miller, <i>et al.</i> , 2000)
pProbeAT'::P <sub>alg</sub>	Stable gfp expressed under alginate promoter with constitutive ampicillin (carbencillin) resistance	(Miller, <i>et al.</i> , 2000)
pUC57	Transport vector for <i>bfp</i> [AAV]	Evrogen‡
pGEM®-T Easy	Confirmation and sequencing vector, single 3' T-overhangs, P <sub>lac</sub>	Promega§
pHERD30T	pUCP30T P <sub>lac</sub> replaced with 1.3-kb AflIII-EcoRI fragment of <i>araC</i> -P <sub>BAD</sub> cassette	(Qiu, <i>et al.</i> , 2008)

† Aamir Ghafoor, Institute of Fundamental Sciences, Massey University, New Zealand.

‡ Evrogen JSC, Moscow, Russia.

§ Promega, Madison, USA.

**Table 2.3. Oligonucleotides used in this study**

Oligonucleotide	Sequence (5' – 3')	Reference
<i>HcRed</i> Forward	AAAGAATTCGGATCCTTTATAAGGAGGAA AACATATGGTGAGCGGCCTGCTGAAGGA	This study
<i>HcRed</i> Reverse	AAAAAGCTTATTATCAGTTGGCCTTCTCGG GCAGGTCGCTGTACCGGGCCACG	This study
<i>HcRed</i> [AAV] Forward	AAAGAATTCGGATCCTTTATAAGGAGGAA AACATATGGTGAGCGGCCTGCTGAAGGA	This study
<i>HcRed</i> [AAV] Reverse	TCTAAGCTTCGATCGATTAATAACTGCTGCAG CGTAGTTTTCGTCGTTTGTGCGTTGGCCTT CTCGGGCAGGTCGC	This study
<i>bfp</i> Forward	AAAGAATTCGGATCCTTTATAAGGAGGAA AACATATG	This study
<i>bfp</i> Reverse	AAAAAGCTTATTATTAATTCAGCTTGTGGC CCAG	This study

## 2.2 Media

Media was autoclaved at 121°C for 20 min (up to one litre volumes) or one hour (up to seven litre volumes). Agar was made with the addition of 1.5% (w/v) bacterial agar. The following media and antibiotics were used in this study:

### 2.2.1 Luria-Bertani (LB) medium (Sambrook, 1989)

NaCl	171 mM
Tryptone	10% w/v
Yeast Extract	5% w/v

### 2.2.2 Mineral salt medium (MSM) (Schlegel, *et al.*, 1961)

NH <sub>4</sub> Cl	18.7 mM
KH <sub>2</sub> PO <sub>4</sub>	11.0 mM
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	5.2 mM
Fe(III)NH <sub>4</sub> -Citrate	4.3 μM
Supplement solution SL6 (2.2.4)	10% v/v

### 2.2.3 Pseudomonas isolation medium (PIM) (Remminghorst & Rehm, 2006)

K <sub>2</sub> SO <sub>4</sub>	57.4 mM
MgCl <sub>2</sub> ·6H <sub>2</sub> O	6.9 mM
Triclosan (Irgasan)	86.3 μM
Peptone from meat	20% w/v
Glycerol	2% v/v

## 2.2.4 Supplement solution SL6

H <sub>3</sub> BO <sub>3</sub>	0.5 mM
CoCl <sub>2</sub> ·6H <sub>2</sub> O	84.1 μM
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	34.8 μM
NiCl <sub>2</sub> ·6H <sub>2</sub> O	8.4 μM
CuCl <sub>2</sub> ·2H <sub>2</sub> O	5.9 μM
MnCl <sub>2</sub> ·4H <sub>2</sub> O	5.8 μM

*Autoclave, then add sterile, filtered:*

NaC <sub>6</sub> H <sub>11</sub> O <sub>7</sub>	45.8 mM
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.8 mM
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.1 mM

## 2.2.5 X-Gal (bromochloroindoxyl galactoside) medium (Horwitz, *et al.*, 1964)

LB Medium

*Autoclave, then add sterile, filtered:*

IPTG (Isopropyl β-D-1-thiogalactopyranoside)	1.0 mM
X-Gal	97.9 μM

### 2.2.6 Antibiotics

Antibiotic stock solutions were prepared by filter sterilisation (0.22 µm) and stored at -20°C. Antibiotics were added to autoclaved media as required at the following concentrations;

	Stock solutions (mg/mL)	Final concentrations (µg/mL)	
		<i>E. coli</i>	<i>P. aeruginosa</i>
Ampicillin (Na salt)	75	75	-
Carbencillin (Na salt)	300	-	300
Gentamicin (sulfate)	10	10	-
	300	-	300

Table 2.4. Antibiotic concentrations used in this study

### 2.2.7 Strain cultivation and storage

Desired strains were cultivated to early-stationery phase by overnight growth in LB medium (2.2.1) supplemented with appropriate antibiotics (2.2.6). Long-term storage of strains was achieved by mixing 7% DMSO (70 µL) with 1 mL of culture liquid and freezing in liquid nitrogen before storage at -80 °C.

## **2.3 DNA manipulation**

The following methods were applied where DNA manipulation was required. Where commercially produced kits or equipment was used, the manufacturer's protocols were followed.

### **2.3.1 Plasmid DNA isolation and storage**

Plasmid DNA was isolated from bacterial cell cultures grown overnight in LB medium (2.2.1) with the Roche® High Pure Plasmid Isolation Kit and stored at -20°C in sterile Milli-Q water.

### **2.3.2 Determination of DNA concentration**

The concentration of DNA in preparations and solutions was determined by using the Qubit® fluorometer to detect fluorescence of a DNA-binding dye The Quant-iT™ dsDNA BR assay (double-stranded DNA, broad-range, Invitrogen). This assay uses a dye that is selective for DNA, minimising the effect of contaminants on quantitative readings. DNA concentration data is accurately generated based on the relationship between the two standards used in the calibration.

### **2.3.3 Agarose gel electrophoresis**

DNA fragments were confirmed and isolated by agarose gel electrophoresis on horizontal gels. Agarose gels were made at a concentration of 1% and placed in Tris/Borate/EDTA (TBE) buffer (2.3.3.3). Stop-mix loading dye (2.3.3.5) was added to DNA samples at a concentration of 1x before loading into wells. The Pstλ molecular weight standard (2.3.3.4) was loaded into a separate well to determine DNA fragment size. Agarose gel electrophoresis was carried out at room temperature in TBE buffer at 90-110 V for 30-60 min, depending on the length of the gel and the separation required.

### 2.3.3.1 Confirmation of DNA fragment size

Where confirmation of fragment size only was required, gels were run by agarose gel electrophoresis (2.3.3) and stained for at least 30 min in ethidium bromide solution (2  $\mu\text{L}/\text{mL}$ ) before being rinsed for at least 30 min in water. The DNA fragments were then visualised using a UV transilluminator ( $\lambda=254$ ) (BioRad Gel Doc 2000).

### 2.3.3.2 Isolation of DNA fragments

Where fragment isolation was required, SYBR<sup>®</sup> Safe gel stain (Invitrogen<sup>™</sup>, 10000 x concentrated) was added to the agarose at a final concentration of 1x. DNA fragments were visualised using a UV transilluminator ( $\lambda=280$ ) (BioRad<sup>™</sup> Gel Doc 2000). The required DNA fragment was cut from the gel using a sharp scalpel. DNA was isolated from agarose using the PureLink<sup>®</sup> Quick Gel Extraction Kit (Invitrogen<sup>™</sup>).

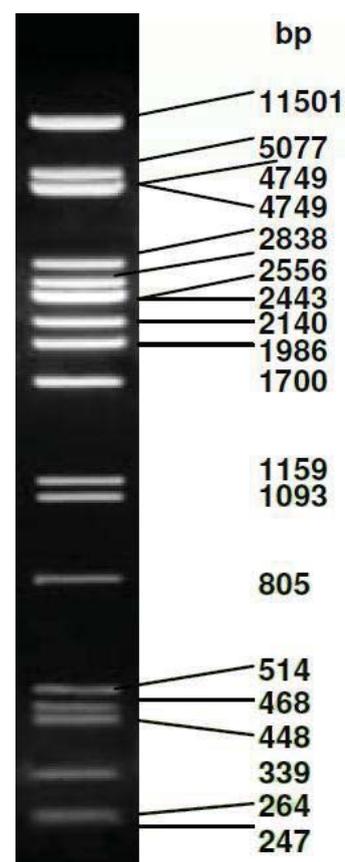
### 2.3.3.3 TBE buffer

Component	Concentration
Tris-HCl	50 mM
Boric acid	50 mM
EDTA	2.5 mM
pH 8.0	

### 2.3.3.5 Stop-Mix loading dye (6x concentrated)

Component	Concentration
Tris-HCl	60 mM
EDTA	60 mM
Glycerol	60 % (v/v)
Orange G	0.2 % (w/v)
Xylen Cyanol FF	0.05 % (w/v)
pH 8.0	

### 2.3.3.4 Pst $\lambda$ DNA Ladder



### 2.3.4 Polymerase chain reaction (PCR)

PCR reactions were performed in a Biometra personal thermocycler (Whatman Biometra<sup>®</sup>, Germany) with the following settings: 1. Primary denaturation (94°C for 240 s). 2. Denaturation (94°C for 15s). 3. Annealing (54°C for *bfp*, 65°C for *HcRed* for 30 s). 4. Extension (68°C for 60 s). Steps 2-4 were repeated for 30 cycles before the mixture was held at 10°C.

#### 2.3.4.1 *Pfx* DNA Polymerase

The proof-reading *Pfx* DNA Polymerase was used to provide high fidelity amplification of targets for subsequent cloning methods:

Component	Concentration	Volume
<i>Pfx</i> Amplification Buffer	10X	10 $\mu$ L
MgSO <sub>4</sub>	50 mM	2 $\mu$ L
dNTPs	10 mM	3 $\mu$ L
Primer mix	10 $\mu$ M each	2 $\mu$ L
Template DNA	10 pg-200 ng	-
<i>Pfx</i> DNA polymerase	-	2 $\mu$ L
Autoclaved, Milli-Q H <sub>2</sub> O	-	to 100 $\mu$ L

**Table 2.5. *Pfx* DNA polymerase PCR reaction mixture**

#### 2.3.4.2 *Taq* DNA Polymerase

*Taq* DNA Polymerase was used to rapidly amplify targets for verification purposes:

Component	Concentration	Volume
PCR Buffer	10X	10 $\mu$ L
MgCl <sub>2</sub>	50 mM	3 $\mu$ L
dNTPs	10 mM	2 $\mu$ L
Primer mix	10 $\mu$ M each	2 $\mu$ L
Template DNA	-	2 $\mu$ L
<i>Taq</i> DNA polymerase	5U/ $\mu$ L	0.4 $\mu$ L
Autoclaved, Milli-Q H <sub>2</sub> O	-	to 100 $\mu$ L

**Table 2.6. *Taq* DNA Polymerase PCR reaction mixture**

### 2.3.5 A-Tailing

Where PCR fragments were being inserted into the pGEM<sup>®</sup>-T Easy vector, A-tailing was performed prior to ligation to increase efficiency. The pGEM<sup>®</sup>-T Easy vector contains a single 3'-T overhang at the insertion site to minimise recircularisation. The A-tailing mix was incubated at 70°C for 15-30 min.

Component	Concentration	Volume
Purified PCR fragment	-	7 µL
<i>Taq</i> polymerase buffer	10 x	1 µL
dATP	2 mM	1 µL
<i>Taq</i> DNA polymerase	5 units/µL	1 µL
	Total	10 µL

**Table 2.7. A-tailing reaction mixture**

### 2.3.6 Preparation of electrocompetent *P. aeruginosa* cells

Competent *P. aeruginosa* cells were prepared using a microcentrifuge-based method as described previously (Choi, *et al.*, 2006). Strains required for electroporation (2.3.7) were grown overnight in LB medium (2.2.1) before centrifugation of 6 mL of culture medium for 2 min at 16000 g. The cell pellets were washed twice with a solution of 300 mM sucrose before being combined and resuspended in 100 µL of 300 mM sucrose. Suspended cells were used immediately for cloning purposes.

### 2.3.7 Electroporation

Electroporation of electrocompetent *P. aeruginosa* cells was performed as described previously (Choi, *et al.*, 2006). 100 µL of electrocompetent cells suspended in 300 mM sucrose was mixed with 500 ng of plasmid DNA and transferred to a sterile 2 mm electroporation cuvette. After electroporation at 2.5 kV, 1 ml of LB medium (2.2.1) was added aseptically and the contents transferred to a sterile bijou bottle and incubated on a shaker for 2 h at 37°C. The suspension was then centrifuged and the cell pellet was plated using aseptic technique onto the appropriate selective media.

### 2.3.8 The mini-Tn7 transposon system

DNA sequences encoding fluorescent proteins were introduced into neutral sites of the target *P. aeruginosa* bacterial chromosome with the use of the mini-Tn7 transposon system (Koch, *et al.*, 2001). The Tn7 transposon inserts at high frequency in one orientation as a single copy into a site named *attTn7*. Insertion is directed with addition of the helper plasmid pUX-BF13 to an area downstream of the *glmS* gene, encoding glucosamine synthetase, an essential growth factor in most bacteria. The fluorescent protein-encoding Tn7 delivery and helper plasmids (Lambertsen, *et al.*, 2004) were propagated in *E. coli* TOP10 cells before isolation (2.3.1) and introduction into the electrocompetent *P. aeruginosa* PDO300 double-knockout mutant (2.3.6). For this method, 30  $\mu$ L of electrocompetent *P. aeruginosa* cells (2.3.6) were mixed with 30  $\mu$ L of the plasmid containing the fluorescent protein. An equal volume of 600 mM sucrose was added to make a final concentration of 300 mM. This mixture was then subject to electroporation (2.3.7) before plating onto the appropriate media. The following labelled strains were generated using this method:

Strain	Tn7 delivery plasmid	Labelled strain
PDO300 $\Delta$ <i>pslA</i> $\Delta$ <i>pelF</i>	miniTn7(Gm)P <sub>A1/04/03</sub> <i>gfp</i>	PDO300 $\Delta$ <i>pslA</i> $\Delta$ <i>pelF</i> :: <i>gfp</i>
PDO300 $\Delta$ <i>pelF</i> $\Delta$ <i>alg8</i>	miniTn7(Gm)P <sub>A1/04/03</sub> <i>HcRed-a</i>	PDO300 $\Delta$ <i>pelF</i> $\Delta$ <i>alg8</i> :: <i>HcRed</i>
PDO300 $\Delta$ <i>pslA</i> $\Delta$ <i>alg8</i>	miniTn7(Gm)P <sub>A1/04/03</sub> <i>ecfp-a</i>	PDO300 $\Delta$ <i>pslA</i> $\Delta$ <i>alg8</i> :: <i>ecfp</i>

**Table 2.8. Tn7 transposon delivery plasmids and strains used to label *P. aeruginosa* PDO300 double-knockout mutants.**

### 2.3.9 Restriction endonuclease digests

DNA was digested with the appropriate restriction endonuclease (Invitrogen; Roche; New England Biolabs) according to the manufacturer's protocol. Generally, digests were prepared as follows:

Component	Concentration	Volume
DNA	2.5 µg	-
SuRE/Cut Buffer	10x	2.5 µL
Restriction Enzyme(s)	10U/µL	0.5 µL
Sterile water		Up to 25 µL

**Table 2.9. Restriction endonuclease reaction mixture.**

The reaction was incubated at 37°C for one hour before the addition of Stop-Mix loading dye (2.3.3.5). Digested fragments were isolated by agarose gel electrophoresis (2.3.3) and gel purification (2.3.3.2).

### 2.3.10 DNA ligation

T4 DNA ligase (Invitrogen, USA) was used to ligate DNA fragments into appropriate plasmid vectors. Fragments and vectors were first digested with the appropriate restriction endonucleases (2.3.9) and purified using agarose gel electrophoresis (2.3.3). The concentrations of the respective DNA fragments were determined (2.3.2) before ligation was performed as per the manufacturer's protocol. A vector to insert DNA molar ratio of 1:3 (w/w) was used in a total volume of 10 µL. Reactions were incubated overnight at 4°C in microcentrifuge tubes floating in large beakers of water (to allow slow cooling to 4°C). For ligations involving pGEM-Teasy, the control insert was ligated to the vector and this ligation mixture was used as a positive control during transformation. The ligation mixture was subsequently transformed into *E. coli* TOP-10 competent cells (2.3.11).

### 2.3.11 Preparation of competent *E.coli* cells (Hanahan, 1983)

*E. coli* TOP10 cells were grown at 37°C in 50 mL LB until an OD<sub>600</sub> of 0.3 was reached. After 10 min incubation on ice the cells were centrifuged at 16 000 g for 15 min at 4°C. The cell sediment was suspended in 18 mL RF1 solution (2.3.11.1), and incubated on ice for 30 min. The cells were again centrifuged under the same conditions and the cell sediment suspended in 4 mL RF2 solution (2.3.11.2). 200 µL aliquots of the suspended cells were dispensed into sterile microcentrifuge tubes and frozen in liquid nitrogen. The competent cells were then stored at -80°C until required.

#### 2.3.11.1 RF1 solution:

Component	Concentration
RbCl	100 mM
MnCl <sub>2</sub>	50 mM
CH <sub>3</sub> CO <sub>2</sub> K	30 mM
CaCl <sub>2</sub> ·6H <sub>2</sub> O	10 mM

#### 2.3.11.2 RF2 solution:

Component	Concentration
RbCl	10 mM
MOPS buffer	10 mM
CaCl <sub>2</sub> ·6H <sub>2</sub> O	75 mM
Glycerol	15% (v/v)

### **2.3.12 *E. coli* transformation**

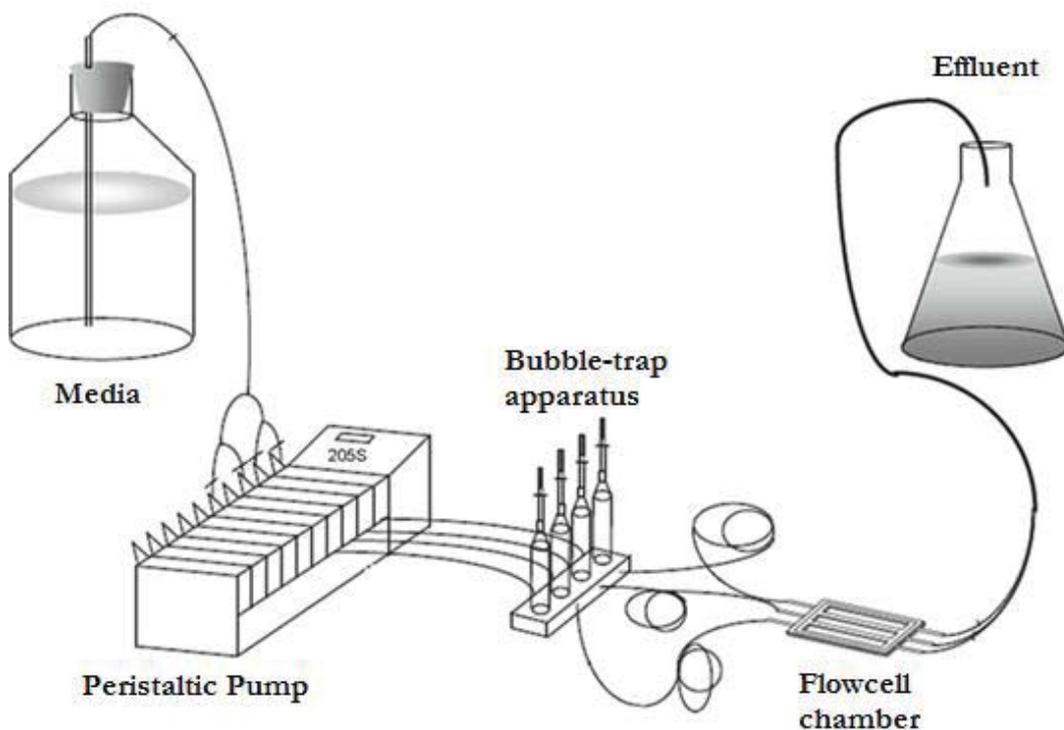
DNA was introduced into *E. coli* for the purposes of propagation and analysis using a modified heat-shock method. The entire ligation reaction mixture (2.3.10) or 2 µg of plasmid DNA was added to 200 µL of *E. coli* competent cells (2.3.11). These cells were then incubated on ice for 1 h. The cells were heat-shocked for 90 s at 42°C, and incubated on ice for a further 5 min. After the addition of 800 µL of LB medium (2.2.1) to the cells, they were incubated for 1 h at 37°C. The cells were centrifuged at 16 000 g for 3 min, and the cell pellet suspended in 300 µL LB medium (2.2.1). Various dilutions of this suspension were then plated onto the appropriate solid media containing antibiotics to select colonies containing the plasmid of interest. As a negative control, cells subjected to the same method without added DNA were plated onto the same selective media.

### **2.3.13 DNA sequencing**

DNA sequencing was carried out by the Massey Genome Service using the BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit. The result was analysed using the ABI 3730 Genetic Analyser (Applied Biosystems). Sequence data was analysed using Vector NTI Software to find homology between expected and obtained results and identify correctly synthesised DNA fragments.

## 2.4 Continuous-culture biofilm system

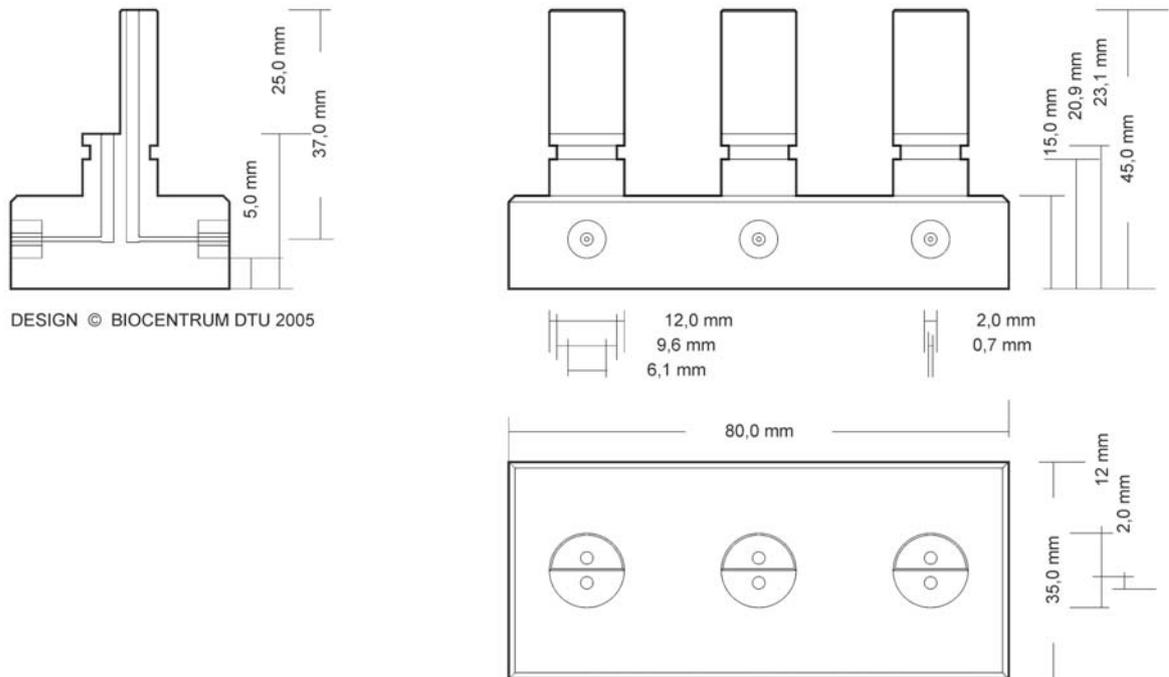
The continuous-culture biofilm apparatus is a closed system which allows viable biofilm structures to be grown and analysed under controlled conditions. Components of this system are connected with silicone tubing and connectors where the media flow is split before the peristaltic pump (Fig. 2.1). Media flow is controlled with the use of a peristaltic pump and bubbles are removed from the system through the use of bubble traps (2.4.1). Once media flows through the flow cell chamber (2.4.2) where the biofilms are grown, it is deposited into an effluent container for disposal. This method allows a continuous flow of nutrients to the biofilm-associated cells and also ensures consistency between results. Each biofilm in this study has been produced in triplicate (i.e. biological replicates) and data shown in this report are representative of this.



**Figure 2.1. The continuous-culture biofilm system (Jakobsen, *et al.*, 2011).**

This closed system allows control of the medium flow speed with the use of a Peristaltic Pump. The introduction of bubbles into the system is minimised with the inclusion of a bubble-trap system (2.4.1) before the medium reaches the flowcell chamber (2.4.2). Waste is deposited into the effluent bottle before being autoclaved and disposed.

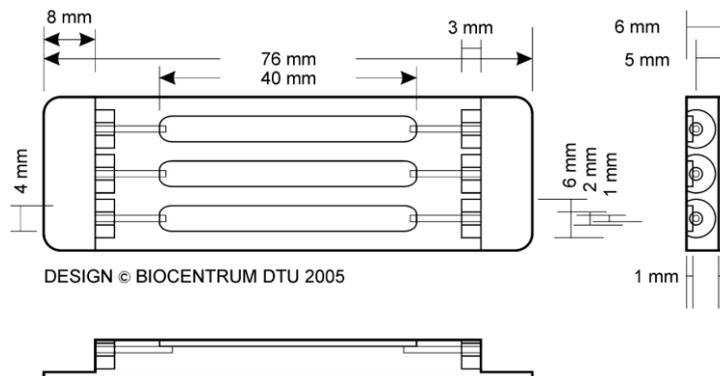
## 2.4.1 Bubble trap apparatus



**Figure 2.2.** The dimensions of the bubble trap apparatus (Nielsen, *et al.*, 2011).

5 mL syringes are attached to the bubble traps and filled with medium to trap any bubbles which might enter the system before the flow cells (2.4.2).

## 2.4.2 Flow cell chamber



**Figure 2.3.** The dimensions of the flowcell chamber (Nielsen, *et al.*, 2011).

A coverslip is attached to the surface with silicone glue and tubing is inserted at each end to facilitate media flow.

### 2.4.3 Cleaning procedure

In between each biofilm experiment the coverslip was removed from the flow cell chamber (2.4.2) and a new one attached using silicon-based adhesive as previously described (Nielsen, *et al.*, 2011). Once dry, the flow cell chamber was attached to the system as shown (Figure 2.1). Flow of 1.0% sodium hypochlorite was started for 5 min at minimum speed (0.3 mL/min) to fill the system and then stopped to incubate the system for 1 h. This step was then repeated. The system was then flushed for 15 min at high speed (5 mL/min) with a 1% Decon 90<sup>®</sup> solution. Sterile water was then run through the system for 15 min at high speed (5 mL/min). The pipes were then filled with the sodium hypochlorite solution and incubated overnight. Before bacterial inoculation, sodium hypochlorite was removed by flushing with sterile distilled water for 15 min at high speed (5 mL/min). Media flow of MSM media (2.2.2) was then initialised and the system checked for loose connections and bubble formation before bacterial inoculation (2.4.4).

### 2.4.4 Biofilm inoculation and incubation

Desired strains were cultivated to early-stationary phase ( $OD_{600}=0.8$ ) by overnight incubation at 37°C in LB medium (2.2.1) supplemented with appropriate antibiotics (2.2.6). The tubes either side of the flow cell were clamped and 500  $\mu$ L of the desired bacterial strain was aseptically introduced into the flow chamber with a pipette at the closest connector. As the pipette tip was inserted into the tubing, a clamp was removed to allow the suspension to enter by capillary flow. This clamp was reattached before the tubing was pinched to remove the pipette tip and reattach the connector with minimal introduction of air bubbles. Once the bacteria were introduced, the tubes connected to the flow chamber were clamped and inverted for 4 h without flow to allow settling and attachment of the bacterial cells to the glass coverslip. Following incubation, clamps were removed from the tubing and flow of the growth medium was initiated at minimum speed (0.3 mL/min). The closed system was then incubated at 37°C for the desired period of time before staining (2.5.1) and analysis by CLSM (2.5.2).

## 2.5 Microscopy techniques

### 2.5.1 Biofilm structure staining

Biofilm structure was visualised with one of two fluorescent nucleic-acid stains, dependent on which fluorescent protein signal was being detected. Where the fluorescence of HcRed was observed, biofilm cells were stained with the green fluorescent nucleic acid stain, SYTO9. Where the fluorescence of cfp or gfp was observed, the biofilm cells were stained with the red fluorescent nucleic acid stain, SYTO64. In this way, the signal from the cells expressing the fluorescent protein could be differentially excited through use of a different excitation laser (see Table 2.1). This method eliminates the risk of background fluorescence from the cell stain appearing as the fluorescent protein signal from cells activating the EPS promoter of interest. To stain the cells, the appropriate dye was diluted by placing 5  $\mu$ L of 10000 x concentrated stain into 1 mL of sterile, Milli-Q water. 500  $\mu$ L of the stain solution was introduced with a sterile pipette tip in an identical manner to the bacterial cell inoculation (2.4.4). Cells within the flow chambers were incubated in the dark with the stain for 15 to 20 min before being rinsed with sterile, Milli-Q water for 15 to 20 min. The tubes connected directly to the flow cell (2.4.2) were then clamped and the biofilms transported to the confocal laser scanning microscope to visualise fluorescence within the viable and fully-hydrated biofilm.

### 2.5.2 Confocal laser scanning microscopy

CLSM investigations were performed using the Leica TCS SP5 DM6000B in the Manawatu Microscopy and Imaging Centre (MMIC) with the following fluorescence parameters:

Fluorophore	Excitation peak	Emission peak	Laser used	Excitation used	Emission range observed
eCFP	434 nm	477 nm	Argon	458 nm	468-505 nm
SYTO9	480 nm	500 nm	Argon	488 nm	490-550 nm
eGFP	489 nm	508 nm	Argon	488 nm	500-600 nm
t-HcRed	590 nm	637 nm	DPSS 561	561nm	600-800 nm
SYTO 64	599 nm	619 nm	DPSS 561	561 nm	609-700 nm

**Table 2.10. Fluorescence parameters for CLSM.**

Fluorescence data were collected by setting the required laser power to 50% and taking images at a resolution of 1024x1024 pixels. For image stacks, a vertical step size of 1  $\mu\text{m}$  was used and images were taken to a height greater than that of the biofilm. Data were collected at 200 x – 400 x zoom with an oil immersion lens.

### **2.5.3 Image analysis software**

Images and image stacks were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland). Briefly, image stacks were imported into the program and layered to create 3-D images. False colours were added to signals to distinguish staining from bacterial fluorescence and localisation of signals was achieved by taking 2-D cross-sectional images from the stacks.

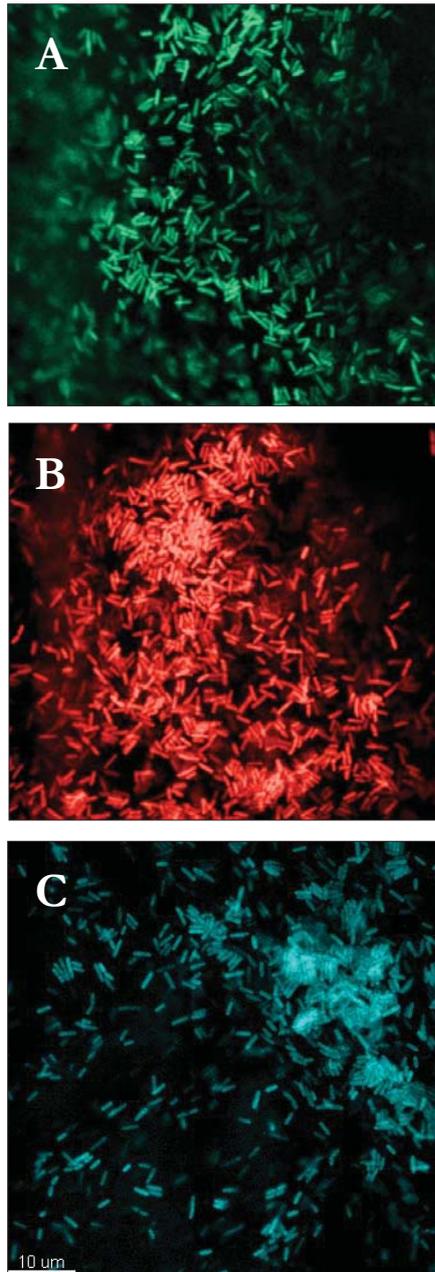
## 3.0 Results

### 3.1 Introduction

In this study, the spatial and temporal localisation of EPS production in mucoid and non-mucoid *P. aeruginosa* biofilms was investigated. *P. aeruginosa* has been extensively utilised as a model organism for the study of biofilm dynamics, due to its close association with a poor clinical prognosis in CF patients (Pedersen, *et al.*, 1992). PDO300 is a mucoid variant of the prototypic PAO1 strain which is routinely isolated from CF patients colonised with *P. aeruginosa*. PDO300 has a knockout mutation within the regulatory pathway of alginate, thus allowing a significant amount of this EPS to be produced for relevant biofilm studies (Mathee, *et al.*, 1999). This strain has been used to investigate the first two aims of this study. The wild-type *P. aeruginosa* PAO1 strain was used in addressing the third aim of this study, to allow the investigation of EPS localisation throughout the development of a non-mucoid biofilm.

### 3.2 Fluorescent protein labelling of PDO300 double-knockout mutants

To investigate the effect of alginate production on bacterial cell localisation, three variants of the *P. aeruginosa* PDO300 strain were used, each with two of three essential EPS-producing genes (*alg8*, *pslA* & *pelF*) knocked out, leaving them able to produce only one of the three EPSs being investigated (Ghafoor, *et al.*, 2011). Alginate production was inhibited by introducing a mutation in the *alg8* gene, a glycosyltransferase which is essential for alginate polymerisation (Remminghorst & Rehm, 2006). Psl production has been inhibited by knocking out *PslA*, an essential gene in the synthesis of this EPS (Overhage, *et al.*, 2005). PelF is a UDP-glucose dehydrogenase as demonstrated in a recent study (Ghafoor, *et al.*, 2013) and a deletion in the *pelF* gene has been shown to inhibit Pel production (Ghafoor, *et al.*, 2011). Chromosomal labelling of each double EPS-knockout mutant was achieved using the mini-Tn7 transposon system (2.3.8). This technique allowed the introduction of genes encoding fluorescent proteins into a neutral site of the PDO300 chromosome by electroporation with the helper plasmid, pUX-BF13 (Koch, *et al.*, 2001). The fluorescence of these strains was confirmed using fluorescence microscopy (Fig. 3.1).

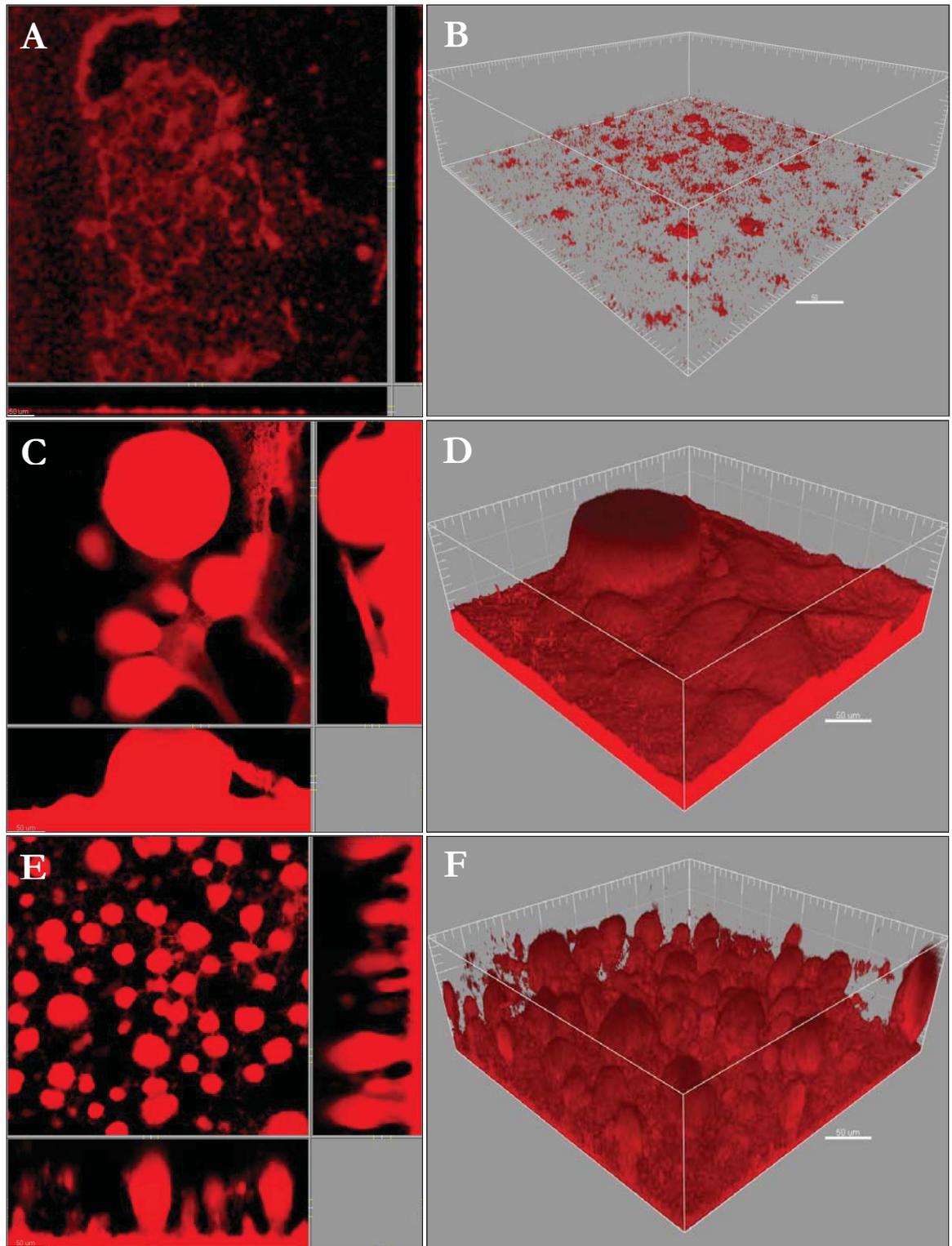


**Figure 3.1. Confirmation of the fluorescence of the double-knockout, single-EPS-producing *P. aeruginosa* PDO300 strains.**

Cells of each fluorescently-labelled *P. aeruginosa* PDO300 double-knockout strains (Table 2.5) grown in LB overnight were placed on a slide and covered with a coverslip, before being subjected to CLSM (2.5.2). The fluorescence of the following strains was confirmed; PDO300 $\Delta pslA\Delta pelF::gfp$  (A), PDO300 $\Delta pelF\Delta alg8::HcRed$  (B) & PDO300 $\Delta pslA\Delta alg8::cfp$  (C). Images were generated using IMARIS software (2.5.3). Scale bar shown is 10  $\mu\text{m}$ .

### 3.3 Effect of EPS production on biofilm structure

To observe the effect of a single EPS production on biofilm structure, the fluorescently-labelled, double-knockout mutants were grown into individual biofilms using the continuous-culture biofilm system as described previously (2.4). Some important characteristics of how EPSs contribute to biofilm architecture can be observed by examining the biofilm structure produced by these mutants of the *P. aeruginosa* PDO300 strain. The alginate-producing *P. aeruginosa* PDO300 double-knockout mutant (*P. aeruginosa* PDO300 $\Delta$ *pslA* $\Delta$ *pelF*) was incapable of forming any three-dimensional structure (Figs. 3.2A & 3.2B) when grown using the continuous-culture biofilm system (2.4). This result further highlights the essential role of the Psl and Pel EPSs in *P. aeruginosa* biofilms (Ma, *et al.*, 2006, Colvin, *et al.*, 2011). Here, the Psl-producing mutant has formed a distinct biofilm architecture characterised by wide microcolonies and significant biomass (Figs. 3.2A and 3.2B) when compared to the biofilm of cells only producing Pel (Figs. 3.2C and 3.2D). In contrast, it was observed that the Pel-producing mutant formed a biofilm structure with numerous, smaller microcolonies and significant inter-colony bridging (Figs. 3.2E and 3.2F). The Pel-producing mutant does not appear to be impaired in surface attachment or biofilm formation, as has been suggested previously (Overhage, *et al.*, 2005, Ma, *et al.*, 2006).

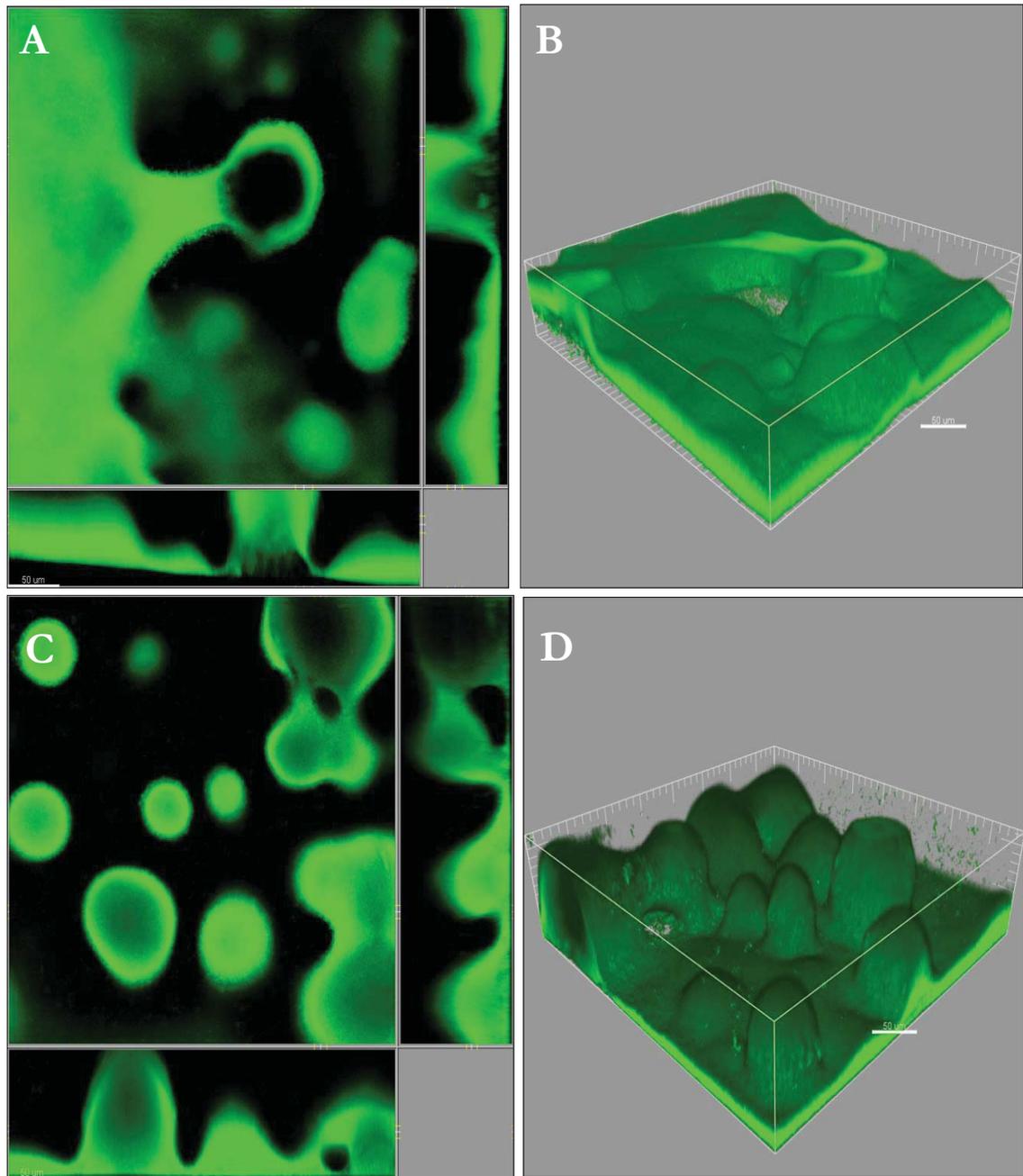


**Figure 3.2. Biofilm structure produced by *P. aeruginosa* PDO300 double-knockout mutants.**

The alginate-producing *P. aeruginosa* PDO300 $\Delta pelF\Delta psLA$  (A & B), Psl-producing *P. aeruginosa* PDO300 $\Delta pelF\Delta alg8$  (C & D) and Pel-producing *P. aeruginosa* PDO300 $\Delta psLA\Delta alg8$  (E & F) strains were grown into a mature biofilm using the continuous-culture biofilm system (2.4). All bacterial cells were then stained with the red nucleic acid stain SYTO64 (2.5.1) and subjected to CLSM (2.5.2) after 96 hours growth in MSM medium (2.2.2). Images were generated using IMARIS software (2.5.3). Scale bars shown are 50  $\mu\text{m}$ .

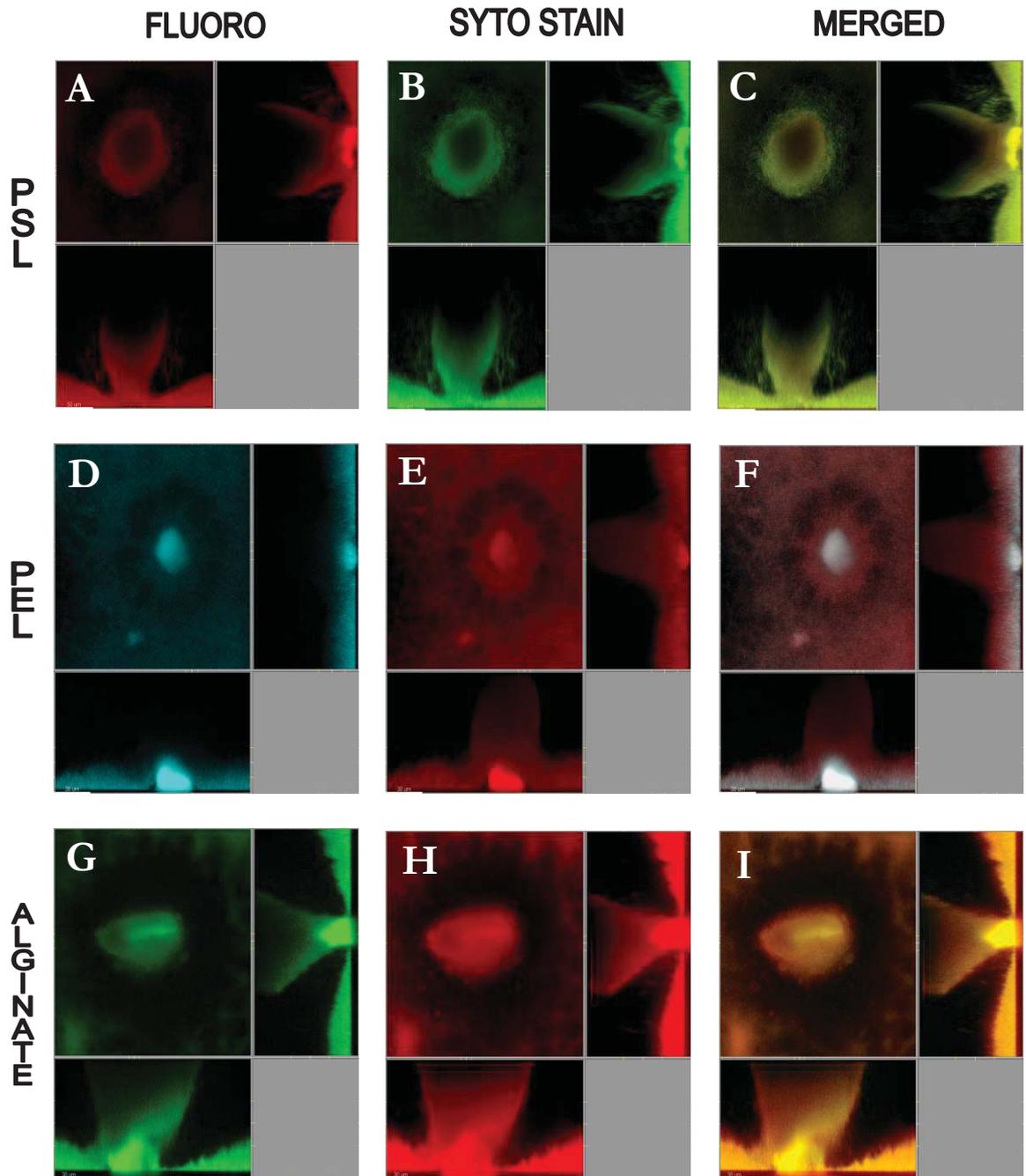
### 3.4 Spatial localisation of PDO300 strains in mature, mucoid biofilms.

In order to investigate the effect of EPS production on the localisation of *P. aeruginosa* within the biofilm matrix, a biofilm consisting of an equal mixture of the three, single EPS-producing, double-knockout mutants of the mucoid *P. aeruginosa* PDO300 strain were grown for 96 h using the continuous-culture biofilm method (2.4) and visualised using CLSM (2.5). The mixture for the mixed mutant biofilm was prepared by measuring the absorbance ( $OD_{600}$ ) of an overnight growth of each strain, then determining the percentage of each strain required to establish an equivalent population using the equation:  $[(1/\text{absorbance}) = 1:\text{strain ratio}] (\text{strain ratio} / \text{total ratio}) \times 100 = \% \text{ strain in final mixture}$ . The localisation of the double-knockout PDO300 mutants within the biofilm structure was achieved by using different CLSM settings (Table 2.1) to differentiate the signals emitted by each individual fluorescently-labelled protein. To ensure the results were representative of the biofilm architecture produced by the isogenic parent strain, a suspension of PDO300 was grown simultaneously under the same conditions as the mutant strains. The overall structure of the mixed mutant biofilm was similar in architecture and biomass to that of the PDO300 wild-type biofilm (compare Figs. 3.3A & 3.3B with Figs. 3.3C & 3.3D). By observing the individual signals from each fluorescently-labelled mutant strain within this biofilm, the differential localisation of each EPS-producing population could be observed. This localisation has been shown compared to the overall biomass which is shown by staining all bacterial cells with the corresponding nucleic acid stain (Figs. 3.4B, 3.4E & 3.4H). Figures have been merged to further localise the fluorescent bacteria within the biofilm structure (Figs. 3.4C, 3.4F & 3.4I). It is clear that the Psl and alginate-producing cells are evenly distributed throughout the biofilm structure (Figs. 3.4A & 3.4C and 3.4G & 3.4I), while the Pel-producing population is predominantly localised to the cells attached to the coverslip surface (Fig. 3.4D & 3.4F).



**Figure 3.3. The mature biofilm structure formed by wild-type *P. aeruginosa* PDO300 compared with the mixed mutant biofilm.**

The wild-type *P. aeruginosa* PDO300 strain (A & B) was grown into a mature biofilm under the same conditions as an equivalent mixture of three single-EPS producing PDO300 mutants (C & D) using the continuous-culture biofilm system (2.4). All bacterial cells were then stained 96 hours post-inoculation with the green nucleic acid stain SYTO9 (2.5.1) and subjected to CLSM (2.5.2). Images were generated using IMARIS software (2.5.3). Scale bars shown are 50  $\mu\text{m}$ .

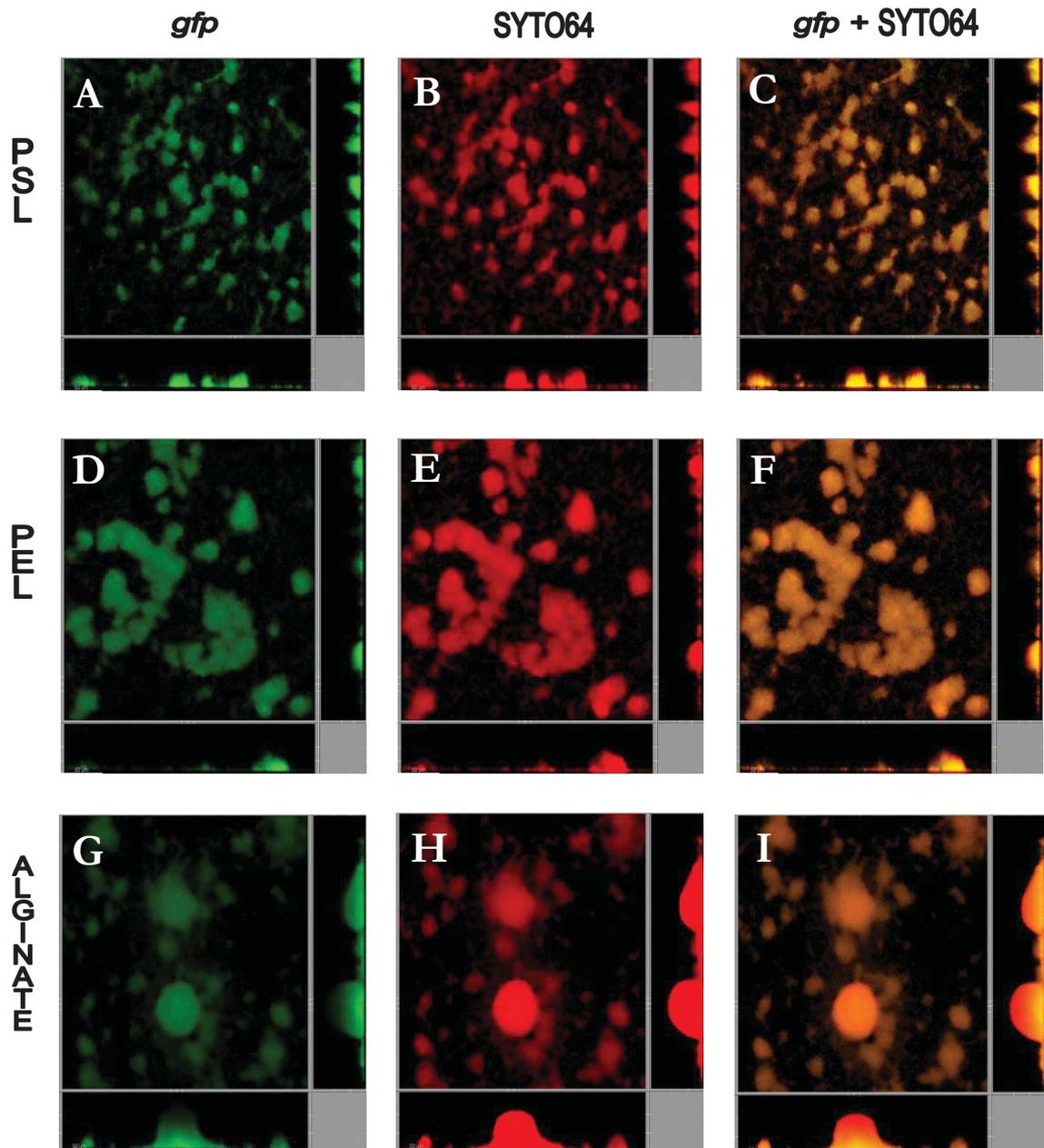


**Fig. 3.4. Spatial localisation of the fluorescently-labelled *P. aeruginosa* PDO300 double-knockout mutants within the mixed mutant biofilm.**

A biofilm consisting of an equivalent inoculation mixture of three fluorescently-labelled *P. aeruginosa* PDO300 double-knockout mutants was grown using the continuous culture flow system (2.4) *HcRed*-tagged Psl-producing PDO300 $\Delta$ *alg8* $\Delta$ *pelF* cells (A) were visualised within a biofilm stained with SYTO9 (B). *Cfp*-tagged Pel-producing PDO300 $\Delta$ *pslA* $\Delta$ *alg8* cells (D) were visualised within a biofilm stained with SYTO64 (E). *Gfp*-tagged alginate-producing PDO300 $\Delta$ *pslA* $\Delta$ *pelF* cells (G) were visualised within a biofilm stained with SYTO64 (H). Images were merged to localise fluorescent bacteria populations within mature biofilms (C, F & I). Image data were acquired by CLSM (2.5.2) 96 hours post-inoculation and images generated with IMARIS software (2.5.3). Scale bars are shown at 50 $\mu$ m.

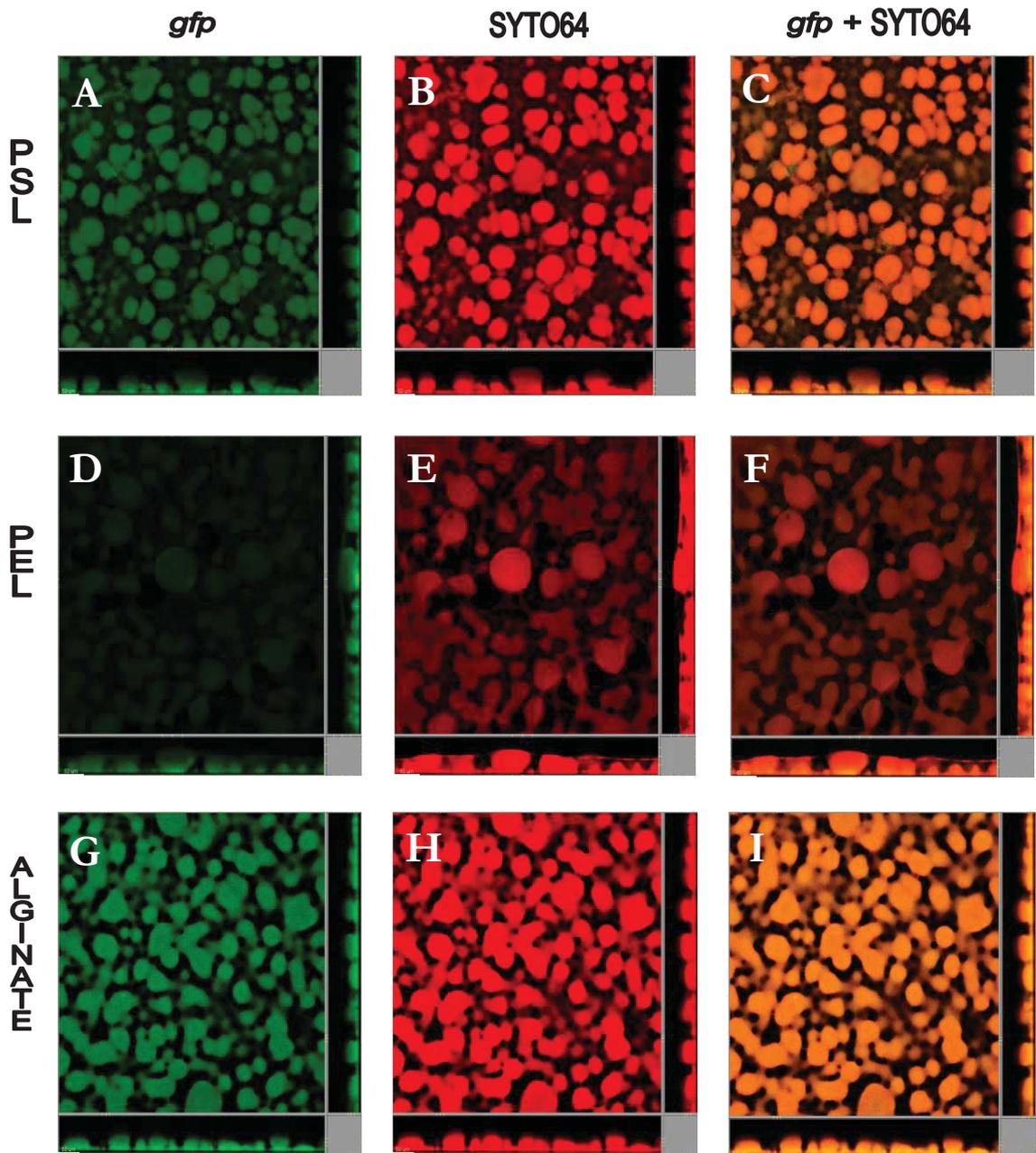
### 3.5 Temporal localisation of EPS gene expression in PAO1 biofilms.

The wild-type *P. aeruginosa* PAO1 strain was used in addressing the third aim of this study, to allow the investigation of EPS localisation throughout biofilm development of a non-mucoid biofilm. As previously mentioned, the mutation which occurs to switch a biofilm to the mucoid phenotype produces the same phenotype as the PDO300 strain, which was used to investigate bacterial localisation in mature, mucoid biofilms. In order to localise EPS gene expression, the broad-host-range promoter-probe vector (pPROBE'-*gfp*[AAV]::Gm) expressing unstable *gfp* under the control of one of the three EPS promoters was used to transform the isogenic *P. aeruginosa* strain, PAO1. The biofilms formed by these transformed strains were grown in MSM medium (2.2.2) for 24, 48, 72 and 96 h before staining with the red-nucleic acid stain SYTO64. *Gfp* expression under the control of each EPS's promoter was observed using the relevant CLSM parameters (Table. 2.1) and images generated with IMARIS software (2.5.3). The *psl* genes were expressed at a high level by *P. aeruginosa* microcolonies 24 hours after biofilm commencement (Figs. 3.5A & 3.5C), with apparent diffusion of *psl* promoter activity over the next 24 h (Figs. 3.6A & 3.6C). After 72 h of biofilm growth, *psl* gene expression was predominantly localised to the periphery of the microcolony structures (Figs. 3.7A & 3.7C) and had declined significantly by day four (Figs. 3.8A & 3.8C). *Pel* was expressed at a medium level by most cells at 24 and 72 h (Figs. 3.5D, 3.5F, 3.7D & 3.7F), with an apparent decrease in expression at 48 and 96 h (Figs. 3.6D, 3.6F, 3.8D & 3.8F). *Alg* expression was contained to the bottom layer of biofilm-associated cells at day one (Figs. 3.5G & 3.5I). Over the course of biofilm development, *Alg* expression is highest at 48 hours (Figs. 3.6G & 3.5I), with cells closer to the coverslip showing a lower level of fluorescence over the next two days (Figs. 3.7G, 3.7I, 3.8G & 3.8I).



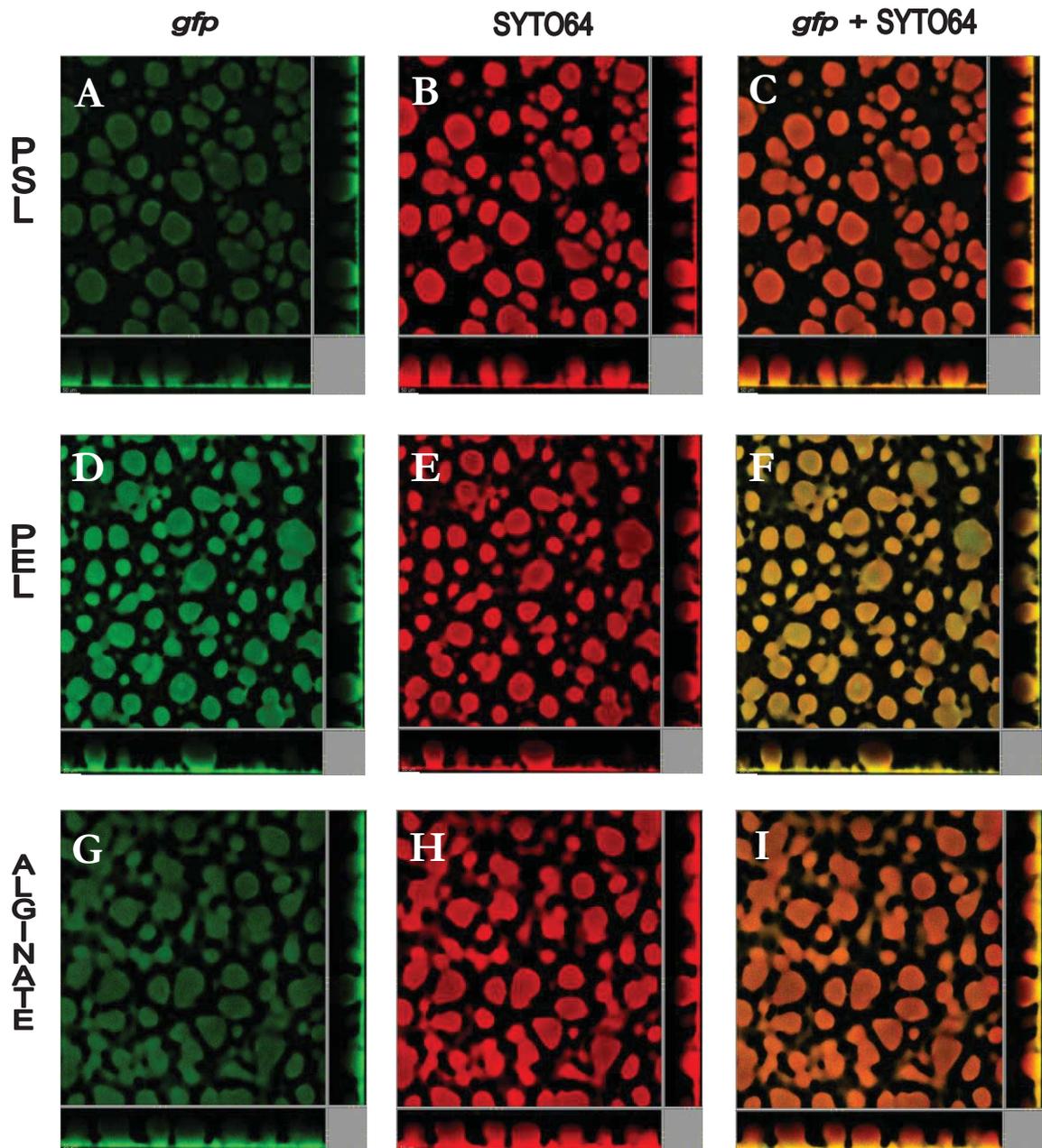
**Fig. 3.5. EPS promoter activity demonstrated with unstable *gfp* expression in 24-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of unstable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 24 hours post-inoculation (scale bars, 50  $\mu$ m) and images generated with IMARIS software (2.5.3).



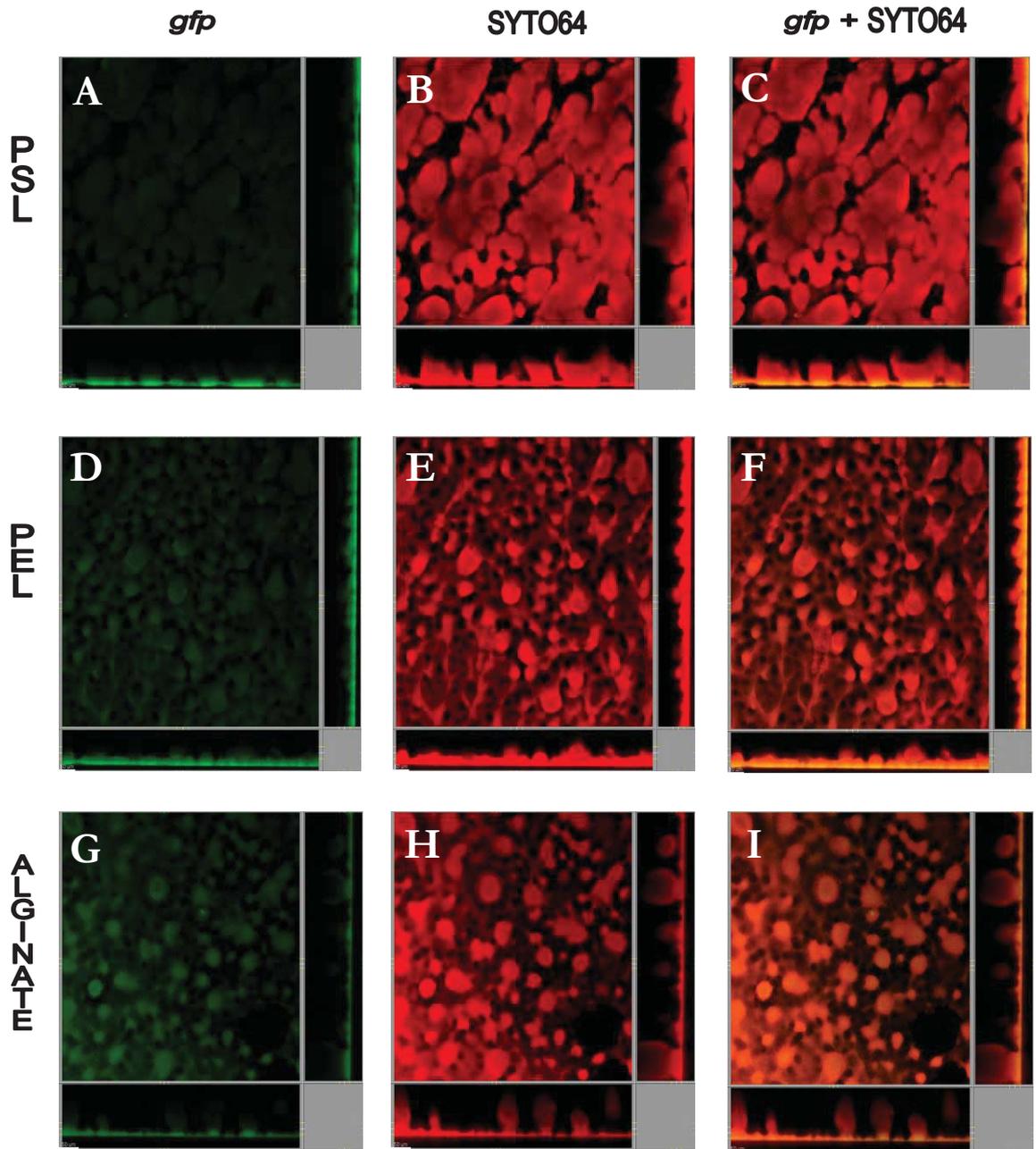
**Fig. 3.6. EPS promoter activity demonstrated with unstable *gfp* expression in 48-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of unstable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 48 hours post-inoculation (scale bars, 50  $\mu$ m) and images generated with IMARIS software (2.5.3).



**Fig. 3.7. EPS promoter activity demonstrated with unstable *gfp* expression in 72-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of unstable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 72 hours post-inoculation (scale bars, 50  $\mu$ m) and images generated with IMARIS software (2.5.3).

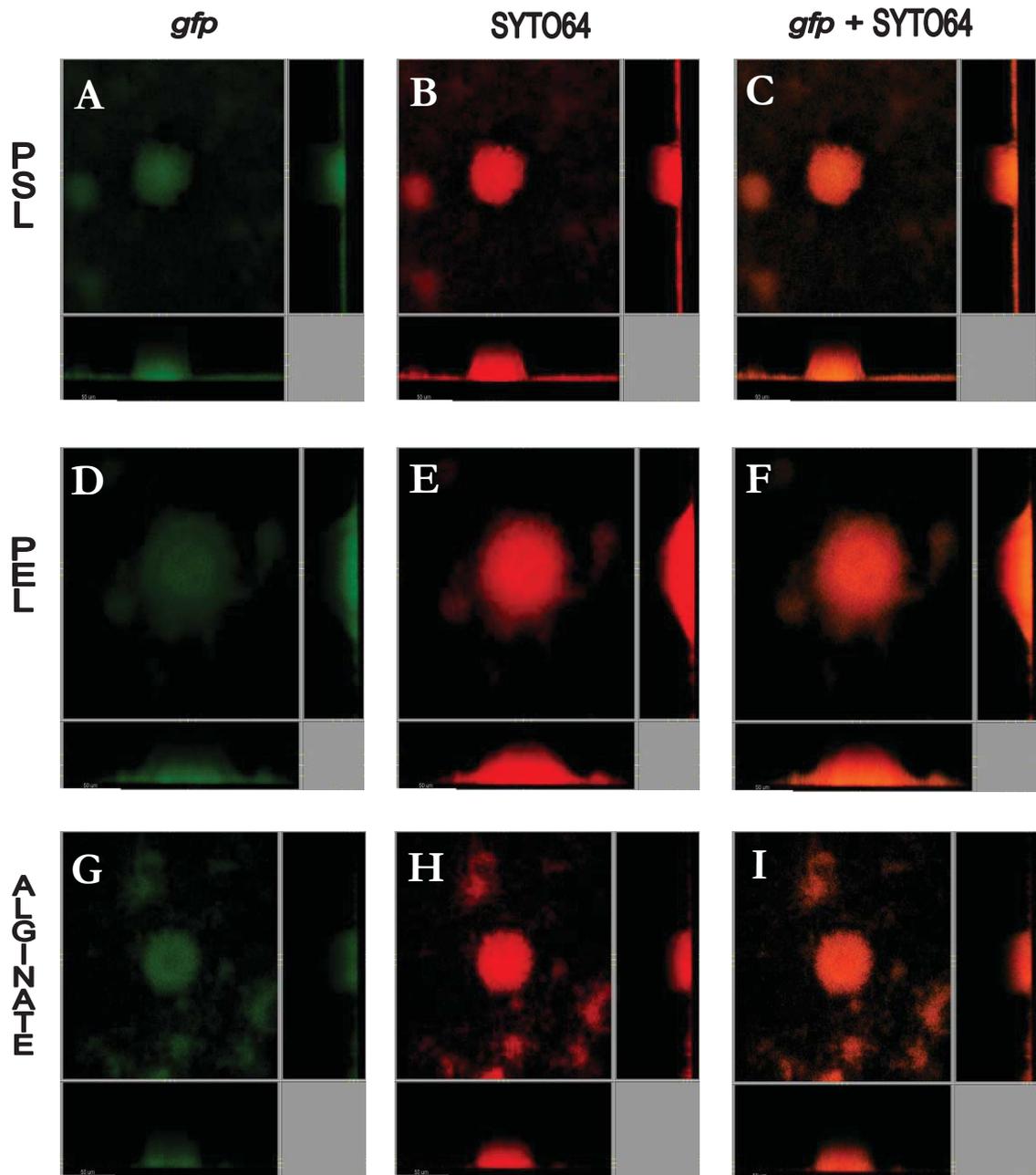


**Fig. 3.8. EPS promoter activity demonstrated with unstable *gfp* expression in 96-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of unstable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 24 hours post-inoculation (scale bars, 50  $\mu$ m) and images generated with IMARIS software (2.5.3).

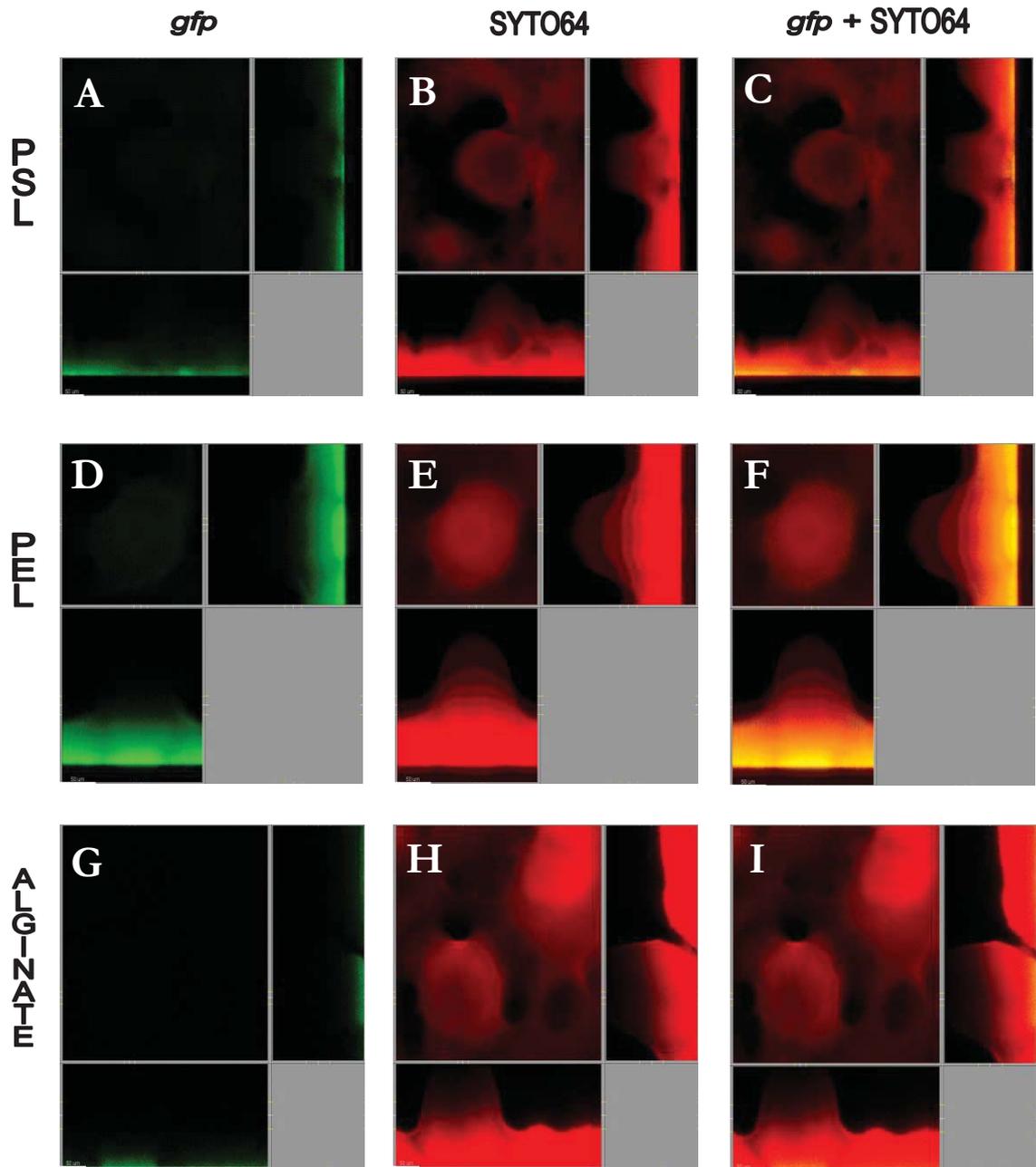
### 3.6 Spatial localisation of EPS gene expression in PAO1 biofilms

In order to meet the fourth aim of this study, isogenic *P. aeruginosa* PAO1 strains were transformed with one of three promoter-probe vectors (pProbeAT<sup>+</sup>::P<sub>*psl*</sub>, pProbeAT<sup>+</sup>::P<sub>*pel*</sub> and pProbeAT<sup>+</sup>::P<sub>*alg*</sub>) expressing stable *gfp* under the control of the Psl, Pel and alginate promoters respectively (Miller, *et al.*, 2000). This method allowed the localisation of EPS gene expression by observing stable fluorescence signals that last for more than 24 hours to see if distinctly localised EPS-producing populations could be observed. After 24 hours, transcription of all three EPS promoters appeared to be localised to the centre of the developing microcolonies (Figs. 3.9A, 3.9C, 3.9D, 3.9F, 3.9G & 3.9I). At 48 hours post-inoculation, *psl* and *alg* were expressed only by the coverslip-associated cells (Figs. 3.10A, 3.10C, 3.10G & 3.10I), while *pel* production was present in over half of the biofilm-associated cells (Figs. 3.10D & 3.10F). On day three, expression of *psl* and *pel* was localised to the centre of the microcolonies (Figs. 3.11A, 3.11C, 3.11D & 3.11F), while *alg* production was evenly distributed throughout the bottom half of the biofilm cells (Figs. 3.11G & 3.11I). At 96 hours, it was observed that both *psl* and *pel* expression was localised to the periphery of the microcolonies (Figs. 3.12A, 3.12C, 3.12D & 3.12F), while alginate production was present only in the surface-associated cells in the centre of the microcolony (Figs. 3.12G & 3.12I).



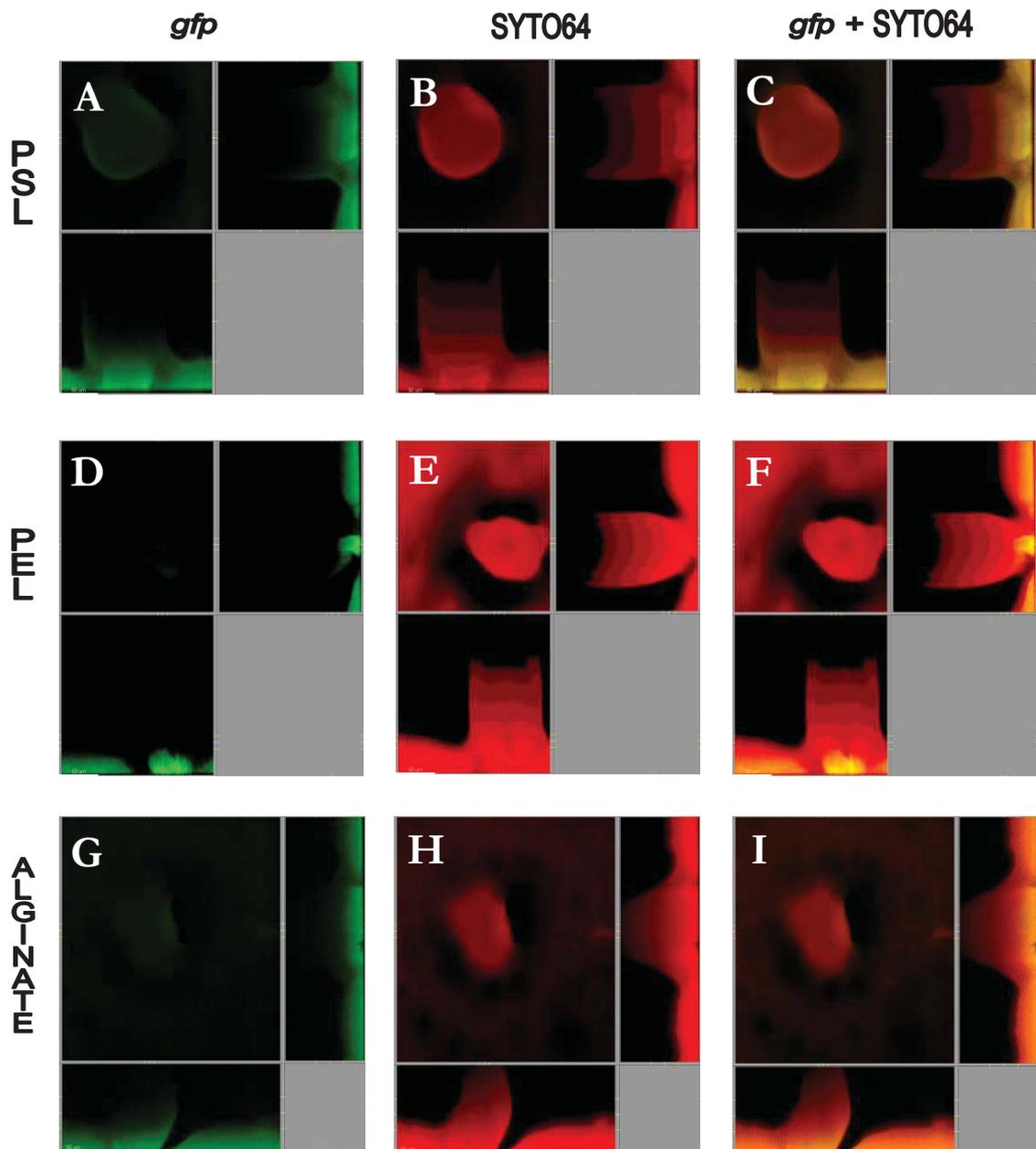
**Fig. 3.9. EPS promoter activity demonstrated with stable *gfp* expression in 24-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of stable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 24 hours post-inoculation (scale bars, 50  $\mu\text{m}$ ) and images generated with IMARIS software (2.5.3).



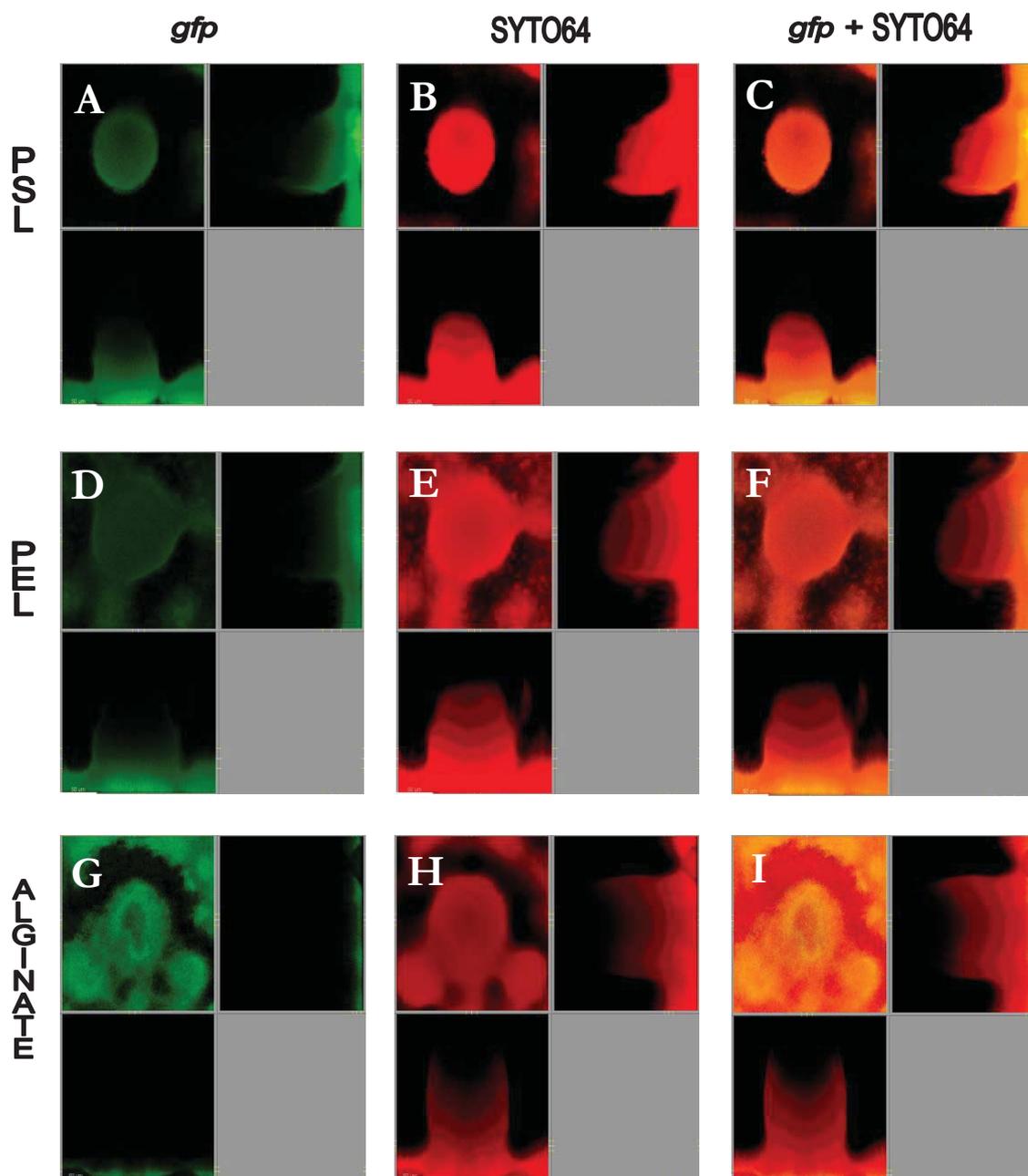
**Fig. 3.10. EPS promoter activity demonstrated with stable *gfp* expression in 48-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of stable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 48 hours post-inoculation (scale bars, 50  $\mu$ m) and images generated with IMARIS software (2.5.3).



**Fig. 3.11. EPS promoter activity demonstrated with stable *gfp* expression in 72-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of stable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 72 hours post-inoculation (scale bars. 50 $\mu$ m) and images generated with IMARIS software (2.5.3).



**Fig. 3.12. EPS promoter activity demonstrated with stable *gfp* expression in 96-hour-old *P. aeruginosa* PAO1 biofilms.**

Expression of stable *gfp* in transcriptional fusions with Psl (A), Pel (D) and alginate (G) promoters is shown. Biofilms were grown using the continuous-culture biofilm system (2.4) and structure was visualised with the use of SYTO64 stain (B, E and H). Images have been merged to localise EPS gene expression within the mature biofilm structure (C, F and I). Image data were acquired by CLSM (2.5.2) 96 hours post-inoculation (scale bars, 50 $\mu$ m) and images generated with IMARIS software (2.5.3).

### 3.7 Attempted generation of blue and red unstable fluorescent proteins

In order to investigate the fifth aim of this study, an attempt was made to generate unstable versions of blue and red fluorescent proteins to allow concurrent visualisation of the three EPS promoters being investigated. The cloning strategy for this objective involved the construction of a multi-promoter/reporter gene construct once lability of the fluorescent proteins had been confirmed. The method used was based on the technique used by Andersen *et al.* to generate unstable variants of GFP by the addition of an amino acid tail to the 3' end of the *gfp* gene. In generating the unstable *gfp*[AAV] gene used in this study, a C-terminal peptide tag was shown to increase the rate at which proteins are degraded by intracellular proteases (Andersen, *et al.*, 1998). High-fidelity PCR (2.3.4) was used to modify the C-terminus of the HcRed fluorescent protein gene using extended primers to encode new peptide tails, while the modified *bfp* gene was constructed *in silico* and ordered from Evrogen©. The sequence of the *bfp*[AAV] gene is shown in Figure 3.13. These genes were confirmed by diagnostic restriction endonuclease digest (2.3.9) and DNA sequencing (2.3.13) before ligation (2.3.10) into the pHERD-30T vector. This plasmid contains the *araC* P<sub>BAD</sub> cassette and has been used successfully to demonstrate arabinose-dependent alginate production in *P. aeruginosa* PAO1. For this study, pHERD-30T was used to facilitate inducible expression of the novel fluorescent protein genes through the addition of 1% arabinose to the growth media. Unfortunately, this method did not result in visible fluorescence of the cells which were successfully transformed with the confirmed plasmids. Since this research was concluded, unstable fluorescent proteins have been made commercially available and could therefore be utilised for further research in this area.

```
ATGAGCGAGCTGATTAAGGAGAACATGCACATGAAGCTGTACATGGAGG
GCACCGTGGACAACCATCACTTCAAGTGCACATCCGAGGGCGAAGGCAAG
CCCTACGAGGGCACCAGACCATGAGAATCAAGGTGGTTCGAGGGCGGCC
CTCTCCCCTTCGCCITTCGACATCCTGGCTACTAGCTTCCTCTACGGCAGCAA
GACCTTCATCAACCACACCCAGGGCATCCCCGACTTCTTCAAGCAGTCCCTT
CCCTGAGGGCTTCACATGGGAGAGAGTCCACCACATACGAAGACGGGGGC
GTGCTGACCGCTACCCAGGACACCAGCCTCCAGGACGGCTGCCTCATCTA
CAACGTCAAGATCAGAGGGGTGAACITTCACATCCAACGGCCCTGTGATGC
AGAAGAAAACACTCGGCTGGGAGGCCTTCACCCGAGACGCTGTACCCCGCT
GACGGCGGCCTGGAAGGCAGAAACGACATGGCCCTGAAGCTCGTGGGC
GGGAGCCATCTGATCGCAAACATCAAGACCACATATAGATCCAAGAAACC
CGCTAAGAACCTCAAGATGCCTGGCGTCTACTATGTGGACTACAGACTGG
AAAGAATCAAGGAGGCCAACAAACGAGACCTACGTCGAGCAGCACGAGGT
GGCAGTGGCCAGATACTGCGACCTCCCTAGCAAACITGGGGCACAAGCTTA
AAGGCCTGCAGCAAACGACGAAAACACTACGCTGCAGCAGTTAAT
```

**Fig. 3.13 Synthesised blue fluorescent protein gene sequence (blue) with AAV tag (red).**

This sequence was designed by removing the stop codon of the blue fluorescent protein gene (Evrogen<sup>®</sup>) and adding a C-terminal peptide sequence encoding the following amino acids: RPAANDENYAAAV. This sequence was custom-made by GenScript<sup>®</sup>.

## 4.0 Discussion

In addressing the first aim of this study, the effect of EPS production on the structure of *P. aeruginosa* biofilms was investigated. It was found that *P. aeruginosa* PDO300 mutants able to produce alginate only could not form any biofilm structure (Appendix 2), while the Psl and Pel-producing mutants both formed viable biofilms with significantly different biofilm mass and shape (Compare Figs. 3.2A & 3.2B with 3.2C & 3.2D). It has been previously indicated that Psl production is essential for biofilm attachment (Jackson, *et al.*, 2004) and development (Ma, *et al.*, 2006), however these findings appear to contradict this by showing that the Pel-producing mutant can also form a viable biofilm structure. The biofilm formed by the strain able to produce only the Pel EPS had the characteristic ‘mushroom’ microcolony structures of the wild-type (Figure 3.2C & 3.2D). These data suggest that Pel has a role in facilitating increased surface area during development of the biofilm, while the Psl EPS appears to enhance intercellular interactions which facilitate the adherence of more cells, leading to an increased biomass of the biofilm. This finding is consistent with the current understanding of Pel as a fundamental component of cell-cell interactions within the surface-associated microcolony, as demonstrated by studies showing *pel*-deficient mutants form biofilms which are smaller in mass and less tightly aggregated than the wild-type strains (Colvin, *et al.*, 2011).

In addressing the second aim of this study, fluorescently-labelled *P. aeruginosa* PDO300 double-knockout mutants were localised within a mixed-mutant, mucoid biofilm model. The overall structure of the mixed-mutant biofilm was comparable to that of the wild-type PDO300 biofilm (compare Figs. 3.3A & 3.3B with 3.3C & 3.3D). Within the mixed mutant biofilm, it was observed that Psl and alginate-producing bacteria were diffusely located throughout the microcolonies (Figs. 3.4A and 3.4G), while the Pel-producing cells were predominantly localised toward the coverslip surface (Fig. 3.4D). This result suggests that production of Pel influences the localisation of *P. aeruginosa* within the biofilm structure. The Pel polysaccharide has been implicated in the attachment of cells to a solid surface, however there appears to be an adaptive response whereby type IV pili can rapidly compensate for the loss of *pel* expression, making this function somewhat redundant (Vasseur, *et al.*, 2005). From this result it can be

concluded that the development of the characteristic mushroom microcolony structures is regulated by cells expressing *pel* at the surface on which the biofilm is attached.

In order to spatially and temporally resolve EPS gene expression within the biofilm, vectors were used to generate unstable and stable *gfp* expression under the control of each EPS operon promoter. Gfp was used as it has been shown that expression of *gfp* did not affect the three-dimensional structure of biofilms in fluorescent protein-expressing bacteria (Nivens, *et al.*, 2001). For temporal localisation of EPS gene expression, *P. aeruginosa* was transformed with a vector encoding an unstable version of the *gfp* gene under control of the EPS promoter activity being visualised. Gfp[AAV] has a half-life of approximately 19 h in the bacterial cell (Andersen, *et al.*, 1998), so if expression from the EPS promoter is stopped, the signal (GFP protein) is relatively quickly degraded. This method has allowed temporal resolution of EPS promoter activity for this study as any observed fluorescent signals are indicative of recent promoter activation. In observing the spatial localisation of EPS promoter activity, a similar vector was used to transform *P. aeruginosa* PAO1 cells, instead with a stable version of the *gfp* gene. The logic behind this method is that an EPS-producing bacterial population will continue to fluoresce and if they are localised to a particular part of the biofilm, this will be observed as a distinct signal. From the data collected through these methods, it is clear that all three EPSs are being produced by most of the biofilm-associated cells after 24 h (Figs. 3.5A, 3.5D & 3.5G). *Pel* expression appears to drop after 48 h and increase again after 72 h growth (Figs. 3.6D & 3.7D). The observation that *pel* promoter activity was reduced is consistent with a recent study showing that while Pel is integral in intercellular interactions involved in initiation of the biofilm structure, continuous expression is not required to maintain these interactions as is the case with *psl* (Colvin, *et al.*, 2011). The increase in expression on day three could be linked to the finding that Pel has a role in increasing the surface area of the biofilm (Fig. 3.2B). With regard to the spatial localisation of *pel* expression, fluorescence remains localised to the bottom layer of biofilm-associated cells throughout the time course experiment (Figs. 3.9D, 3.10D, 3.11D & 3.12D). The surface-associated expression of *pel* is consistent with the observation that Pel-producing cells were localised to the surface to which the bacterial biofilm is attached (Fig. 3.4D).

With regard to the temporal regulation of the Psl EPS, expression levels appear to be at their highest 24 h post-inoculation, with an apparent decrease in fluorescence over the unstable *gfp* time course experiment. This result contrasts literature that has suggested *psl* expression is constitutive in *P. aeruginosa* (Ma, *et al.*, 2006). The current observation could be due to the loss of vector-containing cells as the biofilm medium did not contain a selective antibiotic. The spatial pattern of *psl* promoter activity within the biofilms observed here is predominantly surface-associated during the first two days of biofilm development (Figs. 3.9A & 3.10A). This is consistent with literature which shows Psl is retained on surfaces and can act as a recruitment molecule for planktonic cells (Ma, *et al.*, 2006). In both the temporal and spatial investigations, *Psl* expression is subsequently localised to the periphery of mature microcolonies on day three (Figs. 3.7A & 3.11A). This finding supports a previous investigation by Ma, *et al.* (2009) which found that a distinctly higher level of Psl promoter activity is present in the periphery of mature microcolonies. This finding can be linked to interactions with extracellular DNA and the necessity of cavity formation for planktonic cell dispersal (Ma, *et al.*, 2009). Conversely, another study found that *psl* expression was localised to the centre of microcolonies, with a proposed role in cell differentiation during maturation of the biofilm (Overhage, *et al.*, 2005).

With regard to the regulation of alginate production, promoter activity appeared to be predominantly localised to the surface-associated cells (Figs. 3.9G, 3.10G, 3.11G & 3.12G), with expression levels appearing highest at 48 hours post-inoculation (Fig. 3.6G). It is known that alginate is not produced in significant quantities by PAO1 strains when grown as biofilms (Wozniak, *et al.*, 2003), which may explain the low levels of expression seen in this study. Although alginate is not necessary to initiate biofilm development, its production does have a significant impact on the structure of mature *P. aeruginosa* biofilms (Hentzer, *et al.*, 2002). Alginate is essential in protecting the biofilm-associated cells from opsonisation and phagocytosis during infection with mucoid strains (Stapper, *et al.*, 2004). Surprisingly, a higher level of fluorescence was observed during the biofilm experiments than expected based on current literature, which may suggest alginate plays a more significant role than is currently understood.

## 5.0 Conclusions

Overall, through observations of biofilms produced by single EPS-producing mutants of *P. aeruginosa*, a novel role has been proposed for Pel in increasing the surface area of the biofilm. In further observing the localisation of the Pel-producing population within the biofilm structure, this study provides speculation that the production of this EPS may be influenced by a different signalling mechanism to the other EPSs. These data also indicate that the ability of *P. aeruginosa* to produce EPSs has an effect on the localisation of cells within the mature biofilm. In further resolving EPS gene expression in *P. aeruginosa* biofilms, this study has both supported and contradicted current literature about the functions of these three EPSs. Contrary to the current understanding that production of a Psl EPS is essential for biofilm development in *P. aeruginosa* (Ma, *et al.*, 2006), the current study presents further information which suggests production of the Pel EPS alone can facilitate the formation of viable biofilm structures. If this finding can be confirmed through further research, therapies aimed at targeting EPS production to inhibit biofilm formation in *P. aeruginosa* will need to be aimed at ceasing production of both the Psl and Pel EPSs. The discovery of novel functions of EPS molecules during the process of biofilm formation should help to guide further studies of EPS production in bacterial biofilms. Undoubtedly, future studies will expand on concepts developed here in the hopes of advancing the development of EPS-directed therapies against pathogenic *P. aeruginosa*.

## 6.0 Limitations and future directions

Major limitations were present in this study with regard to the fluorescence data obtained by CLSM and images generated using IMARIS software. CLSM provides a number of limitations in terms of background “noise” and the consistency of data collected. The first limitation in analysis of image data is the variance associated with the use of IMARIS software to generate images. Altering the intensity of fluorescent signal data is required to remove any background fluorescent “noise” appearing in the final images, and can therefore be a significant source of variance between images. Steps were taken to limit this variance by eliminating background noise during collection. By reducing the gain signal to zero with the excitation laser switched off, background signals present in CLSM data were minimised before beginning data collection with the appropriate excitation laser switched on. This study was also limited by the fact that no quantitative data analysis could be performed with the IMARIS software available for use in this study. Additionally, there is a possibility of preferential selection of non-vector containing cells within the biofilm. As it was not economically viable to supplement a large volume of biofilm medium with antibiotics, some loss of the fluorescent-gene-encoding plasmids may have occurred within the biofilm-associated *P. aeruginosa* population over the time-course experiment. Quantitative data would be useful in determining the relative levels of EPS gene expression in biofilm-associated bacteria, and could be achieved through gene expression profiling methods such as real-time PCR or RNA sequencing to quantify EPS expression levels at different stages of biofilm development. These methods could also be used to confirm the finding of this study that *P. aeruginosa* can form viable biofilms without the presence of the Pel EPS. Overall, this study has demonstrated both the applications and limitations of fluorescent label-based approaches in the *in situ* localisation of EPS production throughout biofilm formation. Probably the most limiting factor of this study was the fact that the data collected are almost completely qualitative, therefore limiting the information which can be gathered from the images generated.

## 7.0 Publication

Data from this study was used for the following publication:

Ghafoor, A., Holbrook, S.A.K., Rehm, B. (2013) Temporal-spatial regulation of exopolysaccharides in mucoid and non-mucoid *Pseudomonas aeruginosa* biofilms. In preparation for submission.

## 8.0 References

- Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M & Molin S (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**: 2240-2246.
- Balasubramanian D, Kong KF, Jayawardena SR, Leal SM, Sautter RT & Mathee K (2011) Co-regulation of  $\beta$ -lactam resistance, alginate production and quorum sensing in *Pseudomonas aeruginosa*. *J Med Microbiol* **60**: 147-156.
- Beyenal H, Donovan C, Lewandowski Z & Harkin G (2004) Three-dimensional biofilm structure quantification. *J Microbiol Methods* **59**: 395-413.
- Billings N, Millan MR, Caldara M, Rusconi R, Tarasova Y, Stocker R & Ribbeck K (2013) The extracellular matrix component Psl provides fast-acting antibiotic defense in *Pseudomonas aeruginosa* biofilms. *PLoS pathogens* **9**: e1003526.
- Boyd A & Chakrabarty AM (1994) Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **60**: 2355-2359.
- Caldwell DE, Korber DR & Lawrence JR (1992) Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. *Journal of Microbiological Methods* **15**: 249-261.
- Cammarota MC & Sant'Anna GL (1998) Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. *Biotechnology Letters* **20**: 1-4.
- Choi KH, Kumar A & Schweizer HP (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Methods* **64**: 391-397.
- Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC & Parsek MR (2011) The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog* **7**: e1001264.
- Costerton JW (2007) Springer Series on Biofilms. (Costerton JW, ed. eds.), p. pp. Springer-Verlag, Berlin.
- D'Argenio DA & Miller SI (2004) Cyclic di-GMP as a bacterial second messenger. *Microbiology* **150**: 2497-2502.
- Davies DG, Chakrabarty AM & Geesey GG (1993) Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **59**: 1181-1186.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW & Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**: 295-298.

De Kievit TR, Gillis R, Marx S, Brown C & Iglewski BH (2001) Quorum-Sensing Genes in *Pseudomonas aeruginosa* Biofilms: Their Role and Expression Patterns. *Applied and Environmental Microbiology* **67**: 1865-1873.

Deretic V, Gill JF & Chakrabarty AM (1987) Gene *algD* coding for GDPmannose dehydrogenase is transcriptionally activated in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* **169**: 351-358.

Drenkard E & Ausubel FM (2002) *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**: 740-743.

Frederick MR, Kuttler C, Hense BA & Eberl HJ (2011) A mathematical model of quorum sensing regulated EPS production in biofilm communities. *Theor Biol Med Model* **8**: 8.

Friedman L & Kolter R (2004) Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. *J Bacteriol* **186**: 4457-4465.

Ghafoor A, Hay ID & Rehm BH (2011) Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl Environ Microbiol* **77**: 5238-5246.

Ghafoor A, Jordens Z & Rehm BH (2013) Role of PelF in Pel Polysaccharide Biosynthesis in *Pseudomonas aeruginosa*. *Applied and environmental microbiology* **79**: 2968-2978.

Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS & Lory S (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* **7**: 745-754.

Govan JR & Deretic V (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* **60**: 539-574.

Hay ID, Remminghorst U & Rehm BH (2009) MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **75**: 1110-1120.

Hay ID, Gatland K, Campisano A, Jordens JZ & Rehm BH (2009) Impact of alginate overproduction on attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa* strain. *Appl Environ Microbiol* **75**: 6022-6025.

Henrici AT & Johnson DE (1935) Studies of Freshwater Bacteria. *Journal of Bacteriology* **30**: 61-93.

- Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M & Parsek MR (2001) Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol* **183**: 5395-5401.
- Hentzer M, Riedel K, Rasmussen TB, *et al.* (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* **148**: 87-102.
- Holloway BW, Matsumoto H & Phibbs PV, Jr. (1986) The chromosome map of *Pseudomonas aeruginosa* PAO. *Acta Microbiol Pol* **35**: 161-164.
- Horwitz JP, Chua J, Curby RJ, *et al.* (1964) Substrates for Cytochemical Demonstration of Enzyme Activity. I. Some Substituted 3-Indolyl- $\beta$ -D-glycopyranosides1a. *Journal of medicinal chemistry* **7**: 574-575.
- Huang CT & Stewart PS (1999) Reduction of polysaccharide production in *Pseudomonas aeruginosa* biofilms by bismuth dimercaprol (BisBAL) treatment. *J Antimicrob Chemother* **44**: 601-605.
- Irie Y, Starkey M, Edwards AN, Wozniak DJ, Romeo T & Parsek MR (2010) *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol Microbiol* **78**: 158-172.
- Jackson KD, Starkey M, Kremer S, Parsek MR & Wozniak DJ (2004) Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. *J Bacteriol* **186**: 4466-4475.
- Jakobsen T, Gennip M, Christensen L, Bjarnsholt T & Givskov M (2011) Qualitative and Quantitative Determination of Quorum Sensing Inhibition In Vitro. *Quorum Sensing*, Vol. 692 (Rumbaugh KP, ed.^eds.), p.^pp. 253-263. Humana Press.
- Kirisits MJ, Prost L, Starkey M & Parsek MR (2005) Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **71**: 4809-4821.
- Klausen M, Heydorn A, Ragas P, Lambertsen L, Aes - Jørgensen A, Molin S & Tolker - Nielsen T (2003) Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Molecular microbiology* **48**: 1511-1524.
- Koch B, Jensen LE & Nybroe O (2001) A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. *J Microbiol Methods* **45**: 187-195.
- Lambertsen L, Sternberg C & Molin S (2004) Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. *Environ Microbiol* **6**: 726-732.

- Lawrence JR, Korber DR, Hoyle BD, Costerton JW & Caldwell DE (1991) Optical sectioning of microbial biofilms. *J Bacteriol* **173**: 6558-6567.
- Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y & Lory S (2007) A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol Microbiol* **65**: 1474-1484.
- Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR & Jeffers AK (2005) The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN- $\gamma$ -mediated macrophage killing. *J Immunol* **175**: 7512-7518.
- Linker A & Jones RS (1966) A new polysaccharide resembling alginic acid isolated from pseudomonads. *J Biol Chem* **241**: 3845-3851.
- Lory S, Merighi M & Hyodo M (2009) Multiple activities of c-di-GMP in *Pseudomonas aeruginosa*. *Nucleic Acids Symp Ser (Oxf)* 51-52.
- Ma L, Jackson KD, Landry RM, Parsek MR & Wozniak DJ (2006) Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* **188**: 8213-8221.
- Ma L, Conover M, Lu H, Parsek MR, Bayles K & Wozniak DJ (2009) Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* **5**: e1000354.
- Mathee K, Ciofu O, Sternberg C, *et al.* (1999) Mucoïd conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* **145 ( Pt 6)**: 1349-1357.
- Matsukawa M & Greenberg EP (2004) Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **186**: 4449-4456.
- Merritt JH, Ha DG, Cowles KN, *et al.* (2010) Specific control of *Pseudomonas aeruginosa* surface-associated behaviors by two c-di-GMP diguanylate cyclases. *MBio* **1**.
- Miller WG, Leveau JH & Lindow SE (2000) Improved gfp and inaZ broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* **13**: 1243-1250.
- Mulcahy LR, Isabella VM & Lewis K (2013) *Pseudomonas aeruginosa* Biofilms in Disease. *Microbial ecology* 1-12.
- Myszka K & Czaczyk K (2009) Characterization of adhesive exopolysaccharide (EPS) produced by *Pseudomonas aeruginosa* under starvation conditions. *Curr Microbiol* **58**: 541-546.
- Nielsen MW, Sternberg C, Molin S & Regenberg B (2011) *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* biofilm in flow cells. *Journal of visualized experiments: JoVE* e2383.

Nivens DE, Ohman DE, Williams J & Franklin MJ (2001) Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J Bacteriol* **183**: 1047-1057.

Ohman DE (2009) *Alginate Gene Regulation*. Springer, Berlin.

Overhage J, Schemionek M, Webb JS & Rehm BH (2005) Expression of the psl operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. *Appl Environ Microbiol* **71**: 4407-4413.

Pedersen SS, Hoiby N, Espersen F & Koch C (1992) Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. *Thorax* **47**: 6-13.

Phillips P, Sampson E, Yang Q, Antonelli P, Progulske-Fox A & Schultz G (2009) Bacterial biofilms in wounds. *Wound Healing Southern Africa* **1**: 10-12.

Qiu D, Damron FH, Mima T, Schweizer HP & Hongwei DY (2008) PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. *Applied and environmental microbiology* **74**: 7422-7426.

Remminghorst U & Rehm BH (2006) *In vitro* alginate polymerization and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **72**: 298-305.

Sakuragi Y & Kolter R (2007) Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa*. *J Bacteriol* **189**: 5383-5386.

Sambrook J, Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: a laboratory manual*. Planview, New York.

Sauer K, Camper AK, Ehrlich GD, Costerton JW & Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* **184**: 1140-1154.

Schlegel HG, Kaltwasser H & Gottschalk G (1961) [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. *Arch Mikrobiol* **38**: 209-222.

Shih PC & Huang CT (2002) Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J Antimicrob Chemother* **49**: 309-314.

Stapper AP, Narasimhan G, Ohman DE, *et al.* (2004) Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. *J Med Microbiol* **53**: 679-690.

Tamayo R, Pratt JT & Camilli A (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* **61**: 131-148.

- Tsuneda S, Aikawa H, Hayashi H, Yuasa A & Hirata A (2003) Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiol Lett* **223**: 287-292.
- Vasseur P, Vallet-Gely I, Soscia C, Genin S & Filloux A (2005) The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. *Microbiology* **151**: 985-997.
- Ventre I, Goodman AL, Vallet-Gely I, *et al.* (2006) Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**: 171-176.
- Webb JS, Thompson LS, James S, *et al.* (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. *J Bacteriol* **185**: 4585-4592.
- Wei Q & Ma LZ (2013) Biofilm Matrix and Its Regulation in *Pseudomonas aeruginosa*. *International journal of molecular sciences* **14**: 20983-21005.
- Wozniak DJ, Wyckoff TJ, Starkey M, Keyser R, Azadi P, O'Toole GA & Parsek MR (2003) Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* **100**: 7907-7912.