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^R	ampicillin resistance
bfp	blue fluorescent protein
bp	base pair(s)
c-di-GMP	cyclic diguanylate monophosphate
Cb	carbencillin
Cb^R	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
gfp	gene encoding green fluorescent protein
gfp	green fluorescent protein
Gm	gentamicin
Gm^R	gentamicin resistance
h	hour(s)
HcRed	*Heteractis crispa* 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_{600}	optical density at 600 nm
pel	gene encoding Pel (pellicle-forming) exopolysaccharide
Pel	exopolysaccharide encoded by *pel* gene
PDE	phosphodiesterases
psl	polysaccharide synthesis locus
Psl	exopolysaccharide encoded by *psl* 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 *Pseudomonas aeruginosa* 2

1.3 *P. aeruginosa* biofilms in cystic fibrosis patients 3

1.4 Discovery of EPSs in *P. aeruginosa* 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 *In vitro* 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, *et al.*, 2009). 2

Figure 2.1. The continuous-culture biofilm system (Jakobsen, *et al.*, 2011). 29

Figure 2.2. The dimensions of the bubble trap apparatus (Nielsen, *et al.*, 2011). 30

Figure 2.3. The dimensions of the flowcell chamber (Nielsen, *et al.*, 2011). 30

Figure 3.1. Confirmation of the fluorescence of the double-knockout, single-EPS-producing *P. aeruginosa* PDO300 strains. 35

Figure 3.2. Biofilm structure produced by *P. aeruginosa* PDO300 double-knockout mutants. 37

Figure 3.3. The mature biofilm structure formed by wild-type *P. aeruginosa* PDO300 compared with the mixed mutant biofilm. 39

Fig. 3.4. Spatial localisation of the fluorescently-labelled *P. aeruginosa* PDO300 double-knockout mutants within the mixed mutant biofilm. 40

Fig. 3.5. EPS promoter activity demonstrated with unstable *gfp* expression in 24-hour-old *P. aeruginosa* PAO1 biofilms. 42

Fig. 3.6. EPS promoter activity demonstrated with unstable *gfp* expression in 48-hour-old *P. aeruginosa* PAO1 biofilms. 43

Fig. 3.7. EPS promoter activity demonstrated with unstable *gfp* expression in 72-hour-old *P. aeruginosa* PAO1 biofilms. 44

Fig. 3.8. EPS promoter activity demonstrated with unstable *gfp* expression in 96-hour-old *P. aeruginosa* PAO1 biofilms. 45

Fig. 3.9. EPS promoter activity demonstrated with stable *gfp* expression in 24-hour-old *P. aeruginosa* PAO1 biofilms. 47

Fig. 3.10. EPS promoter activity demonstrated with stable *gfp* expression in 48-hour-old *P. aeruginosa* 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 *P. aeruginosa* 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. *Pfx* DNA polymerase PCR reaction mixture 23

Table 2.6. *Taq* 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 *P. aeruginosa* PDO300 double-knockout mutants 25

Table 2.9. Restriction endonuclease reaction mixture. 26

Table 2.10. Fluorescence parameters for CLSM. 32