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UNIVERSITY OF SOUTHAMPTON
FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
School of Biological Sciences

Persister Cell-Mediated Antimicrobial Tolerance in *Pseudomonas aeruginosa*
Biofilm Populations

by

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BSc (Hons)

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

UNIVERSITY OF SOUTHAMPTON
ABSTRACT
FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
Centre for Biological Sciences
Doctor of Philosophy
PERSISTENT CELL-MEDIATED ANTIMICROBIAL TOLERANCE IN *PSEUDOMONAS*
AERUGINOSA BIOFILM POPULATIONS
by Susanna Jane Sherwin BSc (Hons)

Bacteria preferentially live in biofilms; complex structures of single- or multiple- species of bacteria, surrounded by a self-produced polymeric extracellular matrix and attached to a surface. In this state, they are extremely recalcitrant to antibiotics, in part due to the presence of dormant or very slowly replicating cells called persisters. While lacking specific antibiotic resistance genes, persister cells are able to survive lethal stress due to their slow replication state, and once the antibiotic challenge has been removed, can seed fresh populations of surviving bacteria.

Despite recent progress in understanding molecular determinants of persister cell formation, few studies have examined *in vitro* the evolutionary and ecological drivers that sustain their presence. A functional role for persister cells is hypothesized in deferring replication within bacterial populations that are too crowded to sustain growth. This model predicts that slow-growing lineages with increased persister allocations will arise among density dependent bacterial populations, and that frequent exposure to lethal and non-lethal stresses will impact persister allocation. In support of the model, it was found that flow-cell biofilm culture rapidly resulted in new *P. aeruginosa* lineages with increased persistence, and that exposure to antibiotics can enhance this process. These observations have important clinical implications where chronic biofilm infections are treated with long-term antibiotic exposure.

The second results chapter examines the hypothesis that hypermutable phenotypes of *P. aeruginosa* are a characteristic of persister cell biology. Recent research has shown that key features of the development of bacterial biofilms include increasing mutation rates and increasing numbers of persister cells and the present work addresses whether there may be a relationship between these phenomena. A detailed examination of mutation levels and SOS response-mediated gene expression was carried out in persister cells. Persisters that had been isolated using both DNA damaging (ofloxacin) and non-DNA damaging (ethanol) antimicrobial treatments exhibited significantly increased frequencies of mutation to rifampicin resistance. Gene expression analysis of persister cells isolated following ethanol treatment demonstrated up-regulation of *mutS*, a DNA mismatch repair gene, suggesting that persister cells may undergo elevated DNA damage and mutation that can occur independently of the SOS response.

The third chapter aimed to study the role of persisters in determining the spatial and temporal pattern of biofilm formation following antibiotic treatment. A key feature of biofilms thought to play a role in antimicrobial tolerance is their ability to develop discrete, differentiated microcolony structures during colonization of a surface - these foci within biofilms are highly recalcitrant towards antimicrobials yet the factors that determine their differentiation and growth are poorly understood. This chapter therefore aimed to study the role of persisters in the initiation of microcolony foci and in mediating regrowth of biofilms. In this work, biofilm initiation was studied under a variety of conditions including with or without exposure to lethal or sub-lethal antibiotic challenge and as expected persister cell populations were able to generate significantly more biomass than in biofilms formed from non-persister populations. Dual labelling experiments were also carried out, where mixed persister and non-persister populations were tagged with either red or green fluorescent proteins. These experiments demonstrated that persister-mediated regrowth predominantly occurs as discrete microcolony foci that do not mix with the surviving non-persister cell population, suggesting that persisters are the progenitors of clonal microcolony foci within biofilms that are structurally distinct from the rest of the population of cells within the biofilm.

In summary, this thesis provides new information on the biology of persister cells in *P. aeruginosa* biofilms, and contributes to scientific understanding of the emergence of antibiotic tolerance in bacterial biofilm populations.

LIST OF CONTENTS

ABSTRACT	iii
LIST OF CONTENTS.....	vii
LIST OF FIGURES.....	xi
LIST OF TABLES.....	xvi
DECLARATION OF AUTHORSHIP.....	xvii
ACKNOWLEDGEMENTS	xix
LIST OF ABBREVIATIONS	xxi
CHAPTER 1.....	1
AN INTRODUCTION TO <i>PSEUDOMONAS AERUGINOSA</i> BIOFILM DEVELOPMENT, AND THE CONCEPT OF BIOFILM TOLERANCE TO ANTIMICROBIAL AGENTS.....	1
Biofilm Development	4
Bacterial attachment and adhesion to a surface	6
Quorum sensing in bacterial biofilm development	7
Matrix production	9
Microcolony differentiation	10
Dispersal	11
Bacterial Resistance to Antibiotics	13
Types of antibiotics.....	13
Bacterial resistance mechanisms.....	16
Bacterial Tolerance.....	17
Role of the extracellular matrix	18
Nutrient gradients and location of cells within the biofilm.....	19
Biofilm genetic diversification and heterogeneity	20
Mutation and hypermutation in biofilms.....	21
Persister Cells	23
Persister cells confer tolerance on bacterial populations.....	23
Current hypotheses for mechanisms of persister cell formation	25
Population Models for Processes Underpinning Persister Formation.....	30
Persisters as insurance policy.....	30
Persisters express a trade-off between resource competition and fecundity	30
Persisters as aged cells.....	31
Persisters as non-senescent cells.....	31
Overall Aims and Objectives of Thesis	33
Chapter 2: To determine the ecological and evolutionary influences on persister cell-mediated antimicrobial tolerance in <i>P. aeruginosa</i> biofilms	33

Chapter 3: To determine the role of mutation frequency and SOS response on persistence in <i>P. aeruginosa</i> populations	33
Chapter 4: To determine the role of persistence in microcolony initiation and seeding of new biofilm growth following antibiotic treatment	34
CHAPTER 2	35
ECOLOGICAL AND EVOLUTIONARY INFLUENCES ON PERSISTENT CELL-MEDIATED ANTIMICROBIAL TOLERANCE IN <i>P. AERUGINOSA</i> BIOFILMS	35
Abstract	37
Introduction	38
Experimental Procedures.....	41
Bacterial strains, media and growth conditions	41
Biofilm formation and isolation of mutants.....	41
Growth rates and heritability of growth	41
Motility assays.....	41
Antibiotic killing of planktonic cultures	42
Analysis of biofilm total biomass using COMSTAT	42
Antibiotic killing of biofilm cultures	43
Frequent, intermittent exposure of biofilms to antibiotics.....	43
Statistical analysis	43
Results.....	44
Variation of growth rates and persister levels among biofilm-derived bacterial isolates	44
Morphology and persistence in biofilms seeded from slow-growth isolates MT24 and MT30 ...	48
Frequent treatment of biofilms with antibiotics leads to enhanced persistence	50
Discussion	53
CHAPTER 3	57
ROLE OF MUTATION FREQUENCY AND SOS RESPONSE ON PERSISTENCE IN <i>P. AERUGINOSA</i> POPULATIONS.....	57
Abstract	59
Introduction.....	60
Experimental Procedures.....	63
Bacterial strains, media and growth conditions	63
Antibiotic killing of planktonic cultures	63
Mutation frequency assay	63
Validation of PMA chelation of dead cell RNA	64
Combining PMA and RNALater for preservation of live cell RNA	65
Gene expression of persister cells	65
Calculating relative gene expression	65
Statistics	66
Results.....	67

Determination of persister frequency in wild-type and $\Delta mutS$ <i>P. aeruginosa</i>	67
Effect of ofloxacin and ethanol on persister cell mutation frequencies	68
Gene expression of persister cells after isolation by ofloxacin or ethanol	70
Discussion.....	72
CHAPTER 4.....	77
ROLE OF PERSISTERS IN MICROCOLONY INITIATION AND SEEDING OF NEW BIOFILM GROWTH FOLLOWING ANTIBIOTIC TREATMENT.....	77
Abstract.....	79
Introduction	80
Experimental Procedures	82
Bacterial strains, media and growth conditions	82
Preparation of flow cell inoculum.....	82
Biofilm growth in the absence of antibiotics	82
Biofilm growth in the presence of antibiotics	83
Analysis of biofilm total biomass using COMSTAT.....	83
Transforming <i>P. aeruginosa</i> with green and red fluorescent plasmids.....	83
Growth rates and persister levels of transformant strains	84
2-colour biofilm growth with single antibiotic challenge	84
Statistical analysis.....	84
Results	86
Biofilm growth with no antibiotic stress.....	86
Biofilm growth with single 3-hour treatment of ofloxacin	86
Sub-lethal ofloxacin treatment during biofilm growth.....	91
Fluorescent protein colour tagging of <i>P. aeruginosa</i>	92
Discussion.....	95
GENERAL CONCLUSIONS AND FUTURE WORK	99
Chapter 2 - Ecological and Evolutionary Influences on Persister Cell-Mediated Antimicrobial Tolerance in <i>P. aeruginosa</i> Biofilms	101
Chapter 3 - Role of Mutation Frequency and SOS Response on Persistence in <i>P. aeruginosa</i> Populations	103
Chapter 4 - Role of Persisters in Microcolony Initiation and Seeding of New Biofilm Growth Following Antibiotic Treatment	104
APPENDICES.....	107
Appendix 1 - Characterization of <i>P. aeruginosa</i> Biofilm Colony Variants (Chapter 2).....	109
Mutant colony morphology.....	110
Motility of biofilm isolate mutants MT24 and MT30.....	111
Appendix 2 - Determination of Optimum Rifampicin Concentration for Mutation Frequency Assays (Chapter 3).....	113
Appendix 3 - Isolating Persister RNA (Chapter 3).....	113
PMA should not be used with proprietary solution RNALater	114

Appendix 4 - Relative Expression of Housekeeping Gene rpoD (Chapter 3)116

REFERENCES.....119

LIST OF FIGURES

Figure 1 Schematic of biofilm development, showing the various stages of biofilm formation, including (2) reversible attachment, (3) irreversible attachment and matrix production, (5) quorum sensing, (6) nutrient gradient led cell differentiation, (7) antimicrobial tolerance, and (8) shearing and dispersal of biofilm. Image courtesy of P. Dirckx, Centre for Biofilm Engineering, USA. Permission for use in thesis introduction obtained from P. Stoodley and L. Hall-Stoodley, University of Southampton, UK. 5

Figure 2 Structures of *P. aeruginosa* quorum sensing signalling molecules. PQS is the signalling molecule from the PQS system, C₄-HSL is the AHL signalling molecule from the Rhl QS system, and 3-oxo-C₁₂-HSL is the AHL signalling molecule from the Las QS system. 8

Figure 3 c-di-GMP plays an important role in bacterial dispersal. Intracellular levels of c-di-GMP are controlled by a host of extracellular signals, including NO. c-di-GMP triggers a cascade of proteins that induce changes in cell surface proteins, resulting in the expression of biofilm promoting (e.g. polysaccharide and pili) or virulence and dispersal (e.g. flagella) phenotypes. Figure taken from (McDougald et al., 2011) 12

Figure 4 Chemical structures of antibiotics from a range of classes. Bactericidal antibiotics such as the fluoroquinolone ofloxacin, β -lactams, such as ampicillin and carbenicillin, the aminoglycoside tobramycin, and the bacteriostatic antibiotic tetracycline. 14

Figure 5 Biphasic killing curve seen when *P. aeruginosa* culture is treated with ofloxacin. Mid-exponential phase cultures were exposed to 3 μ g/ml ofloxacin. Most bacteria are killed in the first 30 min of attack, showing exponential decay of normal cells, and then slower decay of persister cells. 24

Figure 6 Diagram of the SOS response in bacteria. Upon DNA damage, RecA surrounds the damage site, and also plays a role in cleaving the transcriptional repressor LexA. Once removed, several SOS response genes are expressed, resulting in the repair of the damaged DNA. Diagram taken from (Justice et al., 2008). 27

Figure 7 *P. aeruginosa* first generation wild-type biofilms give rise to isolates with increased range of growth rates compared to stationary phase planktonic cultures. Isolates collected from first generation wild-type *P. aeruginosa* PA01 biofilms had a wider range of growth rates than isolates collected from stationary phase planktonic culture (Levene's test statistic = 14.48, $P < 0.001$). First generation biofilm isolates $n = 33$, stationary phase planktonic isolates $n = 20$ 45

Figure 8 Representative profiles obtained from slow-growth and fast-growth isolates; biofilm-derived isolates with slow growth showed heritability of growth rate, whilst fast-growth isolates reverted to wild-type growth rates. Heritability of growth characteristics of biofilm-derived isolates MT30 (Slow growth; open squares), MT5 (fast growth; open triangles) and wild-type *P. aeruginosa* PA01 (open diamonds) after A, 3; B, 6; C, 9; or D, 12 subculture passages. Wild-type *P. aeruginosa* PA01 with no passages (filled diamonds) was used in each case as a control. Over 12 subculture passages, MT30

retained its slow-growth characteristics, while fast-growth isolate MT5 reverted to wild-type growth rates. MT30 and MT5 are representative profiles of the data obtained from slow growth and fast growth isolates.46

Figure 9 Slow-growth isolate biofilms give rise to variants with low growth rates compared to first generation wild-type biofilms. Isolates from the second generation biofilm seeded from slow-growth isolates MT24 and MT30 showed significantly slower growth rates than first generation wild-type biofilm isolates ($F_{1, 80} = 105.52, P < 0.001$). Isolates derived from MT24-seeded second generation biofilms had slower growth rates than isolates from the MT30 biofilms ($F_{1, 80} = 5.34, P = 0.023$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. First Generation biofilm isolates $n = 33$, Second Generation slow biofilm MT24 isolates $n = 20$, Second Generation slow biofilm MT30 isolates $n = 30$47

Figure 10 Biofilm-derived slow-growth isolates showed an increased persister cell allocation compared to wild-type and fast-growth isolates. Time dependant killing was carried out at approximate optical densities of 0.6. Isolates were incubated for 3 hours with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin. Solid line indicates wild-type *P. aeruginosa* PA01, while broken lines indicate growth variants. MT5 (filled squares) and MT33 (filled triangles) were fast growth isolates, while MT24 (open triangles) and MT30 (open squares) were slow growth isolates.48

Figure 11 Biofilms generated from slow growth biofilm-derived isolates showed decreased biomass and microcolony size compared to wild-type biofilms. A. Orthogonal contrasts in one-way ANOVA showed that second generation biofilms had less total biomass at 6 days than wild-type biofilms ($F_{1, 91} = 10.16, P = 0.002$). Second generation biofilm seeded from slow isolate MT30 also showed less total biomass than second generation biofilm seeded from slow isolate MT24 ($F_{1, 91} = 4.50, P = 0.037$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. Asterisks indicate outliers. Wild-type biofilm $n = 22$, Second generation biofilm slow (MT30) $n = 37$, Second generation biofilm slow (MT24) $n = 35$. B. **Slow-growth isolate biofilms showed decreased microcolony size compared to wild-type biofilms.** 6 day old biofilms were stained with fluorescent DNA stain SYTO9, and examined using confocal microscopy. Images taken using 200x magnification, and show a scale bar of $150 \mu\text{m}$49

Figure 12 Second generation biofilms exhibited higher survival than wild-type biofilm after exposure to ofloxacin. Orthogonal contrasts in one-way ANOVA showed that after 2 hours of incubation with $192 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin, both slow growth isolates, MT24 and MT30 had higher survival than the wild-type ($F_{1, 5} = 9.81, P = 0.026$). No difference could be detected between the levels of survival shown by the two slow growth isolates ($F_{1, 5} = 1.12, P = 0.339$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. WT $n = 3$, Slow-growth isolate MT30 $n = 2$, Slow-growth isolate MT24 $n = 3$ 50

Figure 13 A. Isolates recovered from biofilms treated with antibiotics exhibited increased persister allocation than isolates recovered from a wild-type biofilm. Orthogonal contrasts in one-way ANOVA showed that after 3 hours of incubation with $400 \mu\text{g}\cdot\text{ml}^{-1}$ tobramycin, isolates from both flow-cells exposed to antibiotics had higher survival than isolates from control flow-cells ($F_{1, 144} = 10.3, P = 0.002$).

There was no difference between bacteriostatic and bactericidal antibiotic treatment of biofilms (tetracycline and ofloxacin respectively, $F_{1, 144} = 1.36$, $P = 0.245$). **B. isolates recovered from biofilms treated with antibiotics exhibited faster growth rates than isolates recovered from a wild-type biofilm.** Orthogonal contrasts in one-way ANOVA showed that isolates from both flow-cells exposed to antibiotics had higher growth rates than isolates from control flow-cells ($F_{1, 144} = 21.65$, $P < 0.001$). There was no difference between bacteriostatic and bactericidal antibiotics ($F_{1, 144} = 2.08$, $P = 0.151$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. Asterisks indicate outliers. Control $n = 51$, Ofloxacin $n = 33$, Tetracycline $n = 63$ 51

Figure 14 No significant difference between persister allocation in the *P. aeruginosa* PA01 wild-type strain and the Δ mutS knockout strain. Survival of Δ mutS and wild-type strains of *P. aeruginosa* PA01 after 3 hour challenge with: A. 3 μ g/ml ofloxacin, or B. 20% ethanol. Box plots show median and inter-quartile ranges; means are shown as encircled crosses. Δ mutS strain $n = 3$, wild-type strain $n = 3$ 67

Figure 15 Ofloxacin-isolated persister sub-populations show increased mutation frequencies compared to non-persister populations in both wild-type and Δ mutS strains. A. Wild-type *P. aeruginosa* PA01. Persisters isolated with 3 μ g·ml⁻¹ ofloxacin, and early log phase populations showed increased mutation frequencies compared to the wild-type control population; persister cells also showed increased mutation frequencies compared to the early log phase population. Persisters $n = 6$, young $n = 5$, control $n = 3$. B. Δ mutS *P. aeruginosa* PA01. Persisters isolated with 3 μ g·ml⁻¹ ofloxacin, and early log phase populations showed no difference in mutation frequencies compared to the Δ mutS control population; persister cells showed increased mutation frequencies compared to the early log phase populations. Persisters $n = 3$, young $n = 6$, control $n = 3$. **Ethanol-isolated persister sub-populations show increased mutation frequencies compared to non-persister populations in only the wild-type strain.** C. Wild-type *P. aeruginosa* PA01. Persisters isolated with 12.5% (v/v) ethanol, and early log phase populations showed increased mutation frequencies compared to the wild-type control population; persister cells also showed increased mutation frequencies compared to the early log phase population. Persisters $n = 3$, young $n = 5$, control $n = 3$. D. Δ mutS *P. aeruginosa* PA01. Persisters isolated with 12.5% ethanol, and early log phase populations showed no difference in mutation frequencies compared to the Δ mutS control population; however, persister cells also showed increased mutation frequencies compared to the early log phase populations. Persisters $n = 3$, young $n = 6$, control $n = 3$. Box plots show median and inter-quartile ranges; means are shown as encircled crosses. 69

Figure 16 Relative expression of SOS and MutS genes in live cells during treatment with ofloxacin or ethanol. Live persister cell RNA was collected during treatment with 3 μ g·ml⁻¹ ofloxacin or 12.5% ethanol, and gene expression was determined by RT-PCR. Gene expression was observed for SOS response genes *recA* and *dinB*, and mismatch repair gene *musS*. Relative expression in this case was calculated by normalising to the expression of the housekeeping gene (see Appendix 4). White bars = *mutS*, hatched bars = *recA*, black bars = *dinB*. 71

Figure 17 Confocal imaging from day 4 of biofilm growth. Four day- old biofilms treated with no antibiotic (top row); a single 3 h treatment of 3 μ g·ml⁻¹ ofloxacin (middle row); or a continual sub-lethal concentration of 1.5 μ g·ml⁻¹ ofloxacin (bottom row) were examined using confocal microscopy. Flow cell

channels contained either persister derived biofilm (right column), biofilm from early log phase cells (middle column), or an equal mixture of the two inocula (left column). Images taken using 200x magnification.87

Figure 18 Confocal imaging from day 6 of biofilm growth. Six day- old biofilms treated with no antibiotic (top row); a single 3 h treatment of $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (middle row); or a continual sublethal concentration of $1.5 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (bottom row) were examined using confocal microscopy. Flow cell channels contained either persister derived biofilm (right column), biofilm from early log phase cells (middle column), or an equal mixture of the two inocula (left column). Images taken using 200x magnification.88

Figure 19 By day 4 of biofilm growth, persister-derived biofilm showed increased development compared to early log phase-derived biofilm during the 3 h ofloxacin treatment. No difference was detected between the mixed channel and the combined persister and early log phase channels. In all 3 hour ofloxacin treatment biofilms, increased growth was seen in the persister channels for both total biomass and average biofilm thickness, while both the control biofilms and the sub-lethal ofloxacin treatment biofilms showed no difference between persister and early log phase channels. Significant differences are marked with a large asterisk. All *P* and *F* values are shown in Table 2 and Table 3. Box plots show median and inter-quartile ranges; means are shown as an encircled cross. Outliers are shown as small asterisks.89

Figure 20 By day 6 of biofilm growth, early log phase-derived biofilm showed increased development compared to persister-derived biofilm during the control treatment. No difference was detected between the mixed channel and the combined persister and early log phase channels. In all control treatment biofilms, significant growth was seen in the non-persister channels for both total biomass and average thickness of biofilms, while both the 3 h ofloxacin biofilms and the sub-lethal ofloxacin treatment biofilms showed no difference between persister and early log phase channels. Significant differences are marked with a large asterisk. All *P* and *F* values are shown in Table 2 and Table 3. Box plots show median and inter-quartile ranges; means are shown as an encircled cross. Outliers are shown as small asterisks.90

Figure 21 Comparison of growth rate and survival to ofloxacin with different isolates from transformation of *P. aeruginosa*. *P. aeruginosa* PA01 wild-type isolates were examined for similarities of growth rate and persister allocation, to allow equality in flow cell growth. Two transformants (circle), one expressing GFP and one expressing the tomato fluorescent protein, were selected for further experimentation because they exhibited both the same growth rate and percentage of persisters.93

Figure 22 Persister-derived cells form more microcolonies than non-persister derived cells. Two-way ANOVA showed that persister-derived biofilms had increased numbers of microcolonies compared to non-persister early log phase-derived biofilms. The coloured tag had no significant effect on the biofilms, and no interaction between the two variables was seen. Box plots show median and inter-quartile ranges; means are shown as an encircled cross.94

Figure 23 Microcolonies are formed by unmixed clonal populations of cells. Biofilms were treated with one single lethal 3 h treatment of $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin. (A) Growth after 4 days. (B) Growth after 6 days. Flow cell channels contained either persister derived biofilm (green cells), or early log phase-derived biofilm (red cells). Images taken using 200x magnification..... 94

Figure 24 Progression of a Biofilm over time. Pictures taken on days 2, 3, 6 and 10, using light microscopy (40 x magnification). On day two, small clumping of bacteria can be seen; on day three, small microcolonies are beginning to form at the edge of the flow cell where shear forces are lowest; day six shows mature microcolonies covering the entire surface of the flow cell; day ten shows further enlargement and maturation of the microcolonies..... 110

Figure 25 Colony morphologies of growth variants, showing either wild type (WT) or small colony variant (SCV) morphologies. A Wild-type (WT) and small colony variant (SCV) colony variants on LB agar photographed using a light box. SCV colonies are smaller than WT colonies due to increased surface tension. B WT and SCV colony variants viewed under phase contrast light microscopy (40x magnification). WT colony shows evidence of growth out from the edges of the colony boundary, which is not observed in the SCV colony..... 111

Figure 26 Biofilm isolated mutants show decreased swim motility. Both A. MT30 and B. MT24 show decreased swimming motility compared to the wild-type *P. aeruginosa* PA01 after overnight growth on specialist 'swim' agar. Each picture is a representative of 6 replicates..... 112

Figure 27 Biofilm isolated mutants show decreased swarming motility. Both A. MT30 and B. MT24 show decreased swimming motility compared to the wild-type *P. aeruginosa* PA01 after overnight growth on specialist 'swarm' agar. Each picture is a representative of 6 replicates..... 112

Figure 28 Rifampicin concentration study. $300 \mu\text{g}\cdot\text{ml}^{-1}$ Rifampicin was considered the best concentration to use in the mutation frequency assay, as it resulted in similar killing in both the wild-type and ΔmutS strains. Each bar is an average of three replicates. ■ = ΔmutS , □ = wild-type. 113

Figure 29 Relative expression of housekeeping gene *rpoD*. Live persister cell RNA was collected during treatment with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin or 12.5% ethanol, and gene expression was determined by RT-PCR. Gene expression was observed for the housekeeping gene *rpoD*. Relative expression of the housekeeping gene was calculated as follows: 116

LIST OF TABLES

Table 1 *Persister cells isolated by time-dependant killing with ofloxacin show hypermutation. All values are averages in mutation frequency of 3-6 replicates of different bacterial sub-populations. All mutation frequencies above 2×10^{-7} are classified as mutators (Mena et al., 2008), and have been marked with an asterix.70*

Table 2 *One-way ANOVAs on days 4 and 6 comparing total biomass of the 50:50 mix control channel with the two treatment channels (persisters or non-persisters). Here, orthogonal contrasts of the pooled groups of early log phase and persisters are shown versus the 50:50 mix, and the early log phase versus the persisters. Significant effects ($P < 0.05$) in bold.91*

Table 3 *One-way ANOVAs on days 4 and 6 comparing average thickness of the 50:50 mix control channel with the two treatment channels (persisters or non-persisters). Here, orthogonal contrasts of the pooled groups of early log phase and persisters are shown versus the 50:50 mix, and the early log phase versus the persisters. Significant effects ($P < 0.05$) in bold.92*

Table 4 *PMA chelates bacterial RNA from cells with comprised membranes. PMA treatment of *P. aeruginosa* PA01 wild-type showed decreased concentrations of RNA after extraction than the control samples, indicating that PMA successfully chelates RNA as well as DNA.114*

Table 5 *Samples treated with a combination of RNALater and PMA showed increased concentrations of RNA compared to those treated with PMA only. This suggests that RNALater affects the binding of PMA, and stops it from chelating RNA from dead cells.115*

DECLARATION OF AUTHORSHIP

I, Susanna Jane Sherwin

declare that the thesis entitled

'Persister cell-mediated antimicrobial tolerance in *Pseudomonas aeruginosa* biofilm populations'

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed:

Date:.....

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AHL	acylhomoserine lactone
ANOVA	analysis of variants
ATP	adenosine triphosphate
CaCl ₂	calcium chloride
CF	cystic fibrosis
CFU	colony-forming unit
CPA	capsular polysaccharide
C _T	cycle threshold
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eDNA	extracellular DNA
EPS	extracellular polymeric substances
ESS	evolutionary stable strategy
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
GOI	gene of interest
GSR	global stress response
HK	housekeeping gene
KH ₂ PO ₄	potassium dihydrogen phosphate
LB	luria broth
M9	minimal medium 9
MDR	multi-drug resistance
MgSO ₄	magnesium sulphate
MIC	minimum inhibitory concentration
MMR	mismatch repair
MP	multiphoton
MT	mutant
NaCl	sodium chloride
Na ₂ HPO ₄	disodium hydrogen phosphate
NER	nucleotide excision repair
NH ₄ Cl	ammonium chloride
NO	nitric oxide

OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIA	polysaccharide intracellular adhesin
PMA	propidium monoazide
PMF	proton motive force
PQS	<i>Pseudomonas</i> quinolone signal
QRDR	quinolone resistant determinative region
QS	quorum sensing
RFP	red fluorescent protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
RPM	revolutions per minute
RT-PCR	real time PCR
SCV	small colony variant
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SP2	spectral photometry 2
TA	toxin-antitoxin
TCS	true confocal scanner
TDK	time dependant killing
WT	wild-type

CHAPTER 1

AN INTRODUCTION TO *PSEUDOMONAS AERUGINOSA* BIOFILM DEVELOPMENT, AND THE CONCEPT OF BIOFILM TOLERANCE TO ANTIMICROBIAL AGENTS

Bacteria possess an extraordinary capacity to adapt and survive within diverse environments. Viable bacteria can be recovered from habitats ranging from moderate environments such as rivers, oceans and soil, through living hosts, to extreme environments such as beneath Greenlandic glaciers (Loveland-Curtze *et al.*, 2009) and deep inside ice cores, thermal springs (Brock and Freeze 1969), deep sea hydrothermal vents (Kashefi and Lovley 2003), and even radioactive waste (Fredrickson *et al.*, 2004). It is currently thought that there are approximately $4-6 \times 10^{30}$ prokaryotic cells worldwide (Whitman *et al.*, 1998), comprising over a third of the world's biomass.

In their natural environment, bacteria are thought to survive preferentially in structures known as biofilms. These are complex three-dimensional constructions, consisting of bacterial cells enveloped in a slime known as the extracellular matrix, and commonly attached to a surface. Once a biofilm has formed on a surface, it is very difficult to remove, as bacteria growing in these structures are typically up to 1000-fold more tolerant to stresses, including antimicrobial compounds, than free-living cells. The recalcitrance of biofilms to removal is of great economic relevance in industrial, environmental and medical settings. Biofilm fouling is a common occurrence in industrial plants, resulting in release of toxic metabolites and corrosion. The metabolic activities of biofilms are not always toxic however, and biofilms are beneficially used to degrade and remove organic waste substances, for example from drinking water during sewage treatment.

Biofilms are also clinically relevant, because it is thought that up to 65% of all infections in developed countries are due to biofilm development (Costerton *et al.*, 1999; Costerton *et al.*, 2003; Lewis 2007; Potera 1999). These infections are wide-ranging, from colonisation of the lungs of patients with cystic fibrosis or chronic obstructive lung disease, to chronic wound infections, and infections of medical implants. Biofilm infections have serious consequences on the patients' prognosis, because bacteria in biofilms are also notoriously recalcitrant to antibiotics.

An important contributor to the recalcitrance of bacteria in biofilms is the presence of large numbers of dormant or slowly replicating cells called persisters. Persister cells are present in all bacterial populations, and due to their slow replication rates are much more recalcitrant to antimicrobial agents, surviving otherwise lethal antibiotic treatment (Balaban *et al.*, 2004; Sufya *et al.*, 2003). Once the antibiotic is removed, persister cells

can re-seed bacterial populations, resulting in recurrent infections. Due to much higher numbers of persisters in biofilms as opposed to planktonic cultures, biofilms are correspondingly more recalcitrant. Therefore, if a biofilm infection is not properly diagnosed, this can subsequently lead to ineffective therapeutic intervention and result in poor clearance of the microcolony, and this in turn can lead to complications through the release of bacteria from the biofilm causing bacteraemia and recurrent symptoms.

This review will detail current understanding of the formation of bacterial biofilms, and theories for the recalcitrance of biofilms when exposed to antimicrobial stress. It will also review the role of persister cells in antimicrobial tolerance, both through mechanistic understanding, and population modelling predictions.

Biofilm Development

Intensive research in the biofilm field has demonstrated that although biofilms can develop from a wide variety of bacterial genera in many different environments, they generally exhibit a common pattern of development (Figure 1). Firstly, free-living planktonic cells find a surface and attach through inter-molecular interactions such as van-der-Waals forces, or electrochemical attraction. This stage is reversible, but the bacteria swiftly show gene up-regulation to allow for the production of various irreversible attachment mechanisms such as several types of pili, and other adhesins. Once a bacterium has become irreversibly attached to a surface, studies show that in *Pseudomonas aeruginosa* the *las* quorum sensing system is activated (Sauer *et al.*, 2002), and, simultaneously, genes for the up-regulation of the extracellular matrix are switched on (Allison and Sutherland 1987). The matrix is a complex assortment of nucleic acids, proteins, polysaccharides such as alginate, and lipids - collectively known as the extracellular polymeric substance (EPS), which envelopes the whole biofilm and can account for between 50-90% of its total organic matter (Flemming and Wingender 2001). While encased in EPS, bacteria replicate swiftly until a mature microcolony has been formed. In a study carried out by Walters *et al.* (2003), *P. aeruginosa* in particular has been shown to have a specific growth rate of 0.66 ± 0.04 cells h⁻¹ in the first 6 hours of biofilm development, compared with a maximum specific growth rate of 0.80 ± 0.04 cells h⁻¹ for the exponential phase of planktonic growth. However, after the biofilm became established, cell replication in the biofilm occurred at a much decreased rate -

an average specific growth rate of 0.013 ± 0.009 cells h^{-1} in the last 24 hours of *P. aeruginosa* biofilm development (Walters *et al.*, 2003).

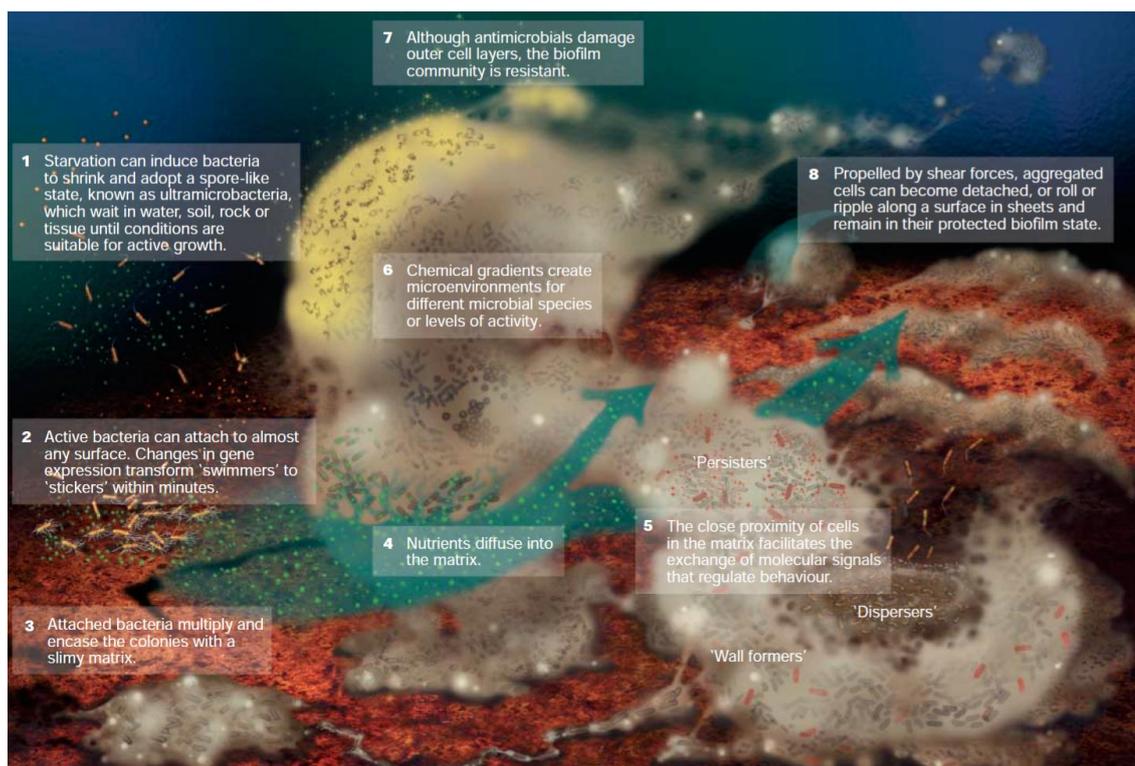


Figure 1 Schematic of biofilm development, showing the various stages of biofilm formation, including (2) reversible attachment, (3) irreversible attachment and matrix production, (5) quorum sensing, (6) nutrient gradient led cell differentiation, (7) antimicrobial tolerance, and (8) shearing and dispersal of biofilm. Image courtesy of P. Dirckx, Centre for Biofilm Engineering, USA. Permission for use in thesis introduction obtained from P. Stoodley and L. Hall-Stoodley, University of Southampton, UK.

Mature biofilms have been observed to show structures such as channels, cavities and pores that facilitate diffusion of chemical nutrients and oxygen (Davey *et al.*, 2003), but due to the density of cells inside a microcolony, steep nutrient and oxygen gradients still exist (Walters *et al.*, 2003). These gradients allow a large amount of genetic variance between cells, and account for the many different mutational and phenotypic variations seen within bacterial species in the biofilm (Boles *et al.*, 2004; Webb *et al.*, 2004). One such phenotypic variant can be seen at late stages of biofilm maturation, as motile bacteria are formed inside the structure of the biofilm. During the stage known as 'dispersal', these motile bacteria are released into the surrounding environment as the biofilm effectively breaks apart to allow for their emission (Purevdorj-Gage *et al.*, 2005; Webb *et al.*, 2003). These bacteria can consequentially attach to another free surface, to allow for colonisation and subsequent biofilm development.

Understanding the steps in biofilm development is crucial for the advancement of medical research, because if developmental mechanisms can be targeted, then biofilm formation may be controlled, potentially halting the movement from susceptible bacteria into recalcitrant biofilms.

Bacterial attachment and adhesion to a surface

Bacteria are able to colonise virtually any surface, from ship hulls and pipelines to rocks, plants and medical implants. Bacterial adhesion occurs in a two-step process, primary (reversible) adhesion, which is then followed by secondary (irreversible) adhesion (Dunne 2002). In some cases, bacterial adhesion is facilitated by surface conditioning, a process where chemicals are adsorbed onto a surface, resulting in increased primary adhesion. Molecules that may bind to surfaces in this way range from water and inorganic salts to albumin or platelets, completely changing the surface properties in some cases, resulting in easier adhesion. Herrmann *et al.* (1988) showed that surface bound fibronectin and fibrinogen on intravenous devices resulted in increased adhesion and infection by coagulase negative *staphylococci*.

In order for primary attachment to take place, any part of the bacterium (e.g. flagella) must come within <1 nm of a surface. This may occur by fluid movement, or by bacterial motility and chemotaxis. Once within range, the adhesion of the bacterium is a result of the net sum of forces acting on the bacteria and the surface. These forces include electrostatic interactions, hydrophobic interactions, and Van-der-Waals forces, with temperature, and steric hindrance also playing a role in this process (An *et al.*, 2000). Electrostatic interactions in most cases favour repulsion, as most inert surfaces have a predominantly negative charge, and most bacteria have a net negative charge making attachment difficult. An exception to this is the bacterium *Stenotrophomonas maltophilia*, which, unusually, has a net positive charge, and is able to bind to negatively charged surfaces such as Teflon (Jucker *et al.*, 1996). In cases where net repulsion occurs, bacteria are able to overcome these forces by means of specific molecular interactions, such as the use of adhesins located on pili.

Once a bacterium has become temporarily attached to a surface, up-regulation of exopolysaccharides, pili and fimbriae occurs, resulting in irreversible attachment to the surface. The cocoon of exopolysaccharides coating the surface of the bacterium also

allow for attachment of other bacteria, forming aggregates at the surface. Bacteria that cause infections that are associated with biomedical devices, such as *Staphylococcus epidermidis* use a range of mechanisms for attachment, including an adhesion-specific antigen called capsular polysaccharide (CPA) as a means of primary adhesion to a surface (Tojo *et al.*, 1988), and subsequently, a polysaccharide antigen called polysaccharide intercellular adhesin (PIA) as a means of allowing cellular aggregation to take place (Mack *et al.*, 1992). *P. aeruginosa* however, has a different means of allowing attachment and aggregation, involving motility and twitching mechanisms. Transposon insertion mutants of *P. aeruginosa* PA14 generated several strains that were surface attachment deficient (*sad*) (O'Toole and Kolter 1998). The first class of mutant had a transposon inserted into an *flgK*-like gene, a gene that codes for the flagellum-associated hook protein 1, essential for flagella operation. Loss of the flagella resulted in no monolayer of cells attaching to a surface. Other insertions in the genes responsible for production of type IV pili resulted in a different type of adhesion loss. The type IV pili is responsible for twitching motility, a mechanism by which cells are able to creep across a surface by means of extension and contraction of the type IV pili. These mutants were able to form a monolayer of cells on a surface, but were unable to aggregate into anything more complex than a monolayer. As the wild-type was able to both form a monolayer on a surface, and then cell aggregation, this work suggests that the flagellum in *P. aeruginosa* is essential for primary adhesion to a surface, and type IV pili are essential for cellular aggregation and biofilm formation.

Once bound to a surface, bacteria begin matrix production, a process by which cells are surrounded in a protective layer of polysaccharide, DNA and proteins (Flemming and Wingender 2001; Friedman and Kolter 2004a; Whitchurch *et al.*, 2002). After this, the bacteria start to form differentiated microcolonies. These processes are heavily dependent on cell-cell communication and co-operation. Several global regulation pathways, known as quorum sensing (QS) systems, have been shown to be responsible for regulating many aspects of biofilm development.

Quorum sensing in bacterial biofilm development

QS systems allow gene expression to be regulated as a response to population density. This occurs by means of a group of self-generated signalling molecules, such as acylhomoserine lactones (AHLs) in many gram negative species. Each cell produces AHLs in small concentrations, which have no effect; however, when a sufficient

population density has been reached, gene activation occurs. *P. aeruginosa* has two AHL-based QS systems Las and Rhl, plus another signalling molecule called the *Pseudomonas* Quinolone Signal (PQS) (De Kievit 2009). The structures of these signalling molecules can be seen in Figure 2 below.

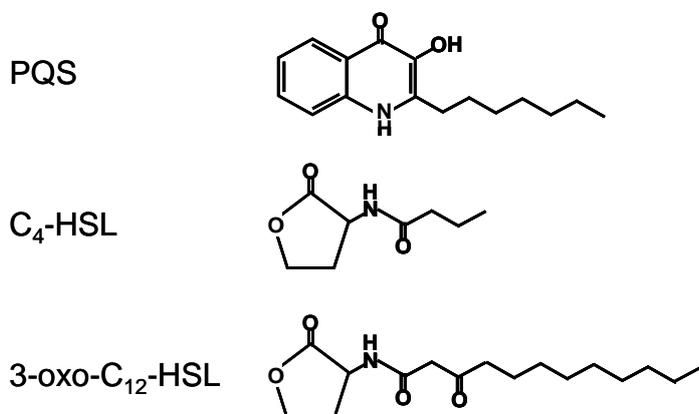


Figure 2 Structures of *P. aeruginosa* quorum sensing signalling molecules. PQS is the signalling molecule from the PQS system, C₄-HSL is the AHL signalling molecule from the Rhl QS system, and 3-oxo-C₁₂-HSL is the AHL signalling molecule from the Las QS system.

The Las system has 3-oxo-C₁₂-HSL signalling molecule that is produced by the AHL synthase LasI, and regulated by the transcriptional regulator, LasR (Gambello and Iglewski 1991; Passador *et al.*, 1993). The Rhl system similarly produces a C₄-HSL that is synthesized by the Rhl AHL synthase RhlI, and regulated by RhlR (Latifi *et al.*, 1995; Ochsner *et al.*, 1994). The PQS system is very slightly different, with the structural genes involved in PQS production being *pqsABCDH*, the transcriptional regulator *pqsR*, and the response effector *pqsE* (Gallagher *et al.*, 2002). Gallagher *et al.* also showed that the structural gene *pqsH* was regulated by LasR, thus linking the two systems. In 1998, a connection was discovered between the QS system and biofilm development, when the *P. aeruginosa* AHL synthase knockout mutant *lasI* was only able to form flat, undifferentiated biofilms, contrasting with the stalk-like microcolonies seen in the wild-type (Davies *et al.*, 1998). Biofilms formed with the *lasI* mutant were also much more susceptible to sodium dodecyl sulphate (SDS) mediated dispersal. Five minutes after treatment with 0.2% SDS, the majority of the *lasI* mutant biofilm had detached from the surface and dispersed (Davies *et al.*, 1998). Thus the Las QS system has a role not only in biofilm development, but biocide tolerance. Similar studies with the Rhl system were unable to produce the same results, indicating that the Rhl system is not involved in the same processes as the Las QS system (Davies *et al.*, 1998).

Matrix production

Once bound to a surface, bacteria immediately begin matrix production. The biofilm matrix is composed of a range of substances, notably exopolysaccharides, protein, extracellular DNA, and lipids. Several gene clusters are involved with the production of extracellular polysaccharides, such as the *alg* biosynthetic genes, plus the *psl* and *pel* operons (Friedman and Kolter 2004a; Friedman and Kolter 2004b). In a mucoid strain of *P. aeruginosa*, a key component of alginate production, *algC*, is activated within 15 minutes of attachment to glass surfaces, and this activity increased for the next 2 hours of incubation (Davies and Geesey 1995). At least part of polysaccharide production is thought to be controlled by QS. This is true of the glucose-rich exopolysaccharide producing *pel* operon, as both *lasI*, and to a lesser extent, *rhlI* mutants show decreased production of these polysaccharides (Sakuragi and Kolter 2007). It is interesting to note that the majority of components of the biofilm matrix are regulated in response to intracellular concentrations of a second messenger molecule, cyclic diguanosine-5'-monophosphate (c-di-GMP) (Harmsen *et al.*, 2010). While low intracellular concentrations promote the planktonic phenotype, high intracellular concentrations of this molecule result in up-regulation of *pel* (Lee *et al.*, 2007), *psl* (Starkey *et al.*, 2009), alginate polysaccharides (Merighi *et al.*, 2007), and type IV pili (Guzzo *et al.*, 2009), all of which are important components of biofilm formation.

Extracellular DNA (eDNA) is an important component of biofilm EPS, and its presence and positioning with the matrix alters as the microcolony matures. eDNA present in *P. aeruginosa* biofilms has been found to be similar to whole genome DNA (Allesen-Holm *et al.*, 2006). It has been shown to be a key component for cell to cell adhesion in the development of biofilms up to 60 hours old, because the addition of DNase I to the system resulted in the halting of biofilm development, and biofilm dissolution within 150 minutes (Whitchurch *et al.*, 2002). This was not seen with mature microcolonies, as these were only 'nominally affected'. This suggests that the role of eDNA in cell-cell adhesion decreases as the microcolony matures, or that other factors become more important in cell-cell adhesion in these stages of microcolony development.

The presence of eDNA within the matrix has been shown to be as a result of the lysis of a subpopulation of bacteria, a process under regulation by QS pathways. A study using three *P. aeruginosa* mutants, *lasI rhlI*, *fliM pila*⁻, and *pqsA*⁻, lacking Las and Rhl

synthases, flagella and pili function, and PQS synthase function, respectively, all showed increased removal by SDS of 2-day old biofilm biomass equivalent to biofilms previously treated with DNase I (Allesen-Holm *et al.*, 2006). This study indicates that all three mutant strains had decreased eDNA production, which increased their susceptibility to SDS. The same study also showed that younger and more mature microcolonies had distinct spatial patterns of DNA organisation. Two day old WT biofilms showed eDNA highly concentrated in the surface of the microcolonies, while more mature 5 day old biofilms showed the eDNA to be concentrated around the stalk and mushroom-cap of the microcolony structure. Again, the three mutant biofilms showed little or no extracellular DNA located on the surfaces of the microcolonies (Allesen-Holm *et al.*, 2006).

Microcolony differentiation

Protected by extracellular matrix, *P. aeruginosa* biofilms grown *in vitro* and in the presence of glucose start to grow into discrete microcolonies with a surface-bound stalk, topped with a mushroom shaped cap (Klausen *et al.*, 2003). Although in single organism microcolonies only one species of bacteria are present, subpopulations of bacteria play different roles in microcolony maturation, allowing the discrete microcolony to form.

Microcolonies are discrete structures that differentiate from the mono-layer at specific points, and cells forming the microcolony are thought to arise from those cells' clonal lineage, rather than aggregation of cells to these focal points (Conibear *et al.*, 2009). While the other biofilm bacteria replicate slowly, cells within microcolonies often proliferate rapidly, resulting in clonal structures (Klayman *et al.*, 2008). Conibear *et al.* (2009) showed that mutation was highly increased in the microcolony structures compared to the surrounding biofilm cells, and that *P. aeruginosa* mutator strains showed increased microcolony initiation and growth, suggesting a role for mutation in the formation and growth of microcolonies within the bacterial biofilm.

While sessile bacteria make up the stalk of the microcolony, twitching motility is essential for forming the mushroom cap. Biofilms grown with *pilA*⁻ mutants lacking type IV pili were able to form stalk shaped structures, but unable to form mushroom cap structures (Klausen *et al.*, 2003). If *pilA*⁻ mutants are combined with the wild-type strain

in flow cells, the motile wild-type bacteria move to form the cap structure of the biofilm, leaving the non-motile bacteria to form stalk structures (Yang *et al.*, 2009). This study also showed that it is essential for stalk bacteria to produce both the iron scavenging siderophore pyoverdine and extracellular DNA (via the PQS regulation pathway) for mushroom caps to be formed, although none of the mushroom cap bacteria need produce these products. It is thought that extracellular DNA is the foundation on which the cap is built, as type IV pili has a high affinity to DNA (Harmsen *et al.*, 2010). In this way, subpopulations of bacteria that would be unable to create complete microcolonies on their own are able to interact and form mature mushroom-shaped microcolonies.

The amphipathic glycolipid surfactant, rhamnolipid, plays multiple roles in the formation and maintenance of microcolony structures in *P. aeruginosa* biofilms. Early on in biofilm formation, low rhamnolipid concentrations can increase hydrophobicity of bacterial cell surfaces, allowing increased attachment of bacteria to hydrocarbon surfaces (Zhang and Miller 1994). Once the microcolony is established, higher concentrations of rhamnolipid facilitates twitching motility and therefore the formation of the microcolony cap (Pamp and Tolker-Nielsen 2007), and it is also essential for maintaining open water channels, as demonstrated by Davey *et al.* (2003). Rhamnolipids have also been found to play a role in the detachment of cells from the biofilm, as a hyper-detachment mutant was discovered to over-express rhamnolipids, but the inactivation of *rhlAB* genes removed the accelerated detachment phenotype (Boles *et al.*, 2005).

Dispersal

During dispersal of planktonic cells from the mature microcolony, several events occur within a narrow time frame. In late stage microcolonies, a layer of sessile cells forms around the mushroom cap, containing a subpopulation of highly motile cells (Purevdorj-Gage *et al.*, 2005). These cells have up-regulated flagella and down-regulated twitching motility, and are subsequently released from the microcolony, allowing bacteria to move to an environment with fresh nutrients, and thus re-seed the microcolony. This only occurs once a critical size has been reached by the mature microcolony (Harmsen *et al.*, 2010).

Cell lysis is a key event in *P. aeruginosa* biofilm dispersal, and is mediated by the induction of a dormant, chromosomal Pf1-like prophage (Webb *et al.*, 2003). Increased

levels of reactive oxygen species (ROS) lead to prophage induction, and phage-mediated lysis occurs within the central portions of the microcolony. Simultaneously, nitric oxide (NO) levels within the biofilm increase to a level that, while non-toxic to the cells, results in planktonic cell dispersal that can also be induced experimentally by the application of exogenous NO to the biofilm (Barraud *et al.*, 2006). The mechanism of NO-induced biofilm dispersal involves modulation of the intracellular concentrations of c-di-GMP - which are decreased following the addition of nitric oxide to the biofilm - and concomitant expression of planktonic and motile cell physiology (Barraud *et al.*, 2009)

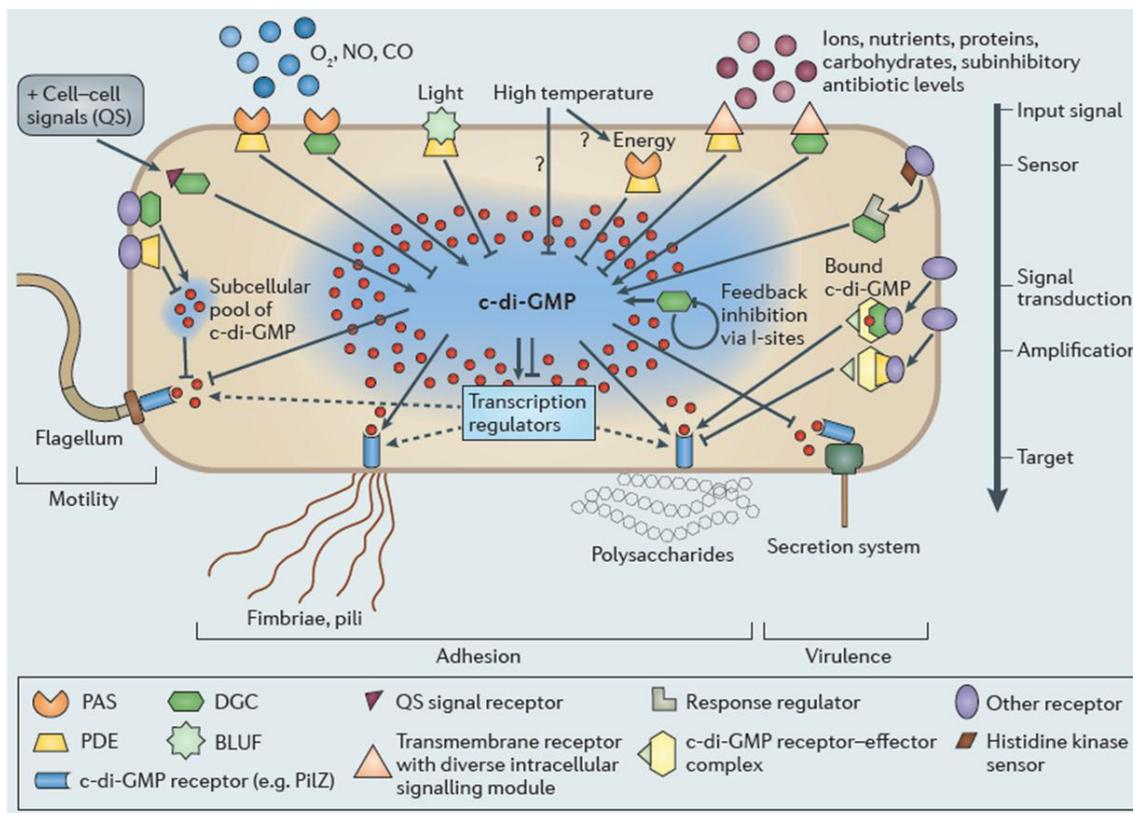


Figure 3 c-di-GMP plays an important role in bacterial dispersal. Intracellular levels of c-di-GMP are controlled by a host of extracellular signals, including NO. c-di-GMP triggers a cascade of proteins that induce changes in cell surface proteins, resulting in the expression of biofilm promoting (e.g. polysaccharide and pili) or virulence and dispersal (e.g. flagella) phenotypes. Figure taken from (McDougald *et al.*, 2011)

In summary, the development and dispersal of microcolony structures within biofilms is a complex process that utilises a wide range of proteins, polysaccharides and lipids, and is dynamically regulated by a number of pathways.

Bacterial Resistance to Antibiotics

Bacteria have developed and used antimicrobial compounds to compete with other bacteria species for nutrients and space for millennia. Resistance to antimicrobials has only become a problem, however, since antibiotics were discovered by humans and developed on a commercial scale. This has resulted in large scale exposure of human pathogens to antibiotics, which, coupled with inappropriate prescription of antibiotics for viral infections, has led to an increase in resistance in pathogenic bacteria. For almost every new antibiotic developed a novel bacterial mechanism of overcoming its lethal action is documented. This section will deal with the general methods of action of antibiotics and bacterial strategies that are used to overcome them.

Types of antibiotics

Although there are many diverse antibiotics available currently, most of them fall into two main categories, depending on the percentage of bacteria in a controlled inoculum that they can kill in 18-24 h. Drugs that can kill >99.9% cells in the given time are classed as ‘bactericidal’ while others (even if they kill between 90 – 99% of the inoculum) are classified as ‘bacteriostatic’ (Pankey and Sabath 2004). Generally, most antibiotics work by attacking the pathogens’ replication mechanisms, as these are sufficiently diverse to minimise cross reaction of the antibiotic with host cell replication mechanisms. If the antibiotic is to work effectively, the cells that it targets must be actively replicating, but as shown later in this review, this is not always the case in bacterial biofilms.

Bactericidal antibiotics

Bactericidal drugs target a wide range of cell replication mechanisms, namely: DNA replication and repair; protein synthesis; and cell wall turnover (Walsh 2000). Bacterial cell walls are made of peptidoglycan, a network of peptide and glycan molecules covalently cross-linked by means of transpeptidase and transglycosylase enzymes. β -lactam antibiotics such as penicillin and cephalosporin act as pseudosubstrates and bind to these enzymes’ active sites with their β -lactam ring, breaking the bond between the C=O and N in the ring, leaving the penicillin bound to the enzymes (Walsh 2000). Due to the length of time it takes for the β -lactam to remove from the enzymes’ active site, crosslinking in the peptidoglycan layer does not occur, leaving the cell weakened and

susceptible to osmotic pressure and lysis. Examples of β -lactam antibiotics are ampicillin and carbenicillin (Figure 4).

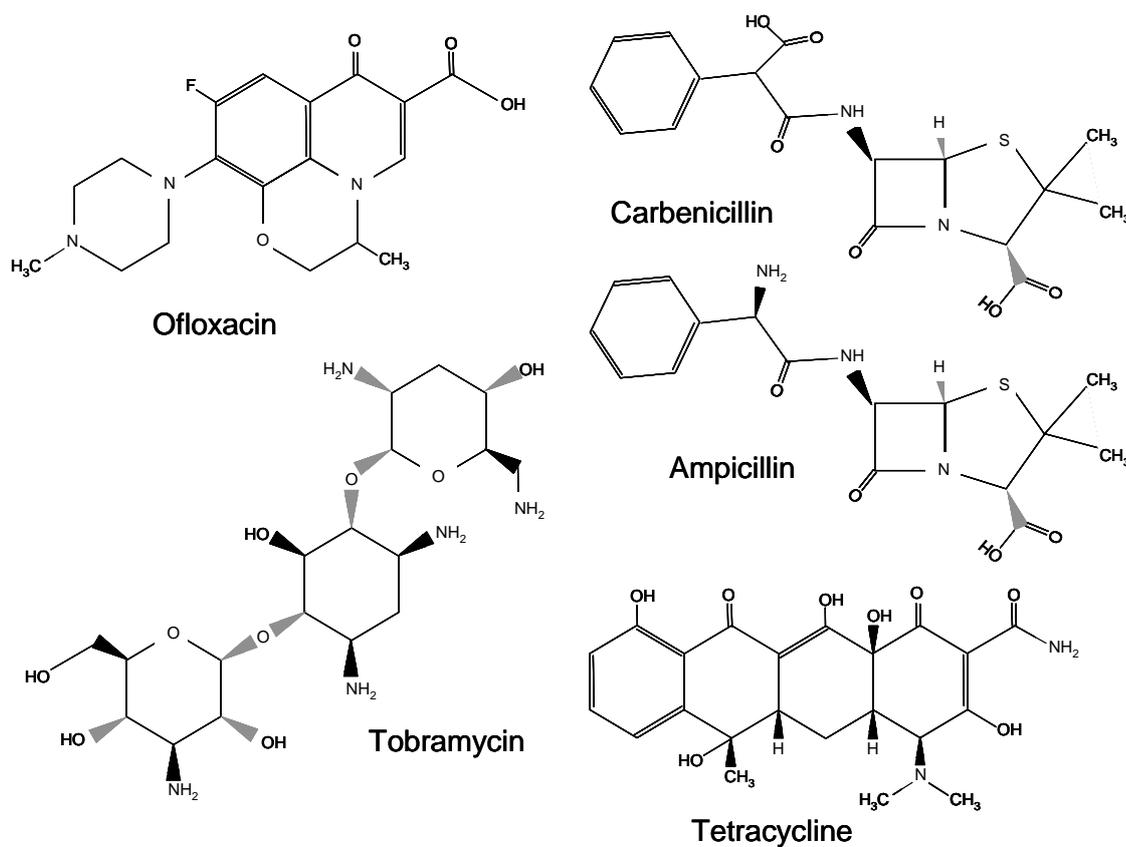


Figure 4 Chemical structures of antibiotics from a range of classes. Bactericidal antibiotics such as the fluoroquinolone ofloxacin, β -lactams, such as ampicillin and carbenicillin, the aminoglycoside tobramycin, and the bacteriostatic antibiotic tetracycline.

Another mechanism by which bactericidal antibiotics cause cell death is by attacking protein synthesis. As prokaryotic protein synthesis is different that of eukaryotic cells in many places, there are correspondingly many sites for antibiotic attack in this procedure (Walsh 2000). Many of the antibiotics targeting protein synthesis are bacteriostatic (see next section). However, the aminoglycoside family can be classed as either bactericidal or bacteriostatic, depending on drug and bacterial species. Aminoglycosides, such as tobramycin (Figure 4) bind to the 30S subunit of ribosomal RNA (rRNA), thus inhibiting protein synthesis. They are also thought to disrupt the integrity of the bacterial cell membrane (Shakil *et al.*, 2008).

Antibiotics also attack DNA replication enzymes, resulting in DNA damage. After bacterial replication the DNA chromosome is supercoiled. In order to uncoil it DNA

gyrase, a type II topoisomerase enzyme, causes transient double stranded breaks in the DNA, allowing a strand of the double helix to pass through the break, and releasing some of the supercoiling (Walsh 2000). Fluoroquinolone antibiotics, such as ofloxacin (Figure 4), bind to the DNA gyrase whilst it is still attached to broken DNA, and form a complex that prevents the cleaved DNA from ligating, resulting in an accumulation of double stranded breaks in the chromosomal DNA. A series of reactions occur as a result of this, leading to the release of superoxide and hydroxyl radicals, resulting in cell death (Dwyer *et al.*, 2007).

Fluoroquinolones are not the only drugs that result in hydroxyl radical mediated cell death, however. A study by Kohanski *et al.* (2007) showed that three major bactericidal drug groups, the fluoroquinolones, β -lactams and aminoglycosides all trigger a common cell pathway leading to hydroxyl radical formation and cell death. The study also showed that knocking out a key protein in the 'SOS' response pathway, RecA (a pathway involved in emergency DNA repair), resulted in increased death in all three bactericidal groups, although it was more pronounced in the fluoroquinolones. This effect was not seen with bacteriostatic drugs, or with rifampicin.

The antibiotic rifampicin is a derivative from an antimicrobial discovered from the bacterium *Amycolatopsis mediterranei* (Floss and Yu 2005). A very large compound, rifampicin inhibits DNA-dependent RNA synthesis because of its strong affinity to the RpoB subunit of the RNA polymerase. Once bound, RNA synthesis is blocked simply due to the size of the compound, and the resulting steric clashes with the growing oligonucleotide (Floss and Yu 2005).

Bacteriostatic antibiotics

Bacteriostatins largely inhibit cell growth by targeting 30S and 50S subunits of the ribosome (Poehlsgaard and Douthwaite 2005). There are a wide range of bacteriostatic classes available, but many of them, such as the sulphonamides, trimethoprim, chloramphenicol and the macrolides are ineffective against *P. aeruginosa* (Pankey and Sabath 2004). The tetracyclines are a class of antibiotic that has been used against *Pseudomonas* of many years. They are all very similar in action and inhibit protein synthesis by binding to the 30S subunit of rRNA (Pankey and Sabath 2004). The structure of tetracycline is shown in Figure 4.

Bacterial resistance mechanisms

When antibiotics are used indiscriminately, due to poor diagnosis, or at sub-lethal concentrations, bacterial resistance soon arises, which has wide-ranging consequences in infection and health (Neu 1992). Bacterial resistance is genetic, non-reversible, and can occur in several ways. Bacteria can have an intrinsic genetic resistance to antibiotics; they can become resistant by direct genetic mutation; or they can acquire resistance horizontally.

Intrinsic resistance in bacteria can occur either by lacking the necessary transporter needed to take the drug into the cell across the cell membrane; by containing an enzyme that automatically breaks down the drug; or by the use of multi-drug resistance pumps, resulting in increased efflux of drug from the cell. In each of these cases, the mechanism for resistance was present before the bacterium came into contact with that particular antibiotic. *P. aeruginosa* is intrinsically resistant to many drugs, which makes treating *pseudomonas* infections particularly problematic. A simple mechanism of resistance to β -lactam antibiotics is the up-regulation of β -lactamase by the bacterium. These enzymes break the β -lactam rings of penicillins before they have even bound to the desired transpeptidases (Walsh 2000). β -lactam antibiotics are now regularly issued with a β -lactamase inhibiting drug such as clavulanic acid to disrupt the binding sites of β -lactamases and allow the antibiotic to work. New, more robust drugs have also been designed, such as carbapenam with harder to cleave β -lactam rings. However, many strains of *P. aeruginosa* contain metallo- β -lactamases, able to break even carbapenam structures, and are resistant to inhibitors (Cornaglia *et al.*, 2011; Sun *et al.*, 2011).

Mutational resistance occurs when bacteria are exposed to sub-lethal doses of an antibiotic over a period of time. This sets up a selection pressure for the bacteria to mutate into slightly altered organisms that are able to survive lethal doses of the same antibiotic. Mutational resistance can result in the drug target in the cell becoming altered slightly at the amino acid level to decrease antibiotic binding while still allowing function of the target. An example of this is rifampicin resistance in bacteria, which occurs at a rate of 10^{-8} or 10^{-9} per bacterium per cell division (Floss and Yu 2005). Resistance in the most part is by single amino acid substitution in the RpoB subunit of the RNA polymerase, resulting in a decrease in affinity of rifampicin for the RNA polymerase, and a corresponding decrease in susceptibility of the bacterium. Less often,

bacteria can gain the *arr* gene, *de novo* synthesis of which creates a protein that cleaves rifampicin into two inactive compounds. A highly resistant strain of *P. aeruginosa* isolated in Thailand which contains this gene has previously been described (Floss and Yu 2005).

Mutational resistance can also occur by altering metabolic pathways and missing out the step that enabled antimicrobial binding, or simply by over-riding the regulation of already present multi-drug resistance (MDR) channels, allowing drug efflux (Walsh 2000). *P. aeruginosa* is able to gain resistance to fluoroquinolones by point mutations in the QRDR (quinolone-resistant-determinative region) motif of DNA gyrase, thought to be its active site (Strateva and Yordanov 2009). Drug resistance in *P. aeruginosa* arises very quickly; in intubated patients hospitalised with *pseudomonas* pneumonia, resistance to the first line of drugs can occur within 10 days of antibiotic initiation (Reinhardt *et al.*, 2007). It is thought that bacteria mutate in response to antibiotic attack because of the levels of ROS that are present in the damaged cell. I have already shown that the major bactericidal antibiotics cause increased intracellular concentrations of hydroxyl radicals (Dwyer *et al.*, 2007; Kohanski *et al.*, 2007), and subsequent study showed that these led to increased mutation and resistance at sub-lethal antibiotic levels (Kohanski *et al.*, 2010).

Lastly, bacterial resistance can be conferred upon bacteria by plasmid or transposon, thus non-resistant bacteria can receive copies of genes from intrinsically resistant cells, and gain resistance themselves. All the above forms of genetic resistance lead to an increase in the Minimum Inhibitory Concentration (MIC) of a specific drug required to kill the bacteria.

Bacterial Tolerance

Contrasting with bacterial resistance is bacterial tolerance. This term has been given to bacteria that seem to be resistant to a broad spectrum of antimicrobial drugs, which occur without apparent specific genetic determinants encoding for that resistance mechanism. This is particularly observed in bacterial biofilms, which exhibit a generalised tolerance to almost all known antimicrobial compounds (Brooun *et al.*, 2000; Gilbert *et al.*, 1997; Gilbert and McBain 2001; Hoyle *et al.*, 1992; Suci *et al.*, 1994). With bacterial biofilms presenting up to 1000-fold more tolerance of extrinsic mortality

factors than their counterpart planktonic cells, much research has been undertaken to determine the cause of this lack of susceptibility. Inherent within a biofilm are factors that affect the pharmacodynamics of an antimicrobial agent with respect to the community response. Factors that can affect the uptake of an agent can be condensed into three main areas: the effect of the matrix on antimicrobial agents; geographical locations of cells within microcolonies; and replication rate of cells and nutrient gradients across the biofilm (Gilbert *et al.*, 2002).

Role of the extracellular matrix

Early papers attributed the majority of biofilm tolerance to the matrix effectively blocking access of antibacterial agents. Antibiotic diffusion studies carried out using alginate gels to represent bacterial EPS showed that aminoglycosides such as tobramycin and gentamicin have diffusion coefficients up to 20% less than those of β -lactam antibiotics such as ceftazidime, cefsulodin and piperacillin (Gordon *et al.*, 1988), and a similar study showed that using a 2% alginate suspension, gentamycin and tobramycin could be completely blocked (Hatch and Schiller 1998). However, Walters *et al.* (2003) showed that when an actual biofilm environment was used, tobramycin was seen to be able to penetrate the biofilm, but at a much reduced ratio and speed compared to fluoroquinolones such as ciprofloxacin. Indeed, recent studies on mucoid *P. aeruginosa* biofilms have shown that the addition of alginate lyase and DNase to the antibiotic mixture enhances aminoglycoside activity (Alipour *et al.*, 2009). Biocides too, are able to penetrate biofilm matrix, as both chlorine and monochloramine have been shown to effectively penetrate *Klebsiella pneumoniae* biofilms (Yu and McFeters 1994). These studies have shown that in most cases, the matrix does not retard antibiotic diffusion enough to account for the extensiveness of biofilm tolerance, although highly charged antibiotics or highly reactive agents such as halogens or peroxygens may be chemically quenched by the matrix during diffusion (Gilbert *et al.*, 2002). In fact, Costerton *et al.* (1994) showed that the water channels present in microcolony morphology increase the permeability of antibiotics to biofilms, yet still the cells remained tolerant. It appears that while the EPS is important in contributing to the defences of a biofilm, it is not solely responsible for the observed recalcitrance, and other factors must play a role in reducing the effectiveness of antibiotics on biofilms.

Nutrient gradients and location of cells within the biofilm

Biofilms show considerable heterogeneity in their make-up, even if the colony was founded by one single bacterial strain. This discovery is crucial to the understanding that antibiotic effectiveness differs across a microcolony. As a biofilm matures and grows, cell density increases, and oxygen and nutrient gradients emerge. These gradients lead to heterogeneity amongst the bacterial population, with cells adapted to survive in their own micro-environments. The spatial location of cells and the differing nutrient gradients that are encountered within a biofilm are two closely linked areas that can affect the uptake of an antibiotic, and therefore the antimicrobial tolerance of microcolonies. Early studies showed that cell density in a biofilm has been seen to affect antibiotic recalcitrance, as older, more dense biofilms showed a higher percentage survival to attack by tobramycin than younger, less dense microcolonies (Anwar *et al.*, 1989).

Studies carried out at a later date were able to build on this observation, and give a more complete understanding of the effect that biofilm density - or more specifically, oxygen gradients and the location of cells within the biofilm - have on antimicrobial recalcitrance. The two distinct populations of the microcolony, the sessile and slowly replicating 'stalk', and the more active 'cap' respond differently to antibiotic attack, due to their different physiological states. For example, when comparing the effects of the antibiotics ciprofloxacin and tobramycin on *P. aeruginosa* biofilms, it was noticed that although both antibiotics penetrated throughout the biofilm, cell death was only noticed at the air interface of the biofilm. After carrying out oxygen concentration profiles of colony biofilms, Walters *et al.* showed that oxygen penetration of a biofilm occurs for only 50 - 90 μm , and this oxygen saturated zone coincided with the zone of antibiotic action (30 - 50 μm) (Walters *et al.*, 2003). The study also showed that the area replete with oxygen was the only section of the biofilm capable of *de novo* protein synthesis, and so was the only area of the microcolony containing actively replicating cells and therefore open to antibiotic attack. In contrast to this, treatment of *P. aeruginosa* biofilms with colistin resulted in only the slow replicating cells being killed (Pamp *et al.*, 2008), suggesting that clearance of bacterial biofilms requires two distinct antibiotics, to target the two distinct biofilm subpopulations present. Thus, the oxygen gradients present across the biofilms have a part to play in the recalcitrance of a biofilm; if an aerobically respiring cell is deprived of oxygen, due to its location deep within the

biomass of a microcolony, it is unable to actively replicate, and as a result is more tolerant of antimicrobial attack.

Anaerobic growth has also been characterised in *P. aeruginosa*. This occurs through a denitrification enzyme pathway, reducing nitrate to intermediates nitrite, nitric oxide, nitrous oxide and finally nitrogen gas (Van Alst *et al.*, 2007). *P. aeruginosa* biofilms have been shown to be capable of growing in extremely hypoxic environments such as mucopurulent masses in cystic fibrosis (CF) airways (Worlitzsch *et al.*, 2002). So far, this review has discussed the factors that affect antimicrobial uptake into the biofilm. However, there are other intrinsic abilities of cells within biofilms that seem to influence the general recalcitrance of the whole.

Biofilm genetic diversification and heterogeneity

A recent and significant advance in biofilm research is the discovery of extensive and biofilm-specific genetic diversity arising during biofilm development. It has been shown that a major source of biofilm recalcitrance can be directly attributed to an enhanced amount of mutation and diversification arising within developing microcolonies (Boles *et al.*, 2004; Conibear *et al.*, 2009; Mai-Prochnow *et al.*, 2006). This diversification is partially as a result of the presence of oxygen and nutrient gradients across the biofilm leading to cells that become adapted to survive in specialist niches (see previous section), but also seems to be heavily dependent on the activity of the DNA repair and maintenance protein RecA. While wild-type bacteria are able to produce several mutant variants after growth in a biofilm, knock-out mutants for the *recA* gene showed a complete lack of heterogeneity (Boles *et al.*, 2004). When wild-type *P. aeruginosa* PA01 is allowed to grow in biofilms, several bacterial sub-populations develop, distinguished by their colony morphology, and their differing phenotypes. Colonies showing smaller than typical diameters on standard agar were seen to enhance biofilm detachment 4-fold, while ‘wrinkly’ colonies caused biofilms that showed accelerated development at every stage, and resulted in biofilms containing around 100-fold more bacteria than wild-type microcolonies. More importantly from the perspective of biofilm tolerance, although the ‘wrinkly’ sub-population of cells within the wild-type accounted for only 12% of a naïve biofilm, after oxidative stress 98% of the surviving cells were the ‘wrinkly’ type, suggesting that while cells may not show resistance *per se*, the ‘wrinkly’ colony variant may be expressing a particular transcriptome that allows for an increased tolerance to antibiotics compared to the wild-type (Boles *et al.*, 2004).

Here then, it is seen that genetic diversity occurs to a large extent in biofilms, and this has directly been observed to increase stress tolerance levels in biofilms.

Mutation and hypermutation in biofilms

Biofilm bacteria show mutation frequencies 100-fold higher than that of planktonic bacteria (Conibear *et al.*, 2009). Indeed, a form of genetic diversification has recently been documented in chronic clinical infections, with some isolates, termed ‘hypermutable’ or ‘mutator’ strains, showing extremely high mutation frequencies compared to other isolates from the same infection. These strains appear with a high frequency (between 30 - 60%) in chronic infections, compared with <1% in acute infections (Ciofu *et al.*, 2005; Henrichfreise *et al.*, 2007; Macia *et al.*, 2005; Oliver *et al.*, 2000). Comparisons of clonal *P. aeruginosa* isolates collected over a period of greater than 5 years from chronically infected CF patients show that mutations arise spontaneously in a wide variety of genes throughout the bacterial chromosome. Due to the redundancy seen in codons coding for amino acids, these mutations can be either synonymous, resulting in no outright protein alteration, but possibly affecting speed of protein translation, or the mutations can be non-synonymous, where base-pair substitutions, insertions and deletions can result in protein truncation and/or loss of function, due to frame-shifts or stop codon generation. Mutations were seen to occur at higher frequencies in genes involved with virulence, such as QS regulation, multi-drug efflux pumps, type III secretion, twitching motility, exotoxin A regulation, and O-antigen biosynthesis (Smith *et al.*, 2006). For the most part, loss of function mutations were seen, for example, mutations occurring in the gene *mexZ*, a negative regulator of the MexXY-OprM efflux pump, result in increased expression of *mexX* and *mexY*, resulting in an increased resistance to aminoglycosides such as tobramycin (Sobel *et al.*, 2003). It was interesting to note however, that loss of function mutations also occurred in the *lasR* gene, a primary regulator of quorum sensing. These mutations directly lead to impairment of biofilm formation and disruption of virulence factor expression. While decreased expression of virulence factors may have the benefit of immune evasion, mutations resulting in decreased biofilm formation seem paradoxical, as the primary form of *P. aeruginosa* in CF airway infection is that of the biofilm.

Mutations to genes such as *mutS* and *mutL*, involved in DNA mismatch repair (MMR), play an important role in the production of hypermutable strains in bacterial biofilms. Mutator strains are designated as isolates that have a frequency of mutation to

rifampicin or streptomycin resistance of $> 2 \times 10^{-7}$, compared to WT mutation frequencies of 9×10^{-10} (Mena *et al.*, 2008). Further examination of these CF isolates showed that out of the 29 patients studied, mutator strains were isolated from 9 patients (31%), 7 of whom had earlier isolates from the same clonal lineage where previous hypermutation had not been seen, suggesting that the hypermutation present was due to mutations that had occurred within the CF biofilm. Mena *et al.* (2008) determined that once non-synonymous mutation has occurred at the *mutS* or *mutL* site, the frequency of mutations increases from a median of 0.25 mutations per year of infection to 3.33 mutations per year of infection, resulting in infectious populations of greater heterogeneity. There was no significant increase in mutations in antimicrobial resistance genes when compared to the non-mutator strains, suggesting that hypermutation has a generalised effect on genetic adaptation, rather than favouring a specific adaptive trait.

An example of heterogeneity in a population that increases the ability of a microcolony to tolerate stress is the presence of dormant or slow growing 'persister' cells. These cells are entirely unaffected by the presence of a wide variety of antibiotic agents, and will be considered in detail in the next section of this review.

Persister Cells

Persister cells have been reported ever since the first widespread use of antibiotics in the mid-1940s, when despite using lethal doses of penicillin, a small proportion of cells were able to survive or ‘persist’ through the challenge (Bigger 1944). Since then, many studies using extensive antibiotics on countless bacterial species have confirmed this ubiquitous phenomenon. Persister cells are a subset of dormant or extremely slow-replicating cells in a bacterial population, which are able to survive exposure to multiple antibiotics (Balaban *et al.*, 2004; Sufya *et al.*, 2003). Prior to antibiotic application, their presence increases in late exponential and stationary phases of population growth (Keren *et al.*, 2004a; Shah *et al.*, 2006). Although many genes may play a role in persister formation, the antibiotic tolerance seen in persisters is not heritable (Keren *et al.*, 2004a), and is distinct in this respect from genetically coded antibiotic resistance elements. Once persister cells revert to normal growth, their offspring are as tolerant as the original bacterial population (Keren *et al.*, 2004b). In this section the role of persisters in antimicrobial tolerance, and the current understanding of the mechanisms by which persisters are formed, will be examined.

Persister cells confer tolerance on bacterial populations

The means by which persister cells form is as yet unknown, but theories for their formation have been presented. Persister cells have been shown to be a pre-existing minority population within a culture, present before attack with antibiotics (Balaban *et al.*, 2004). Persister cells appear to have switched spontaneously from normal cells during late exponential growth and stationary phase (Balaban *et al.*, 2004; Keren *et al.*, 2004a), and thus are present in largest numbers - typically 10^4 colony forming units (CFU) but in some cases reaching a maximum of ~1% of the population - at late exponential and early stationary phase (Keren *et al.*, 2004b; Lewis 2007). Upon treatment with antibiotics, a distinct biphasic killing curve can be observed (Figure 5). Firstly, a fast decay is seen, caused by death of normal cells in the culture. After a few hours, the decay reduces to a sub-exponential rate. This second phase of killing is attributed to the increased tolerance of the persister cells to antibiotics of the persister cell (Balaban *et al.*, 2004). If the antibiotic is subsequently removed from the growth

medium, and the persister cells replicate, the subsequent progeny shows no sign of the persister phenotype and after a lag phase, grow as normal.

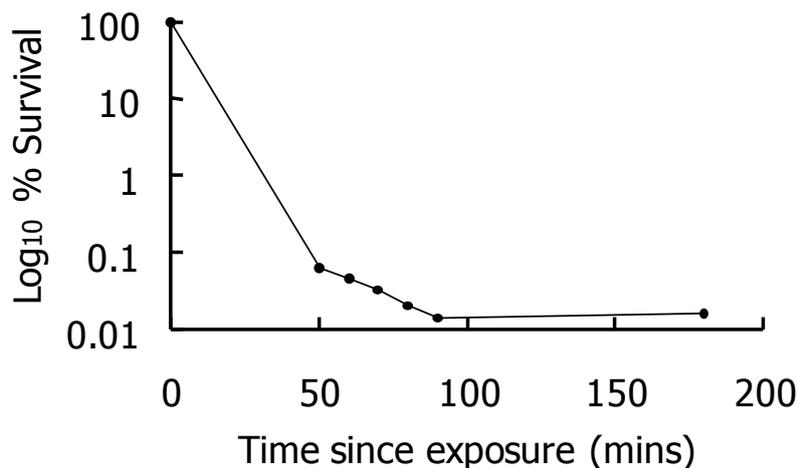


Figure 5 Biphasic killing curve seen when *P. aeruginosa* culture is treated with ofloxacin. Mid-exponential phase cultures were exposed to 3 µg /ml ofloxacin. Most bacteria are killed in the first 30 min of attack, showing exponential decay of normal cells, and then slower decay of persister cells.

Non-heritable tolerance due to low fecundity of persister cells

Despite persister cells surviving antibiotic concentrations much higher than the MIC of bacterial strains, their progeny are as sensitive to the same antibiotic withstood by the persister cell. An explanation was provided by Shah *et al.* (2006), when they revealed a method for physically extracting persister cells from a bacterial population. A bacterial strain was genetically manipulated to contain an unstable form of the green fluorescent protein gene, preceded by a ribosomal promoter. Cells that were actively replicating expressed GFP and consequently fluoresced, while cells that were dormant (alive, but showing low fecundity) did not. After separating cells by fluorescence, both populations were subjected to antibiotic attack, and non-fluorescent cells survived while fluorescent cells did not. Since the vast majority of antimicrobial agents attack bacterial cells via mechanisms involving replication, so cells that are replicating slowly or not at all are much more recalcitrant to antibiotics than actively replicating cells. In other words, persister cells show low fecundity and therefore are not killed by bactericidal antibiotics.

A recent study by Allison *et al.* (2011), demonstrated that *E. coli* persister cells could be killed by allowing them to uptake metabolites such as glucose, mannitol and pyruvate. While not awakening the persister enough to allow cell division, the uptake of these nutrients generated enough proton motive force (PMF) for aminoglycoside antibiotics to

be drawn into the cell, and kill the persister. While these results are interesting, they have limited use clinically, as this effect was only seen with this single class of antibiotic, and each bacterial species required a different substrate to demonstrate the same effects. Although much is known about the population-level effects of persister cells, and their role in antibiotic tolerance, the mechanisms by which persisters are formed, and their functional role in biofilms prior to antibacterial challenges, have yet to be fully understood.

Many hypotheses have been proposed to account for the formation of persister cells. While some concentrate on the genetic mechanisms by which persister cells may arise, others attempt to model the population-dynamic processes that might favour the formation of persister cells. These hypotheses are the products both of experimental procedures and of predictions from mathematical models. First, mechanistic hypotheses will be examined, and then process-based models will be explored in the next section.

Current hypotheses for mechanisms of persister cell formation

Toxin-antitoxin modules

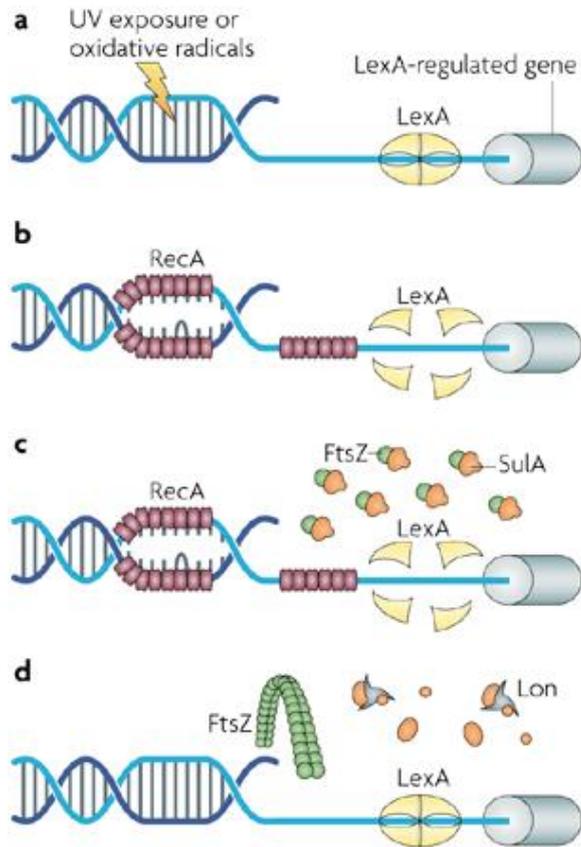
Current understanding of the mechanisms leading to the formation of persister cells suggests that they occur as a result of one or more toxin-antitoxin (TA) complexes switching on, rendering the cell dormant and thus resistant to antibiotics (Shah *et al.*, 2006). TA modules are constitutively expressed, and are a combination of a stable toxin and an easily degraded antitoxin, which together act as a mechanism for the maintenance of cellular DNA. If a cell replicate is produced that lacks the plasmid or gene section on which the TA cassette is located, the cell is condemned, because the moment the present levels of antitoxin drop, the toxin is released to act on an essential target in the cell, subsequently killing it or severely restricting cell growth (Hayes 2003). When first discovered, it was thought that these complexes, if switched on, caused cell death (Sat *et al.*, 2001). More recent studies have shown, however, that some toxin-antitoxin modules merely switch off all cell replication pathways, rendering the cell dormant (Christensen *et al.*, 2003; Pedersen *et al.*, 2002).

It has been shown that the 'high persistence' mutant *E. coli hipA7* over-expresses a non-toxic mutant form of the toxin HipA, and is therefore able to produce about 1000-fold more persister cells than the wild type (Keren *et al.*, 2004b; Korch *et al.*, 2003; Korch

and Hill 2006; Lewis 2007). It is known that bacteria show redundancy for toxin-antitoxin complexes, however; there are >10 TA complexes in *Escherichia coli* (Brown and Shaw 2003), and >60 in *Mycobacterium tuberculosis* (Arcus *et al.*, 2005; Pandey and Gerdes 2005). In contrast, only one chromosomally located TA module has been described in *P. aeruginosa*, with 79% identity to a *P. syringae* ‘prevent host death’ TA module. As it is located in the pf4 prophage section of the chromosome, it is thought to play a role in maintaining the phage in the bacterium (Webb *et al.*, 2004). The overall redundancy of TA modules, however, suggests that knockouts of these complexes will not always result in a phenotypic effect. It is now thought that other bacterial systems such as the SOS response also contribute persister levels in bacterial populations. It is interesting to note that TA modules *symER*, *hokE*, *yafN/yafO*, and *tisAB/istR* all have *lex* boxes upstream of their coding regions, indicating that the so called ‘SOS’ response activates these TA complexes when LexA is cleaved (Dorr *et al.*, 2010).

Effect of SOS response on the formation of persisters

Persister cells have been shown to have some connections to the SOS response pathway. Previously in this introduction it has been shown that the major bactericidal antibiotics kill by high concentrations of hydroxyl radicals (Dwyer *et al.*, 2007; Kohanski *et al.*, 2007). These radicals cause DNA damage, and the SOS response in bacteria is a complex system that coordinates repair responses to damaged DNA. When DNA is damaged, the SOS response protein RecA binds to the ssDNA strand along with several ATP molecules. In this form, RecA becomes an activated co-protease, and cleaves the SOS repressor LexA. When inactivated, LexA becomes disassociated from the ‘lex’ operator box at the start of SOS related genes, which allows these genes to be translated into proteins. The first proteins of the SOS response to be translated are from genes which have low LexA binding to their lex box. Examples of genes that are expressed in under 1 minute of SOS induction are *lexA* (which is rapidly degraded in SOS induced cells), nucleotide excision repair (NER) genes *uvrA*, *uvrB* and *uvrD* (DNA helicase II), and *polB* and *dinG*, which code for Polymerases II and IV respectively. Five minutes after SOS induction, recombination repair genes such as *recA* and *recN* are expressed, while late stage SOS induction allows the expression of mutagenic polymerases such as polymerase V, coded for by *umuDC* (Janion 2008).



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Figure 6 Diagram of the SOS response in bacteria. Upon DNA damage, RecA surrounds the damage site, and also plays a role in cleaving the transcriptional repressor LexA. Once removed, several SOS response genes are expressed, resulting in the repair of the damaged DNA. Diagram taken from (Justice *et al.*, 2008).

Dorr *et al.* were able to show that the SOS response was involved in the production of persister cell, because they found that *E. coli* mutants that were lacking in various essential SOS genes such as *recA*, *recB* or *lexA* showed decreased persister survival in response to ciprofloxacin attack (Dorr *et al.*, 2009). They also demonstrated that fluoroquinolone antibiotics, with their DNA damaging mechanisms, are able to induce persister formation, as they showed that treatment of an *E. coli* culture with sub-lethal concentrations of ciprofloxacin, prior to killing with a lethal dose, resulted in an increased level of persisters compared to the control culture. Increased persisters were also observed when cultures were incubated with the DNA damaging mitomycin C prior to antibiotic killing. Also, by manipulating bacteriophage to knock out the SOS response in bacteria, Lu and Collins (2009) were able to show a significant increase in sensitivity of bacteria to antibiotics, especially fluoroquinolones. A recent study by Kim *et al.* showed that after 5 hours of ampicillin treatment, the majority of an *E. coli*

population had damaged cell membranes, were filamentous and showed high levels of hydroxyl radicals, while the surviving persister fraction had intact cell membranes, were normal sized, and had much lower hydroxyl radical levels (Kim *et al.*, 2011). This confirms the theory that while the greater proportion of the bacterial population dies as a result of increased hydroxyl radicals after antibiotic attack, persister cells have an incremental increase in hydroxyl radicals but escape lethal hydroxyl concentrations.

These studies were able to demonstrate therefore, that persister cells are not just formed by stochastic action of a naive culture, but are able to be induced by DNA damage, and activation of the SOS pathway. Also, they go some way to explaining why persister cells are not killed by hydroxyl damage as normal cells are (Kim *et al.*, 2011), and due to the mutational effect of hydroxyl radicals, may suggest the role of persisters in bacterial mutation (Kohanski *et al.*, 2010). However, this mechanism of persistence, like the TA cassettes mentioned earlier, cannot account for all persisters seen in a population, as knockouts of major SOS response genes did not completely wipe out the persister phenotype seen in the bacterial population.

Other genes implicated in persistence

In order to gain more precise information on the genes involved with the persistence phenotype, various high throughput screening methods have been used to quantify gene up- and down-regulation, or to determine the effects of gene knock-outs on the levels of persisters in a population. Whole genome microarrays of *E. coli* persister cells have revealed up-regulation of ~400 genes, and a corresponding down-regulation of roughly the same number of genes compared to stationary cells (Shah *et al.*, 2006). Notable down-regulated genes were 'energy production' genes and flagellar synthesis, while genes that were up-regulated were components of Toxin-antitoxin modules such as *RelBE*, *MazEF*, *DinJYafQ*, and *YgiU*. However, no data were available to indicate the levels of SOS response genes in these cells. Similarly, in *P. aeruginosa*, screening of a mutant library resulted in the identification of several unrelated genes that either positively or negatively affect the allocation of persisters in a population (De Groote *et al.*, 2009). Knocking out the homolog of the *E. coli* *dinG* gene, a putative DNA-helicase, resulted in 16 times lower persister fraction than the wild-type. This correlates with the theory that persisters are bacteria that have halted growth while DNA repairs take place. Knock-outs of the putative putrescine aminotransferase, *spuC*, resulted in 4.4-fold reductions in persister levels compared to the wild-type. This gene is thought to regulate

the ABC transporter SpuDEFGH, which is essential for the uptake of the polyamine, spermidine. Spermidine, along with putrescine, is involved with many cellular processes, including protecting the cell from external toxic stress. Kwon and Lu (2006) showed that addition of these polyamines exogenously resulted in decreased antibiotic susceptibility to fluoroquinolones, indicating that *spuC* has links to the persister phenotype. A gene that caused a 2.8-fold reduction of the persister fraction was a PaaJ-like acetyl-coA acetyltransferase, which is involved in the metabolism of fatty acids and phospholipids, and as such, it is not certain as yet how this acetyltransferase is involved in the persister phenotype (De Groot *et al.*, 2009). Genes that resulted in 3.3 and 17.2-fold increased persister levels when knocked out include *algR*, and *pilH*, respectively. These genes code for global regulators of two-component regulatory systems, which control many virulence and biofilm-forming components, such as alginate and rhamnolipid production, twitching motility, and synthesis of type IV pili. It is suggested by this study that these regulators also either directly or indirectly regulate persistence also. These studies suggest that there is more to the production of persisters than a SOS response linked, TA mechanism, as so many extra genes are involved in the persister phenotype.

Lewis suggests in his review on programmed cell death that persister cells might arise by a defectiveness in apoptosis, a mechanism by which cells auto-destruct (2000), rather than a resistance to antibiotics, while another theory is that persister cells are turned on by the Global Stress Response (GSR) due to extreme nutritional stress (Gilbert *et al.*, 2002).

At present, therefore, I conclude that although many genetic factors are involved with the phenomenon of persistence, studies have yet to consistently identify a ‘persister regulon’ of genes mechanistically linked to this trait. It is beneficial, therefore, to attempt to study the phenomenon of persistence from the complementary perspective of population ecology, in order to understand the role that persisters have in the bacterial community. This can be performed by attempting to catalogue the evolutionary and ecological drivers that lead to the presence of persisters in a bacterial population.

Population Models for Processes Underpinning Persister Formation

Whilst the last decade has seen intensive research into the genetic mechanisms involved in biofilm formation, and the abilities of persister cells to confer tolerance to a microcolony, a biofilm-specific method for control is still lacking. It may then be beneficial to look at the problem of persister cells from the perspective of their functional role in biofilms, and therefore this section aims to address the presence of persister cells from the viewpoint of their population ecology. This involves using ecological theory to underpin predictive models for the conditions favouring persister cell formation, and to test alternative models with experimental studies.

Persisters as insurance policy

Kussell *et al.* (2005) proposed that persister cells act as an insurance policy for the whole population against catastrophe. They stated that any fitness loss seen by the slow growth of persister cell was compensated for by the decreased risk of extinction to the whole population. Their model suggested that the base-rate level of persisters seen in a wild-type *E. coli* population was suited to an environment where antibiotic stress was rare, whereas the *hipQ* mutant strain of *E. coli*, which has a 1000-fold higher rate of switching from normal to persister cell, is more suited to ‘frequent and intense’ antibiotic stress. Kussell *et al.* conclude therefore that the frequency of switching from ‘normal’ to ‘persister’ cell depends strongly on the frequency of environmental change, rather than the selection pressures of any given environment. This model requires an underlying coordination between cells; otherwise, in periods of plenty, all persisters may disappear from the population. The next model examined explicitly this coordination.

Persisters express a trade-off between resource competition and fecundity

Gardner *et al.* (2007) modelled the allocation of persisters in a bacterial population as a dynamic trade-off between a group benefit of reduced competition for limiting resources and an individual cost of reduced fecundity. By switching to the persister state, cells are able to survive lethal stress, which gives them a ‘selfish benefit’ of survival over other cells in the population. Simultaneously, the normal cells in the population have the indirect or ‘social’ benefit of decreased competition for nutrients that the

persister cell would have otherwise used. Along with the prediction that increased genetic relatedness of the population will positively influence switching to persisters, Gardner *et al.* also predicted the effect that changing the parameters of persistence would have on persister allocation. In a situation where non-persisters were able to survive catastrophe, there would be reduced selection for the evolutionary stable strategy (ESS) of switching to the persister phenotype, as the costs of persistence in reduced fecundity would outweigh the benefits of persistence. In contrast, if persisters were able to replicate and yet retain their tolerant phenotype, the ESS would favour increased persister allocation until all cells were persisters, as they would have the benefits of surviving catastrophe, without the cost of reduced fecundity. This model has been used to predict the role of persisters in factors such as antibiotic attack and nutrient competition. Persisters have also been linked to senescence, both as aged cells, and as those who have postponed ageing to a later date. These models will be examined in the next sections.

Persisters as aged cells

In contrast to the two previous models, Klapper *et al.* (2007) modelled persisters as ageing cells accumulating in a population. Stewart *et al.* (2005) demonstrated empirically that when cells replicate, instead of splitting symmetrically, one of the two daughter cells inherits the effects of senescent ageing, while the other does not. Using this as a basis for their mathematical model, they propose that the slow growth and non-heritable tolerance to antibiotics connected to the persister phenotype, along with persister accumulation in late log and stationary phase of growth, can be explained by an aged sub-population of cells in the culture. However, certain points remain unclear. For instance, if persister cells are very slow replicating highly senescent cells, why are there no cells of intermediate tolerance in the culture that represent cells which are only moderately senescent?

Persisters as non-senescent cells

Theoretical models of senescent ageing in crowded environments suggest an opposite interpretation of the role of senescence in persister formation. Density-dependence in fecundity can stimulate selection on ever-slower senescent ageing when organisms in recruitment-limited populations reduce their intrinsic rate of production of new biomass in favour of a longer lifespan potential (Doncaster 2003; Seymour and Doncaster 2007). This model has selection favouring deferred replication in populations that are too

crowded to sustain fast recruitment. The fitness advantage of increased lifespan then exacerbates crowding, further favouring the long-slow strategy for maximising lifetime reproduction. The model of Seymour and Doncaster (2007) uses a similar mathematical framework to that of Gardner *et al.* (2007) with the difference being conceptually in setting the individual to benefit from reduced senescence, rather than the group to benefit from a social trait for reduced competition. Given that biofilms present a crowded environment to bacterial cells, they may be good examples of ‘*K*-selected’ populations in which fitness depends on lifetime reproductive output (a direct function of the carrying capacity *K*), at the other end of the spectrum from *r*-selection on rate of reproduction (MacArthur 1962; MacArthur and Wilson 1967). As a model system for studying density-dependent selection, biofilms have the additional advantage of expressing high mutation rates and genetic diversification (Allegrucci and Sauer 2008; Boles and Singh 2008; Boles *et al.*, 2004; Conibear *et al.*, 2009).

Overall Aims and Objectives of Thesis

While much research has been done on the physiological mechanisms behind antimicrobial tolerance in bacterial biofilms, and the molecular and genetic processes that activate the production of persister cells, there is a dearth of information to describe the relevance of persister cells in their biofilm population within the abiotic and biotic environment. The overall aims of this thesis therefore, are to examine the functional role of persisters in bacterial populations from a population ecology perspective as well as to determine their impact on the development of biofilm populations. Understanding the evolutionary and population processes behind persister cell formation may lead to the development of new strategies for their control. The following specific aims (by chapter) were identified:

Chapter 2: To determine the ecological and evolutionary influences on persister cell-mediated antimicrobial tolerance in *P. aeruginosa* biofilms

This study aims to test *in vitro* the population model of persisters as non-senescent cells (Seymour and Doncaster 2007). A functional role for persister cells is hypothesized in deferring replication within bacterial populations that are too crowded to sustain growth. In particular:

- This model predicts that slow-growing lineages with increased persister allocations will arise among density dependent bacterial populations. This study will therefore examine whether genetic and phenotypic diversification within biofilms leads to bacterial strains that produce more persister cells.
- Secondly, this model predicts that frequent exposure to lethal and non-lethal stresses will impact persister allocation, so this study aims to determine the effect that frequent antibiotic attack has on selection for persistence in *P. aeruginosa* biofilms.

Chapter 3: To determine the role of mutation frequency and SOS response on persistence in *P. aeruginosa* populations

This study aims to examine the hypothesis that hypermutable phenotypes of *P. aeruginosa* are a characteristic of persister cell biology. Recent research has shown that key features of the development of bacterial biofilms include increasing mutation rates

and increasing numbers of persister cells and I hypothesise that there is a relationship between the two phenomena.

- If the persister phenotype has any connection to mutation in the bacterial biofilm, this may be shown by an increased in mutation frequency in the persister fraction of the bacterial population. This study therefore aims to determine the mutation frequency of whole bacterial populations, and persister sub-populations of both wild-type and $\Delta mutS$ *P. aeruginosa*.
- Both the SOS response pathway and the mismatch repair genes have been linked to mutation in bacterial populations. I therefore aim to determine the expression of SOS-linked (*recA* and *dinB*) and DNA repair (*mutS*) genes in persister cells to further investigate whether persisters have a mutator phenotype

Chapter 4: To determine the role of persistence in microcolony initiation and seeding of new biofilm growth following antibiotic treatment

This study aims to examine whether persisters play a role in the initiation of microcolony foci and in mediating regrowth of biofilms. Recent research has shown that microcolonies develop clonally from single cells in the biofilm monolayer (Conibear *et al.*, 2009). It is therefore hypothesised that persister cells play a role in microcolony initiation, and biofilm re-growth after antibiotic attack.

- I therefore aim to study biofilm initiation of persister and non-persister bacterial populations under a variety of conditions including with or without exposure to lethal or sub-lethal antibiotic challenge, in order to determine the role of persisters in biofilm formation.
- I also aim to examine microcolony growth in both persister and non-persister populations.

CHAPTER 2

ECOLOGICAL AND EVOLUTIONARY INFLUENCES ON PERSISTENT CELL-MEDIATED ANTIMICROBIAL TOLERANCE IN *P. AERUGINOSA* BIOFILMS

Abstract

Persisters are a subset of dormant cells within bacterial populations that are extremely tolerant to antibiotics. A functional role for persister cells is hypothesized in deferring replication within bacterial populations that are too crowded to sustain growth. This model predicts that slow-growing lineages with increased persister allocations will arise among density dependent bacterial populations, and that frequent exposure to lethal and non-lethal stresses will impact persister allocation. Bacterial biofilm communities present an excellent system for studying density dependent selection on persister allocation because they exhibit rapid mutation and genetic diversification. In support of the model, variant *Pseudomonas aeruginosa* strains from PA01 biofilms showed heritable changes in growth rate, with slow-growing isolates producing higher numbers of persister cells than both fast-growing and wild-type *P. aeruginosa*. It was observed that 3-day intermittent exposure of biofilms to both lethal and non-lethal antibiotics gave rise to isolates with increased persister allocation. These data are the first to show that within biofilm culture experiments of short incubation times, biofilm development rapidly results in new *P. aeruginosa* lineages with increased persistence within *in vitro* experiments, and that exposure to antibiotics can induce this process. These observations have important clinical implications where chronic biofilm infections are treated with long-term antibiotic exposure.

Introduction

Persisters are a subset of dormant or extremely slow-replicating cells in a bacterial population, which are able to survive exposure to multiple antibiotics (Balaban *et al.*, 2004; Sufya *et al.*, 2003). Prior to antibiotic application, their presence increases in late exponential and stationary phases of population growth (Keren *et al.*, 2004a; Shah *et al.*, 2006). Although many genes may play a role in persister formation, the antibiotic tolerance seen in persisters is not heritable (Keren *et al.*, 2004a), and is distinct in this respect from genetically coded antibiotic resistance elements.

Although many genetic factors have been shown to be involved with the phenomenon of persistence, studies have yet to consistently identify a ‘persister regulon’ of genes mechanistically linked to this trait. Whole genome microarrays of *E. coli* persister cells have revealed up-regulation of ~400 genes compared to stationary cells (Shah *et al.*, 2006). The latter study and other work suggests a possible role for toxins such as HipA and RelE from the toxin-antitoxin (TA) modules *hipBA* (Moyed and Bertrand 1983) and *relBE* (Lewis 2005). More recently, it has been demonstrated that persistence in *E. coli* depends on the SOS response network (Dorr *et al.*, 2009). In *Pseudomonas aeruginosa*, screening of a mutant library resulted in the identification of several unrelated genes that either positively or negatively affect the allocation of persisters in a population (De Groote *et al.*, 2009). Thus a single mechanistic explanation for the persister trait remains elusive, and the phenomenon is likely to be a complex process involving multiple regulatory networks.

Putative functions for persister cells in bacterial populations have also been explored with theoretical population dynamic models. For example, persisters have been modelled in the context of a population level ‘insurance’ strategy against catastrophe (Kussell *et al.*, 2005), as a social phenomenon that allows for reduced competition under conditions of limiting resource (Gardner *et al.*, 2007), or as a sub-set of senescent, aged cells within the population (Klapper *et al.*, 2007). In this study, It is hypothesised that persisters arise as an adaptation to conditions of dense cellular crowding that can occur in biofilms. This model proposes that selection will favour lineages with deferred replication and increased longevity (i.e. slow growth and reduced senescence) within bacterial populations that are too crowded to sustain fast recruitment. This response to

crowding stimulates selection on ever-slower growth and reduced senescence as organisms decrease their intrinsic rate of production of new biomass in favour of a longer lifespan potential (Doncaster 2003; Seymour and Doncaster 2007). As persisters are dormant cells, with resulting decreased fecundity and reduced senescence, mutations will also be favoured that result in increased allocation of persister cells within a lineage population. The fitness advantage of increased lifespan then exacerbates crowding, further favouring the long-slow strategy with increased persister allocation.

Given that biofilms present a crowded environment to bacterial cells, they may be good examples of ‘*K*-selected’ populations in which fitness depends on lifetime reproductive output, in contrast to *r*-selected populations where fitness depends on rate of reproduction (MacArthur 1962; MacArthur and Wilson 1967). Bacterial biofilms are a helpful model system for studying density-dependent selection on fecundity, because they have been demonstrated to show reduced growth activity after reaching carrying capacity, with the majority of the cells capable of returning to a fast replicating strategy if the carrying capacity increases (Sternberg *et al.*, 1999). Biofilms have the additional advantage of expressing high mutation rates and genetic diversification (Allegrucci and Sauer 2008; Boles and Singh 2008; Boles *et al.*, 2004; Conibear *et al.*, 2009).

Here the model of density-dependent senescence (Doncaster 2003; Seymour and Doncaster 2007) is tested, with the hypothesis that increased persister cell allocation arises in response to crowded conditions as one component of a ‘spreader’ phenotype, characterised by reduced replication (fecundity) and offset by relatively long lifespan. The spreader phenotype contrasts to a ‘compressor’ phenotype of cells that trade shorter lifespan for faster replication in response to unrestricted nutrients and space. The testable components of this hypothesis are firstly that bacterial variant strains with slow-growth phenotypes will emerge from mature biofilm environments. Previous studies have shown that this does indeed occur, as slow growing colonies, known as small colony variants (SCV) have been observed to spontaneously emerge in high frequencies, (often as much as 40% of the biofilm population) in biofilms of many bacterial species including *S. aureus*, *Streptococcus pneumoniae* and *P. aeruginosa* (Allegrucci and Sauer 2007; Haussler *et al.*, 2003; von Eiff 2008). Since the model of Seymour and Doncaster (2007) predicts runaway selection on ever-slower senescence under density-dependent fecundity, the second testable component is that variant bacterial lineages with an increased allocation of persisters will arise spontaneously within bacterial

biofilm populations as a result of the crowded biofilm environment. Mechanistically, it is still unclear whether there is a direct link between slow growth and increased persister allocation, but slow bacterial growth due to nutrient limitation has been previously shown to result in increased persistence in planktonic cultures (Evans *et al.*, 1991; Evans *et al.*, 1990; Finch and Brown 1975; Gilbert and Brown 1978; Sufya *et al.*, 2003). I will test whether processes of diversification within biofilms can lead to slow-growing strains with enhanced persister allocations.

The third testable component of density-dependent senescence is that the type of selection favoured in the biofilm after frequent lethal events will differ from that favoured after non-lethal stresses. Lethal catastrophe such as that caused by bactericidal antibiotics results in increased death of cells other than persisters. Once the stress has passed, the reduced competition between cells for nutrients should favour the fast-growing compressor strategy (Seymour and Doncaster 2007). Conversely, non-lethal stress such as that caused by bacteriostatic agents postpones fecundity, thus favouring spreader phenotypes upon the removal of the stress and resumption of nutrient competition, either as a fitness benefit of increased reproductive lifespan (Seymour and Doncaster 2007) or as a social trait (Gardner *et al.*, 2007). In order to address whether such biofilm-specific population and evolutionary processes can impact on the formation of persister cells *in vitro*, the emergence of lineages with increased allocation of persister cells in biofilms were tested for. I also aimed to determine whether there are differences in the effect of bactericidal and bacteriostatic antibiotics on levels of persisters in mature biofilms, as would be predicted by the deferred replication model. This was done by applying both lethal and non-lethal antibiotic events, in order to distinguish the models of persisters as expressions of the senescent cost to fecundity (Klapper *et al.*, 2007) or deferred fecundity in extended lifespan (Seymour and Doncaster 2007).

Experimental Procedures

Bacterial strains, media and growth conditions

Pseudomonas aeruginosa PA01 (Stover *et al.*, 2000) and biofilm-derived phenotypic variants originating from this organism were used in all experiments. Unless otherwise stated, all bacteria were grown either in Luria-Bertani (LB) broth, or LB agar. Liquid bacterial culture was carried out at 22°C with 150 rpm orbital shaking, and plates were incubated at 37°C unless otherwise stated.

Biofilm formation and isolation of mutants

Biofilms were grown in rectangular three-channel flow cells with individual channel dimensions of 1×4×40 mm and flow rate of 270 $\mu\text{l min}^{-1}$. Biofilm cultures were maintained on M9 medium containing 0.1 mM CaCl_2 , 1.0 mM MgSO_4 , 6.78 $\text{g}\cdot\text{l}^{-1}$ Na_2HPO_4 , 3.0 $\text{g}\cdot\text{l}^{-1}$ KH_2PO_4 , 1.0 $\text{g}\cdot\text{l}^{-1}$ NH_4Cl , and 0.5 $\text{g}\cdot\text{l}^{-1}$ NaCl , with 1.0 $\text{g}\cdot\text{l}^{-1}$ glucose as the sole carbon source. The technique used for culturing biofilms in flow cells is as previously described (Moller *et al.*, 1998; Webb *et al.*, 2003). Bacteria were obtained from the flow cell reactors by collecting cells dispersed from the biofilms via the effluent tubes, diluting in M9 medium, and plating by serial dilution onto LB agar plates. After overnight growth, individual colonies were picked from the plates, grown overnight in LB broth, and frozen down in 13% (v/v) glycerol.

Growth rates and heritability of growth

Each biofilm-derived colony isolate was recovered from frozen stock by growing in LB overnight at 37°C with 250 rpm shaking, and purity was checked by plating out on LB agar, and incubating overnight. Overnight cultures were diluted 10^{-1} and growth curves were measured in replicate in a 96-well plate (Sunrise remote plate reader, Tecan UK Ltd, Theale, Reading). Optical density (OD) measurements (620 nm) were taken every 10 min. Growth rates were calculated by using the following formula $\text{Growth Rate} = (\text{OD } 0.6 - \text{OD } 0.2) / (\text{Time } 0.6 - \text{Time } 0.2)$. Heritability of growth rate was determined by sub-culture over 12 days, with isolate growth rate measurements repeated every 3 days.

Motility assays

Swim plates (10 $\text{g}\cdot\text{l}^{-1}$ tryptone, 5 $\text{g}\cdot\text{l}^{-1}$ NaCl and 0.3% (w/v) agarose) were inoculated with bacteria from overnight culture on LB agar, wrapped in parafilm to prevent

dehydration, and incubated at 30°C for 16 hours. Swarm plates (0.5% (w/v) agar, 8 g·l⁻¹ nutrient broth and 5 g·l⁻¹ glucose) were allowed to dry overnight at room temperature before inoculation from overnight culture on LB plates, and swarm plates were then incubated overnight at 37°C. Twitching plates (10 g·l⁻¹ tryptone, 5 g·l⁻¹ yeast extract, 5 g·l⁻¹ NaCl and 1% (w/v) agar) were dried briefly before stab inoculation with overnight growth of bacteria from LB agar. Incubation occurred for 24 h before measuring the zone of motility at the agar/petri dish interface. These motility assays were modified from a previously reported method (Rashid and Kornberg 2000).

Antibiotic killing of planktonic cultures

Biofilm-derived isolates were recovered from frozen samples by growth in LB overnight at 37°C with 250 rpm shaking. This culture was used to inoculate 100 ml LB at a dilution factor of 10⁻³. Where possible, cultures were grown to OD 0.6 before time dependant killing (TDK) was carried out. When an OD of 0.6 was reached, the culture was separated into 10 ml aliquots for TDK assays in 50 ml falcon tubes, or 200 µl aliquots for 96 well plate TDK assays. Antibiotic killing of planktonic cultures of *P. aeruginosa* was carried out with either ofloxacin (3 µg·ml⁻¹) or tobramycin (400 µg·ml⁻¹) as previously reported (Brooun *et al.*, 2000; Dorr *et al.*, 2009; Keren *et al.*, 2004a; Mulcahy *et al.*, 2010). Aliquots were incubated for 3 hours with antibiotic at room temperature. Control samples were incubated with the equivalent volume of PBS instead of antibiotic. Persister cells were identified as described previously by CFU count after serial dilution in LB broth, and plating on LB agar plates using a drop plate method (Reed and Reed 1948).

Analysis of biofilm total biomass using COMSTAT

Confocal laser scanning images of 6-day old continuous culture biofilms were obtained using a Leica (TCS SP2 MP FCS) upright microscope and x20 objective. Images had 512×512-pixel resolution and used identical gain, offset and pinhole settings for each data collection point. To enable visualization of cells within the biofilm the medium supply to the flow cell was stopped and each channel was stained for 10 min with 1.0 ml of DNA staining solution (5 µM SYTO-9 in M9; Invitrogen, U.K.), followed by a period of 10 min with medium flow to wash out excess stain. A single argon laser line was used with excitation wavelength of 488 nm and using an emission filter with a bandpass of 500–600 nm. Three-dimensional rendering and analysis was performed using COMSTAT software (Heydorn *et al.*, 2000) on the Matlab platform. COMSTAT

analysed each stack for total biomass (μm^3). Values are calculated means of data from several image stacks (an average of 5 image stacks from three different channels in three separate experiments).

Antibiotic killing of biofilm cultures

Biofilms were grown for 6 days before treatment with $192 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin for 2 hours. Whole biofilm biomass was harvested from the effluent tubes (3-cm tube sections, interior diameter 2.6 mm) before and after treatment, and biomass was disrupted by vortexing sample in 10 mls PBS for 2 min in the presence of 1.97 g of 2 mm glass beads. Persister cells were identified by CFU count after serial dilution in LB broth, and overnight incubation on LB agar plates. Three replicate biofilm channels were collected for each treatment.

Frequent, intermittent exposure of biofilms to antibiotics

To assess the persister response of repeated treatments of antibiotic to biofilms, biofilms were grown for 7 days, and then treated with either the bacteriostatic antibiotic tetracycline ($600 \mu\text{g}\cdot\text{ml}^{-1}$) or the bactericide ofloxacin ($192 \mu\text{g}\cdot\text{ml}^{-1}$) over a period of 72 hours. Flow cell reactors were incubated with the appropriate antibiotic for 2 hours, the antibiotic was washed out by turning the peristaltic pump back on, allowing media to recommence flow for 2 more hours. This was repeated 3 times (a total of 12 hours) and the biofilm was fed as normal for the next 12 hours. This 24-hour cycle was repeated over 3 consecutive days, and then biofilm-derived isolates were collected by the method described above. Isolates were tested as described above for variation in growth rate and persister level using the antibiotic tobramycin ($400 \mu\text{g}\cdot\text{ml}^{-1}$) to discount any increased survival being due to genetic resistance mutations as a result of extended exposure to the antibiotics ofloxacin and tetracycline.

Statistical analysis

After normalisation of residuals with \log_{10} transformations of percentage survival and growth rate, one-way ANOVA tested for differences between groups; orthogonal contrasts further tested for an overall difference between two pooled treatments and a control, and for a difference between the two treatments. Levene's test was used to assess homogeneity of variances. The threshold of acceptable Type-I error was taken as $\alpha = 0.05$ throughout.

Results

Variation of growth rates and persister levels among biofilm-derived bacterial isolates

Bacterial isolates collected from mature 10-day old biofilms showed a wide range of growth rates in planktonic culture. Isolates collected from the first generation wild-type biofilm experiment had growth rate s.d. = 0.3008 at $OD_{620} h^{-1}$, compared to isolates collected from stationary phase planktonic growth, with s.d. = 0.0769 at $OD_{620} h^{-1}$ (Figure 7). Fast and slow-growth phenotype isolates were subjected to further studies to assess whether the growth rate changes were heritable genetic changes that may be subject to, or have arisen by evolutionary selection. This was achieved by comparing changes to the isolates' growth rate over multiple passages of the organism. After 12 passages, reversion to the wild-type rate of growth was seen in all five of the fast-growth isolates studied. In contrast, five out of six slow-growth isolates maintained their slow-growth phenotype. Figure 8 shows an example representative of the growth profiles of the fast- and slow-growth variants over the 12-day period. To further assess the heritability of the slow-growth variants, two isolates designated MT24 and MT30 were used to seed flow cells and grown for 6 days. When isolates from these second generation biofilms were studied for growth rate variation, they were seen to have significantly different growth rates compared to those obtained from wild-type biofilms. Compared to wild-type biofilms, slow-growth variant biofilms gave rise to isolates with slower growth rates and that exhibited a narrower range of growth rates (Figure 9).

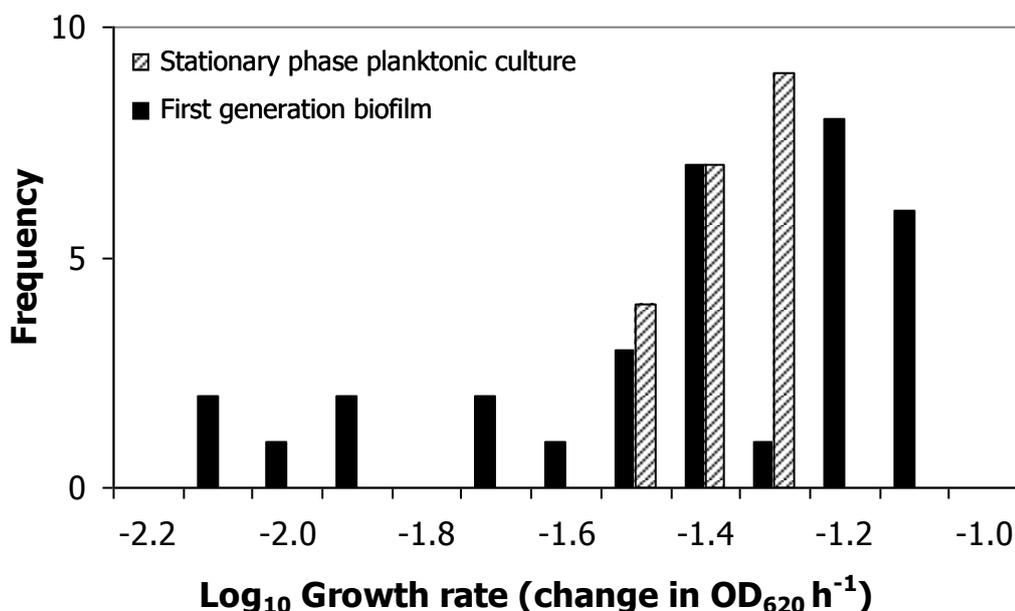


Figure 7 *P. aeruginosa* first generation wild-type biofilms give rise to isolates with increased range of growth rates compared to stationary phase planktonic cultures. Isolates collected from first generation wild-type *P. aeruginosa* PA01 biofilms had a wider range of growth rates than isolates collected from stationary phase planktonic culture (Levene's test statistic = 14.48, $P < 0.001$). First generation biofilm isolates $n = 33$, stationary phase planktonic isolates $n = 20$.

Fast and slow-growing biofilm-derived isolates were examined for persister cell allocation in planktonic populations. Time dependant killing studies were carried out using ofloxacin, and cells surviving 3 hours of incubation at lethal concentrations were designated as 'persister cells' (Keren *et al.*, 2004a). Fast-growing biofilm-derived isolates, MT5 and MT33, were observed to have bi-phasic killing patterns similar to the wild-type biofilm inoculum, while the killing curves exhibited by slow-growth isolates MT24 and MT30 were seen to have a lesser rate of death with time than wild-type and fast-growing isolates. At the end of the study, slow-growing variant strains had higher persister cell levels compared to wild-type or fast-growing isolates (Figure 10).

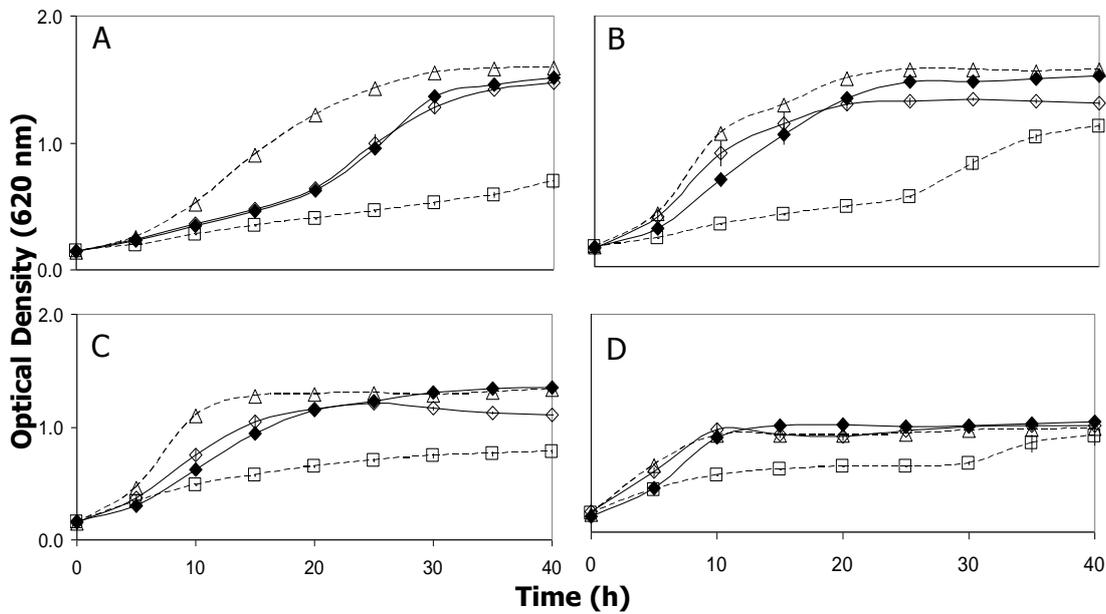


Figure 8 Representative profiles obtained from slow-growth and fast-growth isolates; biofilm-derived isolates with slow growth showed heritability of growth rate, whilst fast-growth isolates reverted to wild-type growth rates. Heritability of growth characteristics of biofilm-derived isolates MT30 (Slow growth; open squares), MT5 (fast growth; open triangles) and wild-type *P. aeruginosa* PA01 (open diamonds) after A, 3; B, 6; C, 9; or D, 12 subculture passages. Wild-type *P. aeruginosa* PA01 with no passages (filled diamonds) was used in each case as a control. Over 12 subculture passages, MT30 retained its slow-growth characteristics, while fast-growth isolate MT5 reverted to wild-type growth rates. MT30 and MT5 are representative profiles of the data obtained from slow growth and fast growth isolates.

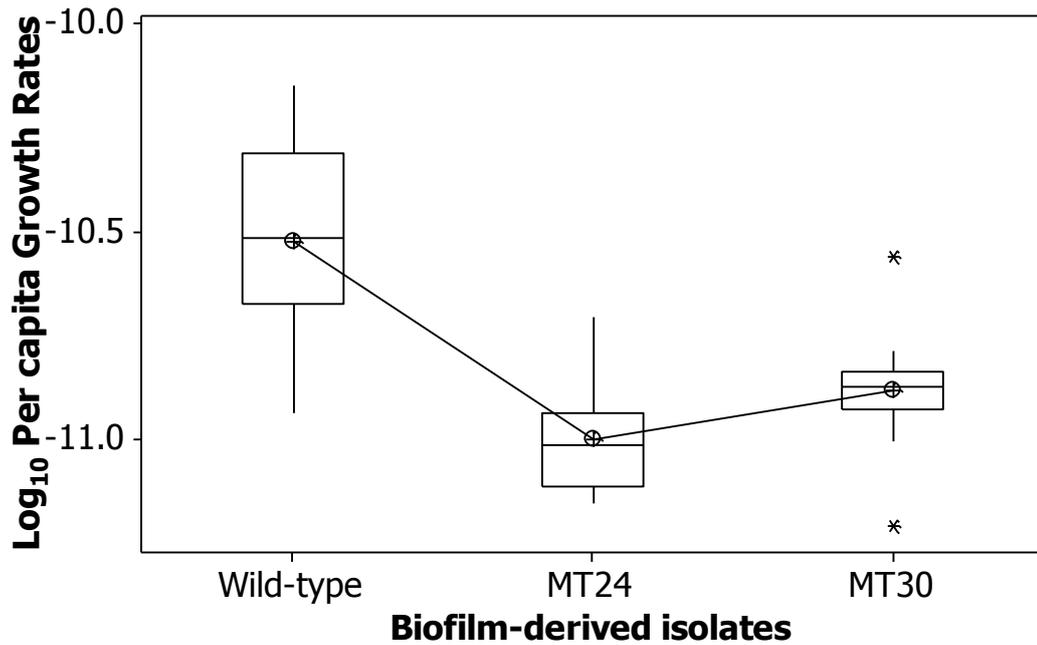


Figure 9 Slow-growth isolate biofilms give rise to variants with low growth rates compared to first generation wild-type biofilms. Isolates from the second generation biofilm seeded from slow-growth isolates MT24 and MT30 showed significantly slower growth rates than first generation wild-type biofilm isolates ($F_{1,80} = 105.52, P < 0.001$). Isolates derived from MT24-seeded second generation biofilms had slower growth rates than isolates from the MT30 biofilms ($F_{1,80} = 5.34, P = 0.023$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. First Generation biofilm isolates $n = 33$, Second Generation slow biofilm MT24 isolates $n = 20$, Second Generation slow biofilm MT30 isolates $n = 30$.

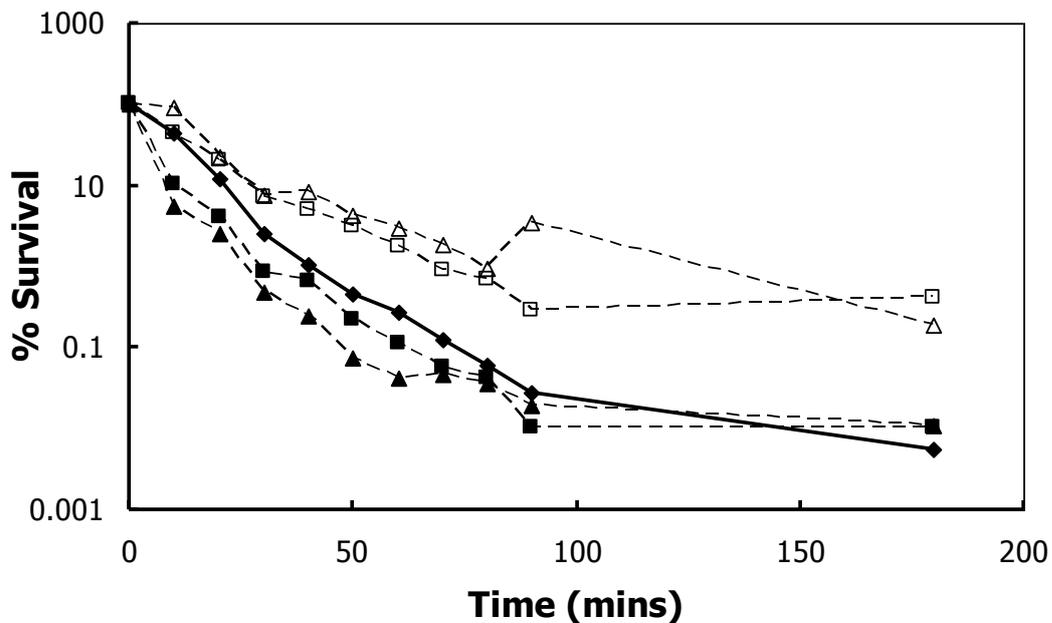


Figure 10 Biofilm-derived slow-growth isolates showed an increased persister cell allocation compared to wild-type and fast-growth isolates. Time dependant killing was carried out at approximate optical densities of 0.6. Isolates were incubated for 3 hours with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin. Solid line indicates wild-type *P. aeruginosa* PA01, while broken lines indicate growth variants. MT5 (filled squares) and MT33 (filled triangles) were fast growth isolates, while MT24 (open triangles) and MT30 (open squares) were slow growth isolates.

Morphology and persistence in biofilms seeded from slow-growth isolates MT24 and MT30

Biofilm growth and persistence of the biofilm-derived slow-growth isolates were examined. At 3 (data not shown) and 6 days of biofilm growth, both slow-growth isolates MT24 and MT30 showed decreased biomass (Figure 11A) compared to wild-type *P. aeruginosa* biofilms. Differences were also observed in the ability to form microcolonies. Slow-growth isolate MT30 did not appear to form structured microcolonies at all, forming instead a relatively flat, interconnected biomass (Figure 11B), whereas MT24 generated many foci from which microcolonies were able to grow, but the microcolonies were much smaller in diameter compared to the wild-type. However, when these two biofilms were treated with the antibiotic ofloxacin, and viable cells from these biofilms were subsequently collected by disruption of biomass, CFU counts showed that both slow-growth isolate biofilms had increased survival compared to the wild-type (Figure 12), with no detectable difference between the two slow-growth isolate biofilms. Further characterisation of biofilm derived colony variants, including swim and swarm assays can be found in Appendix 1 (pages 109 - 113).

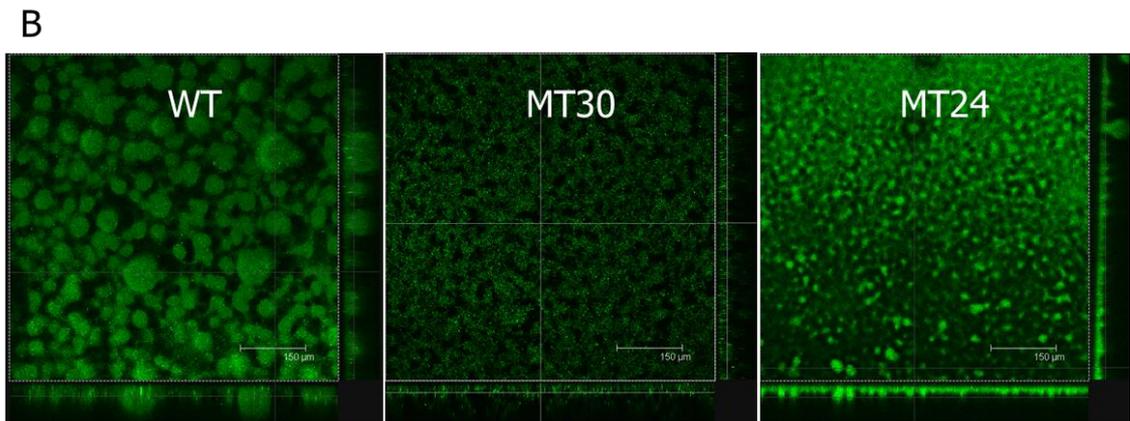
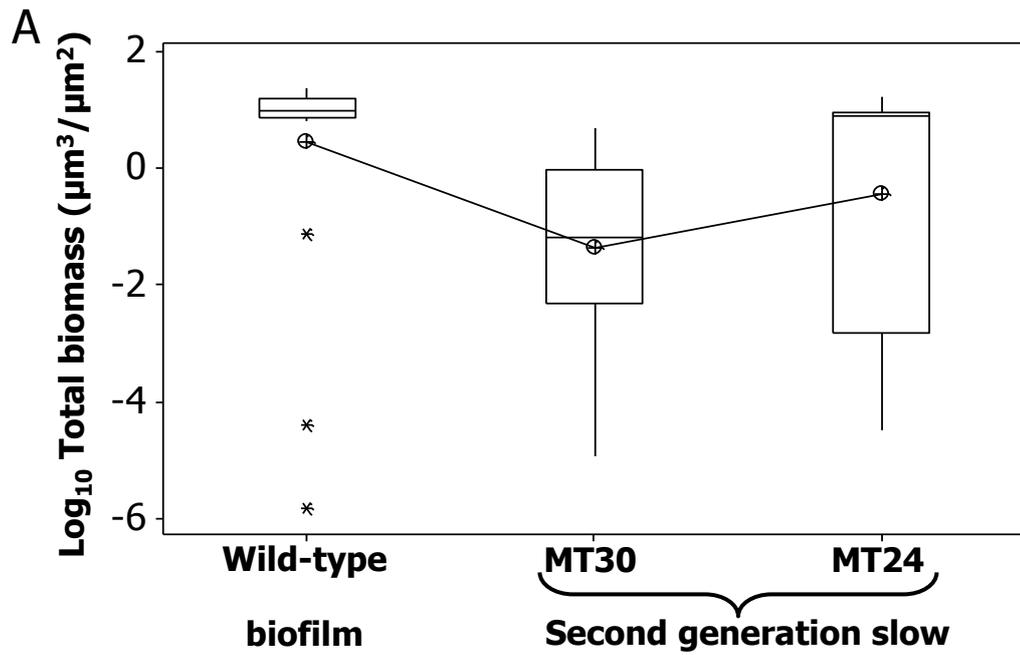


Figure 11 Biofilms generated from slow growth biofilm-derived isolates showed decreased biomass and microcolony size compared to wild-type biofilms. A. Orthogonal contrasts in one-way ANOVA showed that second generation biofilms had less total biomass at 6 days than wild-type biofilms ($F_{1, 91} = 10.16, P = 0.002$). Second generation biofilm seeded from slow isolate MT30 also showed less total biomass than second generation biofilm seeded from slow isolate MT24 ($F_{1, 91} = 4.50, P = 0.037$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. Asterisks indicate outliers. Wild-type biofilm $n = 22$, Second generation biofilm slow (MT30) $n = 37$, Second generation biofilm slow (MT24) $n = 35$. B. **Slow-growth isolate biofilms showed decreased microcolony size compared to wild-type biofilms.** 6 day old biofilms were stained with fluorescent DNA stain SYTO9, and examined using confocal microscopy. Images taken using 200x magnification, and show a scale bar of 150 µm.

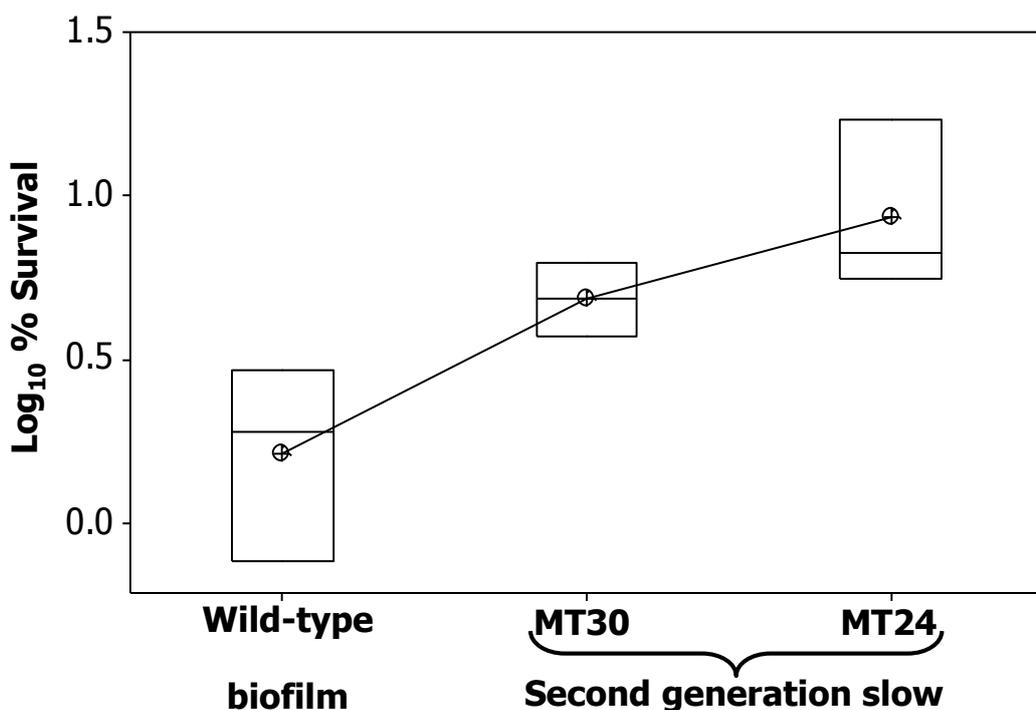


Figure 12 Second generation biofilms exhibited higher survival than wild-type biofilm after exposure to ofloxacin. Orthogonal contrasts in one-way ANOVA showed that after 2 hours of incubation with $192 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin, both slow growth isolates, MT24 and MT30 had higher survival than the wild-type ($F_{1,5} = 9.81, P = 0.026$). No difference could be detected between the levels of survival shown by the two slow growth isolates ($F_{1,5} = 1.12, P = 0.339$). Box plots show median and inter-quartile ranges; means are shown as encircled crosses. WT $n = 3$, Slow-growth isolate MT30 $n = 2$, Slow-growth isolate MT24 $n = 3$

Frequent treatment of biofilms with antibiotics leads to enhanced persistence

Higher levels of persistence arose in biofilms treated with frequent exposure to either bactericidal or bacteriostatic antibiotics (ofloxacin or tetracycline respectively) than in control biofilms, with no detectable difference between the two types of antibiotic exposure (Figure 13A). Due to the extended exposure of biofilms to both ofloxacin and tetracycline in this study, all persistence assays were carried out with the antibiotic tobramycin, to limit the possibility that increased survival may occur due to mutations that confer genetic resistance to ofloxacin or tetracycline. Biofilms that had been treated with either antibiotic exhibited higher growth rates than biofilms with no antibiotics (Figure 13B). The two types of antibiotic had similar influences on the growth rates of the isolates. Growth rate did not influence persister levels in this experiment. For the ofloxacin treatment, persister allocation was not detectably different between small

colony variant and wild-type isolates (mean \pm s.e. of \log_{10} % survival 0.968 ± 0.089 and 1.041 ± 0.093 respectively, $F_{1,56} = 0.032$, $P = 0.575$). The same result was seen for all three treatments (data not shown).

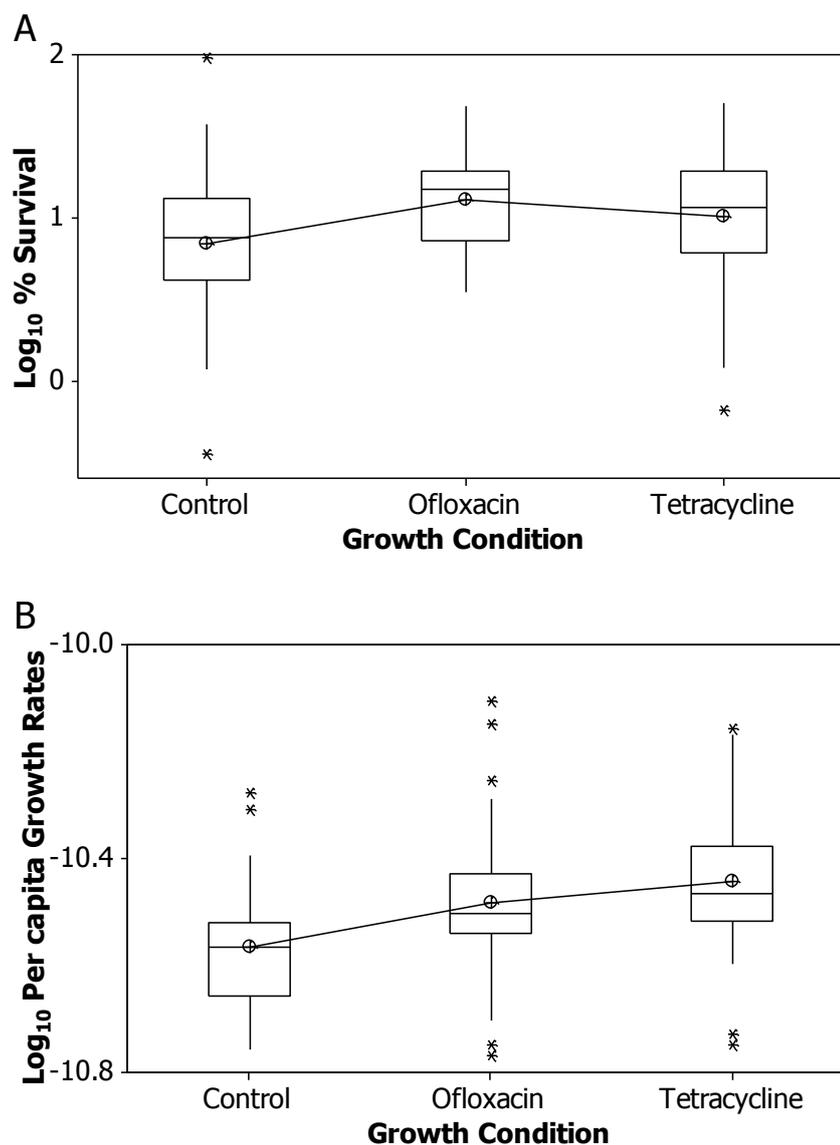


Figure 13 A. Isolates recovered from biofilms treated with antibiotics exhibited increased persister allocation than isolates recovered from a wild-type biofilm. Orthogonal contrasts in one-way ANOVA showed that after 3 hours of incubation with $400 \mu\text{g}\cdot\text{ml}^{-1}$ tobramycin, isolates from both flow-cells exposed to antibiotics had higher survival than isolates from control flow-cells ($F_{1,144} = 10.3$, $P = 0.002$). There was no difference between bacteriostatic and bactericidal antibiotic treatment of biofilms (tetracycline and ofloxacin respectively, $F_{1,144} = 1.36$, $P = 0.245$). **B. isolates recovered from biofilms treated with antibiotics exhibited faster growth rates than isolates recovered from a wild-type biofilm.** Orthogonal contrasts in one-way ANOVA showed that isolates from both flow-cells exposed to antibiotics had higher growth rates than isolates from control flow-cells ($F_{1,144} = 21.65$, $P < 0.001$). There was no difference between bacteriostatic and bactericidal antibiotics ($F_{1,144} = 2.08$, $P = 0.151$). Box plots show

median and inter-quartile ranges; means are shown as encircled crosses. Asterisks indicate outliers. Control $n = 51$, Ofloxacin $n = 33$, Tetracycline $n = 63$.

Discussion

To my knowledge, this study is the first to address experimentally how biofilm-associated ecological and evolutionary processes may impact on the formation of persister cells and on tolerance towards antimicrobials. In contrast to planktonic bacteria, biofilms comprise sessile, structured and crowded communities of cells that exhibit steep chemical and nutrient gradients, and enhanced mutation and genetic diversification. Under such conditions, recruitment limitation may favour selection on spreader mutations for slow reproduction and long lifespan (Seymour and Doncaster 2007). This deferred-replication model is applicable to any organism showing age-structured senescence, including bacterial populations which are now established to display senescent ageing (Stewart *et al.*, 2005).

It was observed that isolates with heritable slow-growth phenotypes showed increased allocation of persisters in planktonic culture compared to wild-type or fast-growing isolates (Figure 10); slow-growing isolates also exhibited increased survival in the face of antibiotic challenges within biofilms (Figure 12). Previous studies have used starvation in planktonic culture as a mechanism for controlling growth rate (Evans *et al.*, 1991; Evans *et al.*, 1990; Finch and Brown 1975; Gilbert and Brown 1978; Sufya *et al.*, 2003), finding that increased persistence can be associated with decreased growth rates. This study is the first to show that biofilm-specific processes of phenotypic variation can rapidly lead to sub-populations of cells with an enhanced ability to form persisters.

While isolates collected from mature biofilms in this study were observed to have a wide range of growth rates (Figure 7), slow-growth isolates showed increased stability compared to fast-growth isolates (Figure 8), and displayed little reversion when seeded to new biofilms (Figure 9). Previous studies have noted the presence of slow-growing small colony variant (SCV) sub-populations in mature biofilm environments (Allegrucci and Sauer 2007; Haussler *et al.*, 2003; Webb *et al.*, 2004). In these studies, SCV isolates collected from biofilms *in vitro*, and also from clinical settings, often exhibit slow-growth, hyper-piliation and increased attachment to surfaces. Thus slow and stable growth phenotypes are consistently recovered from mature biofilms, and may occur as a result of evolutionary pressures within the biofilm that select for increased fitness under density dependant conditions.

It was observed that new *P. aeruginosa* lineages with increased persister allocation arose rapidly in biofilms in response to exposure to antibiotics (Figure 13A). When antibiotics were introduced to the flow cell reactor system intermittently over a 3-day period, it was discovered that treatment with either bacteriostatic or bactericidal antibiotics resulted in the isolation of bacterial lineages with higher levels of persistence compared to isolates from control biofilms. None of the population modelling predictions available for persister allocation fitted wholly to these observations. While an increase in persistence as a response to bacteriostatic antibiotic was predicted by the deferred-replication model (Seymour and Doncaster 2007), the corresponding increase in persisters in response to the bactericidal antibiotic was not an expected outcome. Likewise, the outcomes did not support the prediction from the reduced-competition model (Gardner *et al.*, 2007) that frequent application of a non-lethal antibiotic would result in a decrease in persister allocation. These outcomes are consistent with the model of Kussell *et al.* (2005), hypothesising that the type of environmental stress affecting bacterial populations may be less important than the frequency with which the stress affects the population. This suggests that the relationship between persister allocation and environmental stress is a complex one, where persistence in biofilms may be more strongly related to the frequency of stress, rather than the type of stress *per se*. The outcomes are broadly consistent with a bet-hedging strategy (Kussell *et al.*, 2005) that may be influenced by the senescent cost of reproduction (Klapper *et al.*, 2007) and that promotes a strategy of long-slow life histories in crowded biofilms (Doncaster 2003; Seymour and Doncaster 2007) leading to potential group benefits (Gardner *et al.*, 2007).

In this study it has been shown that antibiotic treatment of biofilms over a 3-day period can lead to increased persister allocation within new biofilm-derived lineages of *P. aeruginosa*. This has important implications in situations where antimicrobials are applied repeatedly over long periods, such as in chronic infections caused by biofilms, because it may lead to enhanced bacterial recalcitrance towards antibiotics. For example, these data may help to explain why late *P. aeruginosa* clinical isolates from CF patients collected over a 96-month period showed 100-fold increased levels of persisters compared with the earlier strains (Mulcahy *et al.*, 2010). The observations in this study may additionally contribute to explaining why *Candida albicans* strains with higher levels of persisters are associated with long-term exposure to the microbiocide chlorhexidine in patients at risk of oral candidiasis (Lafleur *et al.*, 2010).

Interestingly, frequent cyclical exposure to antibiotics also had the unpredicted effect of increasing overall growth rates seen in isolates gathered from mature biofilms. Both antibiotic treatments had the same effect of increasing bacterial growth rate in comparison to the wild-type (Figure 13B). This suggests that persister allocation is not directly linked to growth rate, and that slower growth characteristics do not necessarily result in enhanced persistence as has previously been considered to be the case. For example, a lineage that has mutated to allow a spreader lifestyle and greater persister allocation may exhibit an increased survival advantage over other lineages during antibiotic treatment. Once the antibiotic is removed, the lineage may favour the compressor strategy to enable best use of the nutrients available, while still having an increased persister allocation as a bet-hedging strategy. Further study in this area will be needed to reveal the precise relationship between growth rate variations and persistence. In particular, survivorship curves have yet to be defined that could distinguish between the models of persister cells as expressions of the senescent cost of reproduction (Klapper *et al.*, 2007), or as the end-point of a spreader strategy towards negligible senescence to accommodate deferred reproduction (Seymour and Doncaster 2007).

In summary, this study has shown that increased persistence is linked to heritable variation derived from biofilm-specific strain diversification. It was observed that frequent antibiotic stress resulted in enhanced persister allocation, regardless of whether the antibiotic used is lethal or non-lethal in its mode of action. These observations provide an insight into the importance of ecological influences on persister formation. Further research into the role of environmental selection forces on the allocation of persisters will advance scientific understanding of antimicrobial tolerance in bacterial populations and its control in clinical, environmental and industrial settings.

CHAPTER 3

ROLE OF MUTATION FREQUENCY AND SOS RESPONSE ON PERSISTENCE IN *P. AERUGINOSA* POPULATIONS

Abstract

This chapter examines the hypothesis that the hypermutable phenotype is a characteristic of persister cell biology. A detailed examination of mutation levels and SOS response-mediated gene expression in persister cells was carried out in an attempt to understand the relationship between these phenomena. Persisters that had been isolated using both DNA damaging (ofloxacin) and non-DNA damaging (ethanol) antimicrobial treatments exhibited significantly increased frequencies of mutation to rifampicin resistance, compared to the total bacterial population and compared to young populations with lower persister allocation. The mutation frequencies within these persister fractions were comparable with those of mutator strains ($>2 \times 10^{-7}$). Gene expression analysis of persister cells isolated following ofloxacin and ethanol treatment showed SOS activation in ofloxacin treated cells, but not in ethanol treated ones. Increased levels of *mutS*, a mismatch repair gene, were seen in both ofloxacin and ethanol treated cells. Taken together, these data suggest that persister cells exhibit a hypermutable phenotype that can occur independently of the SOS response.

Introduction

Bacteria in biofilms undergo rapid diversification and are subject to higher mutation rates than planktonic cells (Conibear *et al.*, 2009). The bacterial biofilm is a complex environment, with nutrient gradients, oxygen limitation and water channels creating micro-niches within microcolonies (Davey *et al.*, 2003; Sternberg *et al.*, 1999; Walters *et al.*, 2003). In these microcosms, conditions are such that bacteria may be subjected to varying mutagenic stresses (e.g. damaging reactive oxygen species), or there may be conditions under which a hypermutable phenotype may be advantageous to bacterial survival (Oliver and Mena 2010; Stewart and Franklin 2008). In the case of chronic infection, respiratory bursts from neutrophils, and lysis by complement are just two forms of host immune response that result in the biofilm being under constant stress (Jensen *et al.*, 2010). Subsequently, levels of mutation and diversification in biofilms are high, resulting in bacterial isolates emerging with varied colony morphology, growth rate, virulence factors and resistance mechanisms (Boles *et al.*, 2004; Smith *et al.*, 2006).

Amongst the range of mutants isolated from clinical biofilms, several studies have shown that *ex vivo* isolates commonly exhibit a hypermutable phenotype, called mutator strains (LeClerc *et al.*, 1996; Oliver *et al.*, 2000). Mutator strains, in the majority of cases, have loss of function mutations in DNA mismatch repair (MMR) genes such as *mutS* and *mutL*, which results in an increased number of subsequent mutations in genes involved with virulence, resistance and biofilm formation (Ciofu *et al.*, 2005; Henrichfreise *et al.*, 2007; Macia *et al.*, 2005; Oliver *et al.*, 2000). (See also Introduction section of this thesis, pages 21-22). Various *in vivo* models have also shown that hypermutation may favour the adaptation and persistence of bacterial pathogens. Giraud *et al.* (2001) using a murine model of *E. coli* intestinal colonisation, found that hypermutation was initially beneficial because it allowed a faster adaptation to the mouse gut environment. It has also been shown that the inactivation of the MMR system in *E. coli* favours the persistence of urinary tract infections in a mouse (Labat *et al.*, 2005), and MMR mutants can greatly enhance the rate of acquisition of resistance to many antibiotics (Blazquez 2003; Macia *et al.*, 2005; Sobel *et al.*, 2003).

Recent studies have shown that mutator strains derived from chronic cystic fibrosis lung infection have increased persister levels compared to non-mutator strains (Mulcahy *et al.*, 2010). However, despite the key role of persister cell and mutator phenotypes in bacterial survival of lethal antibiotic stress, no studies have investigated whether persister cells themselves exhibit enhanced mutation frequencies.

Both hypermutation and persister cells are linked to the induction of DNA mutation repair pathways such as the SOS response pathway. Dorr *et al.* (2009) have reported that formation of the persister subpopulation involves induction of the SOS response pathway. The antibiotic group of choice for *in vitro* persister studies in both *E. coli* and *P. aeruginosa* are the fluoroquinolones, and they are routinely used *in vitro* to isolate persister cells (De Groot *et al.*, 2009; Dorr *et al.*, 2009; Keren *et al.*, 2004a). It has long been known that fluoroquinolones inhibit DNA gyrase, resulting in double stranded breaks in the bacterial chromosome, and leading to the release of superoxide and hydroxyl radicals, resulting in cell death (Dwyer *et al.*, 2007). Such pathways have been shown to trigger SOS response pathways, which halt cell replication while repairing damaged DNA. Recent observations that pre-treatment of a bacterial culture with sub-lethal concentrations of ciprofloxacin resulted in an increased number of persister cells (Dorr *et al.*, 2009) are therefore not surprising; likely due to the induction of the SOS response by the DNA-damaging ciprofloxacin. Thus the relationship between antibiotic treatment, induction of the SOS response and persister cells are closely interrelated.

The SOS response system can result in increased levels of mutation within cells. While the SOS response system is the pathway of choice for DNA damage repair, lack of proof-reading capabilities in the SOS response DNA polymerases results in mismatch base pairs, which can lead to mutation if undetected. In lethal antibiotic stress, hydroxyl radicals are formed, causing DNA damage, and resulting in cell death. A very recent paper has shown that while levels of hydroxyl radicals are extremely high in dead cells, the hydroxyl radical concentration in persister cells was only very slightly raised (Kim *et al.*, 2011). This would suggest that the persister does not have lethal hydroxyl radical levels, but possibly enough to induce SOS response-mediated DNA repair. A further paper has shown that it is the presence of hydroxyl radicals in antibiotic surviving cells that result in mutation derived resistance mechanisms arising in bacteria (Kohanski *et al.*, 2010). Therefore in conditions of lethal antibiotic stress, persister cells and mutation may be caused by the same mechanisms.

In this chapter I aimed to examine the inter-relation of mutation and persistence. It was first demonstrated that there is an increase in mutation frequency in the persister sub-population of cells, compared to non-persister cells. It was then investigated whether this increased mutation frequency is dependent on the type of antibiotic used to isolate the persister cells, and whether the induction of the SOS response is necessary for this increase. Overall these data suggest that enhanced mutation levels are an intrinsic property of persister cells.

Experimental Procedures

Bacterial strains, media and growth conditions

Pseudomonas aeruginosa PA01 (Stover *et al.*, 2000) and isogenic hypermutable $\Delta mutS$ strain (Oliver *et al.*, 2004) were used in all experiments. Unless otherwise stated, all bacteria were grown either in Luria-Bertani (LB) broth, or LB agar. Liquid bacterial culture was carried out at 37°C with 150 rpm orbital shaking, and plates were incubated at 37°C unless otherwise stated.

Antibiotic killing of planktonic cultures

P. aeruginosa PA01 wild-type and $\Delta mutS$ strains were recovered from frozen samples by overnight growth in LB. This culture was used to inoculate 100 ml LB at a dilution factor of 10^{-3} . Where possible, cultures were grown to OD 0.6 before time dependant killing (TDK) was carried out. When an OD of 0.6 was reached, the culture was separated into 10 ml aliquots for TDK assays in 50 ml falcon tubes. Antimicrobial killing of planktonic cultures of *P. aeruginosa* was carried out with either ofloxacin ($3 \mu\text{g}\cdot\text{ml}^{-1}$) (Keren *et al.*, 2004a), or ethanol (20% (v/v)). Aliquots were incubated for 3 hours with antimicrobial agent at room temperature. Control samples were incubated with the equivalent volume of PBS instead of antimicrobial agent. Persister cells were identified as described previously by CFU count after serial dilution in LB broth (Keren *et al.*, 2004a), and plating on LB agar plates using a drop plate method (Reed and Reed 1948).

Mutation frequency assay

P. aeruginosa PA01 wild-type and $\Delta mutS$ strains were recovered from frozen samples by overnight growth in LB. This culture was used to inoculate 100 ml or 500 ml LB at a dilution factor of 10^{-3} . For whole population mutation frequencies, cultures were grown in 100 ml LB to OD 0.9 before serial dilution in LB broth and plating on LB agar plates using a drop plate method to determine total CFU ml^{-1} , and plating on LB agar plates with $300 \mu\text{g ml}^{-1}$ rifampicin to determine numbers of cells that have gained the 1bp mutation conferring rifampicin resistance (see appendix 2, page 113, for optimum rifampicin concentration study). Plates were counted at 24 and 48 h, and mutation frequencies were determined by dividing rifampicin resistant cells ml^{-1} by total cell population ml^{-1} (Driffield *et al.*, 2008). For young population mutation frequencies, cultures were grown in 100 ml LB to OD 0.2 before transfer to 50 ml Falcon tubes and

centrifugation using Heraeus Instruments Megafuge 1.0 benchtop centrifuge at 4000 rpm for 20 min. Cells were resuspended in 2 ml PBS and mutation frequencies were determined as above. For persister mutation frequencies, cells were grown in 2 x 500 ml LB to 0.9 OD to allow aeration of culture during growth. The cultures were combined into one flask before antimicrobial treatment for 3 h using either ofloxacin ($3 \mu\text{g}\cdot\text{ml}^{-1}$) or ethanol (12.5%) with intermittent stirring to allow full antibiotic coverage. Each sample was centrifuged in 2 x 450 ml aliquots using the Beckman J2-21 centrifuge at 7000 rpm for 20 min. Samples were fully resuspended in 10 ml PBS and then further concentrated using the Heraeus Instruments Megafuge 1.0 benchtop centrifuge at 4000 rpm for 20 min, and resuspended in 2 ml PBS. CFU counts for each sample were determined before and after antimicrobial treatment, and after centrifugation, and mutation frequencies were determined as described above.

Validation of PMA chelation of dead cell RNA

Ten ml of overnight culture of *P. aeruginosa* was divided between two 20 ml universal bottles and centrifuged at 4000 rpm for 20 min using the Heraeus Instruments Megafuge 1.0 benchtop centrifuge. One sample was resuspended in 10 ml PBS, whilst the other was resuspended for killing in 70% (v/v) ethanol, using 1.97 g of 2 mm glass beads to homogenise the samples. Both samples were vortexed periodically over a 3 h incubation period, before pelleting and resuspending in 5ml PBS. These samples were mixed at varying ratios of live and dead cells, for propidium monoazide (PMA) validation.

Twenty mM PMA (Biotum) was added to 500 μl samples resulting in a final concentration of 50 μM PMA (Rogers *et al.*, 2008). In the case of the controls, an equal volume of PBS was added. After 30 min incubation in the dark, samples were exposed to a 650-W halogen light source (Kaiser Videolight 6; Kaiser Fototechnik, Buchen, Germany) at a distance of 20 cm for 3 min (Parshionikar *et al.*, 2010). After photo-induced cross-linking, cells were centrifuged using the Heraeus Biofuge *pico*, and resuspended in 500 μl PBS to remove unbound PMA, before RNA extraction was carried out using the Invitrogen PureLinkTM RNA mini kit. Extracted RNA concentrations were determined by measurement of 2 μl sample using the Nanodrop ND-1000 spectrophotometer.

Combining PMA and RNALater for preservation of live cell RNA

This validation experiment was carried out as above, but with the added step of centrifuging and resuspending bacteria in 500 μ l RNALater (Sigma), or in PBS, before treatment with PMA. Once photo-induced cross-linking had occurred, cells were centrifuged using the Heraeus Biofuge *pico*, and resuspended in 500 μ l PBS to remove unbound PMA, and RNALater, before RNA extraction using the Invitrogen PureLink™ RNA mini kit.

Gene expression of persister cells

Persister cells were isolated as in the mutation frequency assay, and samples were collected at timepoints 0 min, 10 min, 30 min and 3 h of incubation with either 3 μ g·ml⁻¹ ofloxacin or 12.5% (v/v) ethanol. The samples contained a mixture of live (persister) and dead cells, and dead cell RNA was chelated with PMA as described above. Persister cell RNA was extracted using the Invitrogen PureLink™ RNA mini kit. Sample RNA concentrations were measured, and identical RNA concentrations for all samples were added to the reverse transcription step. Reverse transcription was carried out using the ImProm-II™ kit from Promega, using the Biorad MJmini PCR cycler, according to the reverse transcription protocol. Specialist primer-probe kits from PrimerDesign were created to detect gene expression of the *P. aeruginosa* genes *recA*, *dinB*, *mutS* and *rpoD*. Real time PCR (RT-PCR) was used to detect copy numbers of genes using the Biorad IQ5 cycler.

Calculating relative gene expression

Cycle threshold (C_T) values from the RT-PCR were converted to relative gene expression levels via the following equations. To calculate relative expression of the three genes of interest (GOI) – *recA*, *dinB* and *mutS* - compared to the housekeeping (HK) gene *rpoD*:

1. First calculate ΔC_T values:

$$\Delta C_T = (\text{mean GOI } C_T) - (\text{mean HK } C_T).$$

2. Then calculate relative expression:

$$2 - ([\Delta C_T \text{ treatment samples}] - [\Delta C_T \text{ control samples}])$$

The housekeeping gene expression was calculated as follows:

$$2 - ([\text{mean } rpoD \text{ } C_T \text{ treatment samples}] - [\text{mean } rpoD \text{ } C_T \text{ control samples}])$$

Statistics

After normalisation of residuals with \log_{10} transformations of percentage survival and mutation frequency, one-way ANOVA tested for differences between groups; orthogonal contrasts further tested for an overall difference between two pooled treatments and a control, and for a difference between the two treatments. Levene's test was used to assess homogeneity of variances. The threshold of acceptable Type-I error was taken as $\alpha = 0.05$ throughout.

Results

Determination of persister frequency in wild-type and $\Delta mutS$ *P. aeruginosa*

Bacterial populations of wild-type *P. aeruginosa* and $\Delta mutS$ showed no detectable difference in persister frequencies after antimicrobial treatment for 3 hours. This lack of effect was seen with both the DNA damaging agent ofloxacin ($F_{1,5} = 0.53$, $P = 0.508$) (Figure 14A) and the cell membrane disrupting agent ethanol ($F_{1,5} = 1.10$, $P = 0.353$) (Figure 14B).

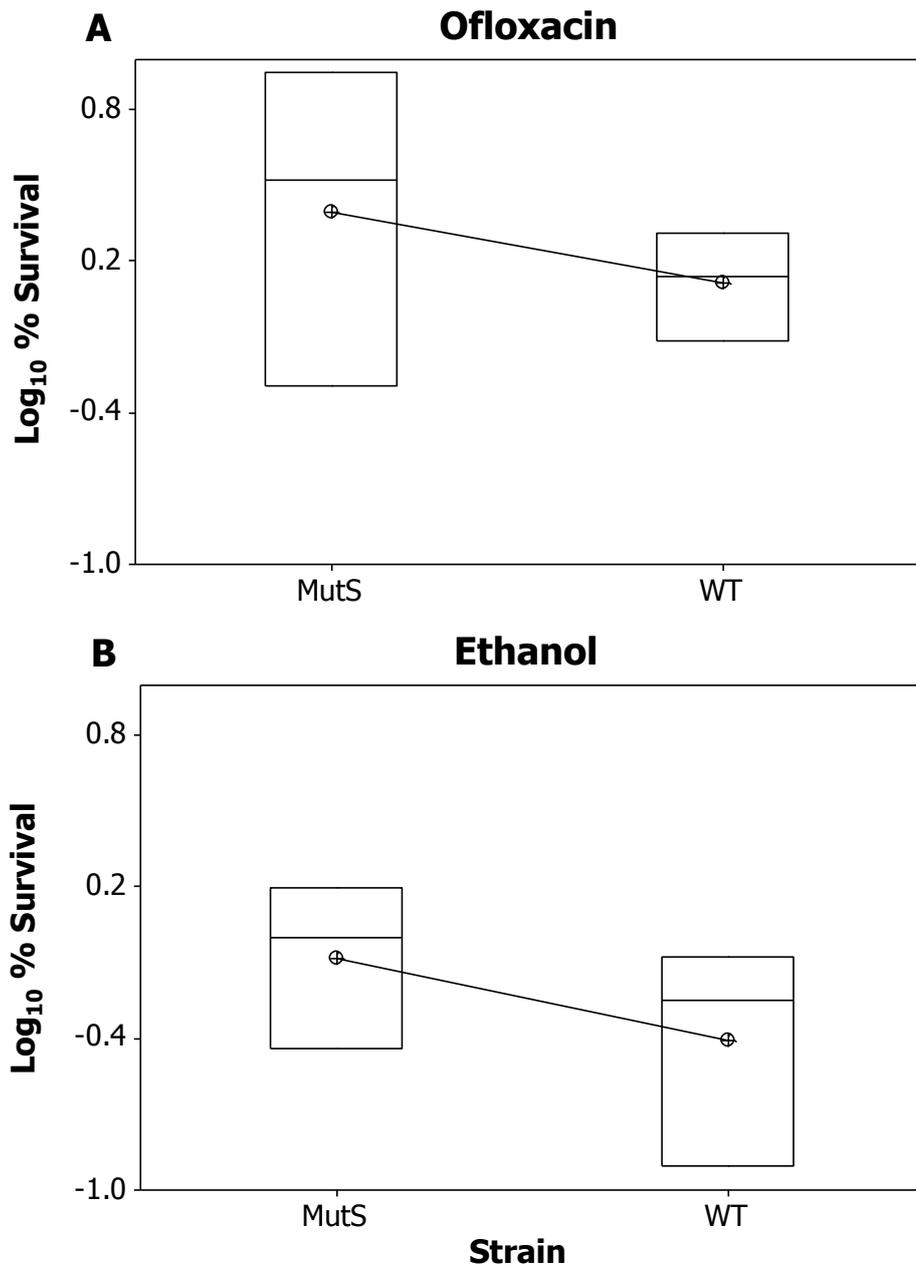


Figure 14 No significant difference between persister allocation in the *P. aeruginosa* PA01 wild-type strain and the $\Delta mutS$ knockout strain. Survival of $\Delta mutS$ and wild-type

strains of *P. aeruginosa* PA01 after 3 hour challenge with: A. 3 µg /ml ofloxacin, or B. 20% ethanol. Box plots show median and inter-quartile ranges; means are shown as encircled crosses. $\Delta mutS$ strain $n = 3$, wild-type strain $n = 3$.

Effect of ofloxacin and ethanol on persister cell mutation frequencies

By isolating persister cells with either DNA-damaging ofloxacin, or non-DNA damaging ethanol, increased mutation frequencies in persister populations compared to early log phase cells were observed. Orthogonal contrasts in one-way ANOVA showed that both persister cells isolated by treatment with ofloxacin, pooled with early log phase cells showed increased mutation frequencies compared to the control whole population of cells in the wild-type strain ($F_{1,11} = 24.89$, $P < 0.001$) (Figure 15A). This effect was not seen in the $\Delta mutS$ strain (Figure 15B), where the pooled persister and early log phase populations showed no difference to the control whole population ($F_{1,9} = 0.33$, $P = 0.578$). When examining differences between ofloxacin-isolated persister populations and early log-phase populations, mutation frequencies were significantly higher in the persister populations, both in the wild-type ($F_{1,11} = 65.66$, $P < 0.001$), and in the $\Delta mutS$ strain ($F_{1,9} = 5.46$, $P = 0.044$).

Due to the DNA damaging effect of fluoroquinolones, this experiment was repeated using ethanol, an antimicrobial agent that kills via membrane disruption (Figure 15C, D). Again, orthogonal contrasts in one-way ANOVA showed that a pooled sample of ethanol-isolated persister cells and early log phase cells showed increased mutation frequencies compared to the control whole population of cells in the wild-type strain ($F_{1,8} = 8.95$, $P = 0.017$). Once again, this effect was not seen in the $\Delta mutS$ strain, where the pooled persister and early log phase populations showed no difference to the control whole population ($F_{1,9} = 0.15$, $P = 0.706$). Despite using non-DNA damaging ethanol to isolate persister cells, Figure 15C showed that the persister cell population in the wild-type strain still showed a significant increase in mutation frequency compared to the early log phase cells ($F_{1,8} = 10.31$, $P = 0.012$). However, this effect was not observed in the $\Delta mutS$ strain ($F_{1,9} = 0.19$, $P = 0.672$, Figure 15D). The increased mutation frequency observed in persister cell fractions of *P. aeruginosa* populations is an exciting discovery, suggesting a possible link between the two distinct cell functions of mutation and persister allocation.

As the $\Delta mutS$ strain is hypermutable, all subpopulations of cells measured for mutation frequency are higher than the designated threshold for mutator strains (Table 1) (Mena *et al.*, 2008). The wild-type strain has a low mutation frequency in the total population, but persister cells isolated with ofloxacin show a 57-fold increase in mutation frequency, so high that they became analogous to that of a mutator strain (Table 1). Although the wild-type ethanol-isolated persister cells do not reach a mutation frequency high enough to be classified as a ‘mutator’, there is still a 6.3-fold increase compared to the total wild-type population, considerably higher than the same experiment in the $\Delta mutS$ strain, which showed only a 0.7-fold increase in persister mutation frequency.

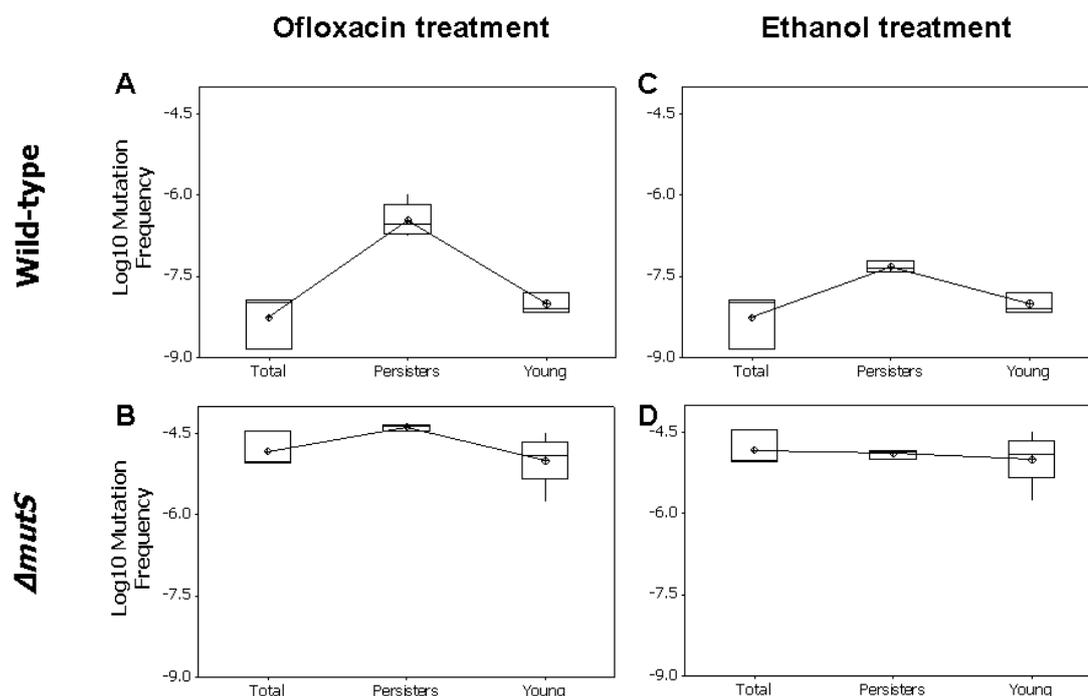


Figure 15 Ofloxacin-isolated persister sub-populations show increased mutation frequencies compared to non-persister populations in both wild-type and $\Delta mutS$ strains. A. Wild-type *P. aeruginosa* PA01. Persisters isolated with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin, and early log phase populations showed increased mutation frequencies compared to the wild-type control population; persister cells also showed increased mutation frequencies compared to the early log phase population. Persisters $n = 6$, young $n = 5$, control $n = 3$. B. $\Delta mutS$ *P. aeruginosa* PA01. Persisters isolated with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin, and early log phase populations showed no difference in mutation frequencies compared to the $\Delta mutS$ control population; persister cells showed increased mutation frequencies compared to the early log phase populations. Persisters $n = 3$, young $n = 6$, control $n = 3$. **Ethanol-isolated persister sub-populations show increased mutation frequencies compared to non-persister populations in only the wild-type strain.** C. Wild-type *P. aeruginosa* PA01. Persisters isolated with 12.5% (v/v) ethanol, and early log phase populations showed increased mutation frequencies compared to the wild-type control population; persister cells also showed increased mutation frequencies compared

to the early log phase population. Persisters $n = 3$, young $n = 5$, control $n = 3$. D. $\Delta mutS$ *P. aeruginosa* PA01. Persisters isolated with 12.5% ethanol, and early log phase populations showed no difference in mutation frequencies compared to the $\Delta mutS$ control population; however, persister cells also showed increased mutation frequencies compared to the early log phase populations. Persisters $n = 3$, young $n = 6$, control $n = 3$. Box plots show median and inter-quartile ranges; means are shown as encircled crosses.

Gene expression of persister cells after isolation by ofloxacin or ethanol

P. aeruginosa PA01 wild-type showed different gene expression profiles of persister cells when treated with different antimicrobial agents. For further details of persister cells RNA extraction, please see appendix 3 (pages 113-115). When ofloxacin was used to isolate persister cells, very little change in gene expression occurred during the first 10 min of treatment for the three genes studied (Figure 16). At 30 min of ofloxacin treatment, increased expression is seen in genes *recA* and *mutS*, and this expression is only slightly reduced at 3 hours of treatment. The SOS response gene *dinB* showed increased expression at only 30 min time point, and to a lower level than the SOS gene *recA*. These data suggest that as expected, the SOS response pathway is triggered by ofloxacin treatment. What was unexpected however was the level of *mutS* expression, as it was present at much higher levels than the SOS response genes *recA* and *dinB*.

Table 1 Persister cells isolated by time-dependant killing with ofloxacin show hypermutation. All values are averages in mutation frequency of 3-6 replicates of different bacterial sub-populations. All mutation frequencies above 2×10^{-7} are classified as mutators (Mena *et al.*, 2008), and have been marked with an asterix.

Antimicrobial Treatment	Bacterial Population Fraction	Average Mutation Frequency	
		Wild-type	$\Delta mutS$
N/A	Total population	7.54×10^{-9}	* 1.82×10^{-5}
N/A	Early log. phase	1.05×10^{-8}	* 1.42×10^{-5}
$3 \mu\text{g}\cdot\text{ml}^{-1}$ Ofloxacin	Persisters	* 4.30×10^{-7}	* 4.16×10^{-5}
12.5% Ethanol	Persisters	4.74×10^{-8}	* 1.29×10^{-5}

In contrast, the persister cells reacted differently to the presence of ethanol. Throughout this treatment, the SOS response genes were not up-regulated at all (Figure 16). This was expected, as ethanol kills by cell membrane damage, but again, the *mutS* gene was up-regulated during the first 30 minutes of the treatment, although the levels had decreased back to control levels by 3 hours. This suggests that MutS has a previously undescribed role during antimicrobial stress. While the up-regulation of MutS during

ofloxacin treatment can be explained by its role in repairing mismatches in DNA (a result of the SOS induction), its presence in ethanol treated cells is less obvious, as the corresponding genes for the SOS response were not up-regulated. For details of housekeeping gene expression, please see appendix 4 (pages 116 -117).

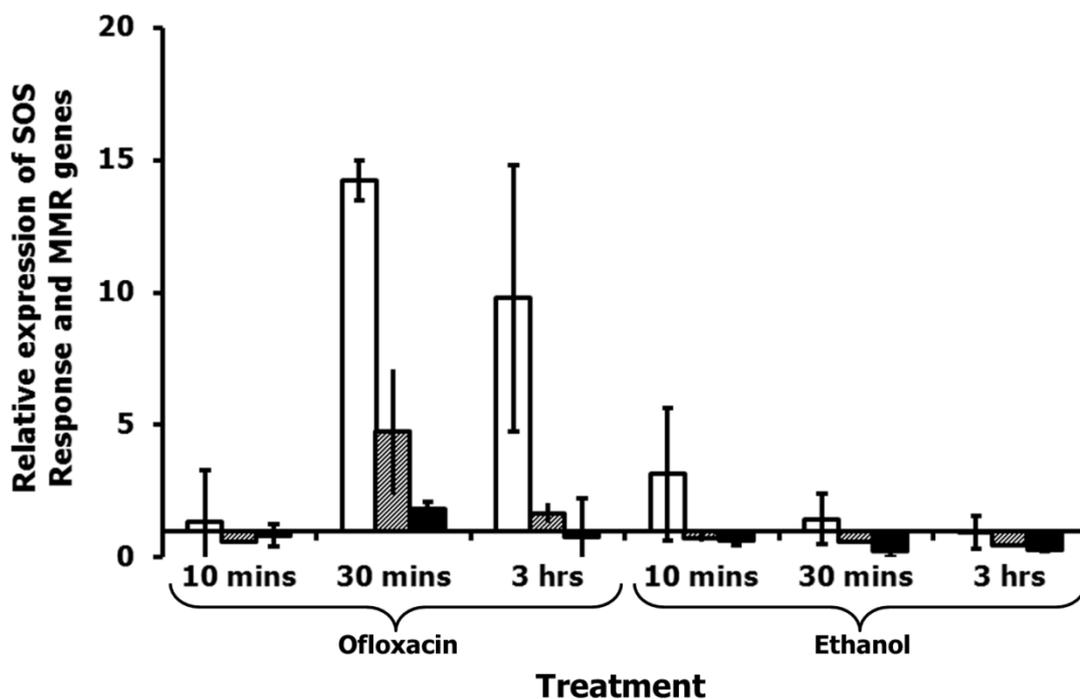


Figure 16 Relative expression of SOS and MutS genes in live cells during treatment with ofloxacin or ethanol. Live persister cell RNA was collected during treatment with $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin or 12.5% ethanol, and gene expression was determined by RT-PCR. Gene expression was observed for SOS response genes *recA* and *dinB*, and mismatch repair gene *mutS*. Relative expression in this case was calculated by normalising to the expression of the housekeeping gene (see Appendix 4). White bars = *mutS*, hatched bars = *recA*, black bars = *dinB*.

Discussion

Bacteria within biofilms exhibit many mechanisms by which they can adapt to changing environments. Two ways in which this can occur are firstly, mutation in response to selective pressures within the microcosm, and secondly, the formation of a persister subpopulation, which are able to tolerate lethal bacterial stresses. This study makes an important association between mutation and persister cells, demonstrating that the two processes may be more closely linked than previously considered.

The levels of persister cells isolated by both ofloxacin and ethanol were surprisingly low in the lab mutator strain compared *ex vivo* mutator strains (Figure 14). Previous studies looking into persister allocation in *ex vivo* hypermutable *P. aeruginosa* strains have shown that 73% have increased persister allocation, compared to non-mutator strains (Mulcahy *et al.*, 2010). This would suggest that the increased persistence seen in the *ex vivo* mutator strains is a direct result of the increased levels of antibiotic and inflammatory stresses present in the cystic fibrosis lung. Our lab strains have been passaged for years in nutrient rich environments, with very little prolonged antimicrobial stress. The persister survival mechanism is not as essential in these environments, and this may be why similar levels of persister frequency were seen in the lab strains, despite the fact that one of these strains is a mutator strain.

It has been shown in this study that persister cells have increased mutation frequencies compared to other cells in the population. Mutation in whole bacterial populations occurs at a very low frequency of approximately 1 in 10^9 cells (Table 1). Mutator strains, such as the $\Delta mutS$ strain, have much higher mutation frequencies, as a result of loss of function in one of the mismatch repair genes (Table 1). However, for both strains, a significant increase in mutation frequency was seen in persister cells isolated from the bacterial population, compared to early log phase populations (Figure 15A, B). This suggests two possibilities: (i) that the persister population has a higher level of mutation frequency than the normal cells in the population or (ii) that the method of persister isolation has the effect of increasing levels of mutation frequencies in bacteria.

It is clear from this study that the means by which persisters are isolated is of vital bearing to the downstream experimental procedure. Persisters isolated with ofloxacin, a

DNA damaging antibiotic, had increased mutation frequencies compared to persisters isolated with non-DNA damaging ethanol (Figure 15, Table 1). While ofloxacin is an excellent lethal antibiotic for the resistant bacteria *P. aeruginosa*, and is used for routine persister cell isolation *in vitro* (De Groote *et al.*, 2009; Dorr *et al.*, 2009; Keren *et al.*, 2004a), it does have an effect on persister cells, due to its mode of action. Like all fluoroquinolones, ofloxacin causes cell death by forming double stranded breaks in DNA. Consequentially, cells that survive in the presence of ofloxacin are more likely to have damaged DNA, leading to up-regulation of the SOS response. SOS-mediated DNA repair is carried out by DNA polymerases with no proof-reading capabilities, resulting in point mutations. These data lead to the conclusion that isolating persister sub-populations using ofloxacin has an effect on the cells' mutation frequencies.

The use of fluoroquinolones in persister cell isolation studies, and in calculating persister allocation in populations, must be re-evaluated due to the effect that these antibiotics have on up-regulating SOS response in persister cells. Previous studies examining gene knockouts that effected persister levels in *P. aeruginosa* populations measured persister cell levels by killing with ofloxacin, and not surprisingly found that knockouts of a putative DNA-helicase resulted in 16 times lower persister fraction than the wild-type (De Groote *et al.*, 2009). The data shown in this study confirm findings that persister cells up-regulate SOS response genes in reaction to the fluoroquinolones (Dorr *et al.*, 2009). Therefore, if investigation is to be carried out on effects downstream of persister isolation, it is necessary to consider the effects of the antibiotic on the study, in the planning stages of the experiment.

Fluoroquinolones are not the only drugs that result in SOS up-regulation, however. A study by Kohanski *et al.* (2007) showed that three major bactericidal drug groups, the fluoroquinolones, β -lactams and aminoglycosides all trigger a common cell pathway leading to hydroxyl radical formation and cell death. The study also showed that knocking out a key SOS response protein, RecA, resulted in increased death in all three bactericidal groups, although it was more pronounced in the fluoroquinolones. This effect was not seen with bacteriostatic drugs, or with rifampicin. This suggests that a connection may be present between persister cells and mutation, if only that of one triggered by antibiotic attack. It may be therefore, that increased mutation is a direct response in part to antibiotic treatment, due to the common mode of death seen by many bactericidal antibiotics.

Notwithstanding the effect of antibiotic on mutation frequencies in persister cell subpopulations, there is an underlying correlation between persister cells and mutation frequency. While fluoroquinolones such as ofloxacin result in DNA damage both by their mechanism of killing, and by the production of hydroxyl radicals, ethanol is known to be a hydroxyl scavenger, and acts as a biocide by damaging bacterial cell membranes. Persister cells isolated by ethanol treatment were also seen to have significantly increased mutation frequencies in the wild-type strain (Figure 15C). Although ethanol is not thought to be DNA damaging, and is used in procedures to minimise the effects of hydroxyl radicals on the cell, there is a possibility that the presence of hydroxyl radicals could oxidise ethanol into hydroxyethyl radicals, and subsequently into acetaldehyde and the superoxide radical, which may result in the increased mutation levels seen in this study. However, during the gene expression study, SOS response was not induced during ethanol treatment, which suggests that these radicals were not present during this experiment (Figure 16). The ability of persisters to show increased mutation frequencies when isolated by a cell membrane disrupting biocide is therefore significant. It suggests that at least some of the persister forming pathways in the cell may also have connections with cell pathways that result in mutation, either directly, or indirectly.

When persisters are isolated by means of different antimicrobials, different genes are up-regulated, suggesting differing pathways to persister formation. In the wild-type strain studied, the SOS response genes were up-regulated during ofloxacin treatment, but not during ethanol treatment (Figure 16). This confirms previous studies that fluoroquinolones induce the SOS response pathway. However, increased mutation frequencies were observed in both ofloxacin and ethanol isolated persister cells (Figure 15A, C), which suggests that the increase in mutation frequency is not solely due to the induction of the SOS response.

The *mutS* gene was highly up-regulated during ofloxacin treatment and up-regulated to a lesser extent during ethanol treatment in the wild-type strain (Figure 16). As the function of MutS is mismatch repair, an up-regulation in gene expression would suggest a corresponding decrease in the appearance of mutations, but mutation frequencies were still increased, and this increase was in proportion with the expression of *mutS* (Figure 15A, C). It was interesting to note that persister cells isolated from the Δ *mutS* strain

with ethanol did not show an increased mutation frequency compared to the total population (Figure 15D), as was seen by the wild-type strain (Figure 15C). Also, the mutation frequency of the *ΔmutS* persister cells isolated with ofloxacin, while significantly elevated, was not increased to the extent of the ofloxacin-isolated persister cells in the wild-type strain (Figure 15, Table 1). This potentially suggests a twofold role of the MutS protein: (i) to reduce mutations during DNA replication (as seen by the increase in mutations overall in the knockout strain), but (ii) upon antimicrobial stress, to have some role in increasing mutations in the surviving persister fraction. How this happens, and whether this effect is purely coincidental, or due to another cell process entirely, must be determined by further study in this area.

Ecologically, there are several selective advantages to persistence being linked with the increased mutation frequency phenotype. Two ways in which selection can favour high mutation rate are as follows: Firstly, if crowding favours selection on ever slower senescence (Seymour and Doncaster 2007) (chapter 2 of this thesis), then a lineage that can adopt a mechanism for increasing the mutation rate may out-compete others if the mutations directly result in more persisters. The model proposed by Seymour and Doncaster (2007) describes how selection on lifetime reproductive success in crowded populations favours an indefinite trading of replication rate for lifespan, with longer and slower life histories gaining over shorter-faster ones. The slower turnover inevitably reduces the rate of evolution as well, unless the mutation rate has a compensating increase. In biofilms it has been seen that mutation in microcolonies is approximately 100-fold higher than planktonic cells (Conibear *et al.*, 2009). Furthermore, this increased mutation arises during long-term chronic infections, as many isolates from clinical biofilm infections have been documented to contain hypermutable strains (Ciofu *et al.*, 2005; Henrichfreise *et al.*, 2007; Macia *et al.*, 2005; Oliver *et al.*, 2000).

More generally, selection can favour high mutation rate wherever crowded conditions favour competitive release. Competitive release is the experience of less strong density impacts from individuals of other genotypes than the majority of the population, giving a Lotka-Volterra competition coefficient < 1 . Competitive release arises out of being different, and by definition, mutants are different. Therefore, even a mutation that is deleterious in a freely growing population can do relatively well in crowded conditions where cell turnover is low. The mutant can afford to be deleterious in terms of having reduced intrinsic net rate of reproduction (the fitness criterion for free-growing

populations), i.e. a higher persister fraction in its lineage, provided it has increased lifetime output (the fitness criterion under density-dependent fecundity) allowing it to spread in crowded conditions. This type of lineage is called a slow-dominant lineage, as it counteracts having a slow intrinsic rate of reproduction by having competitive superiority over other lineages. Therefore, evolutionary theory suggests an adaptive advantage to mechanisms that link persister cells with high mutation rate. Ecologically then, as well as mechanistically, there is a role for the connection between persister cells and increased mutation.

In conclusion, I was able to show in this study that persister cells have increased mutation frequencies compared to whole cell populations, and that this was seen with both DNA damaging and non-damaging antimicrobial agents. Contrary to current opinion, the genes most up-regulated during ofloxacin attack are not the SOS response genes, but *mutS*, which was also up-regulated during both ofloxacin and ethanol treatment. It may be that there is a connection between mutation and persistence. Future work is needed to tease out the interconnections between these two bacterial processes.

CHAPTER 4

ROLE OF PERSISTERS IN MICROCOLONY INITIATION AND SEEDING OF NEW BIOFILM GROWTH FOLLOWING ANTIBIOTIC TREATMENT

Abstract

Bacterial biofilms cause many recurrent problems of contamination or infection because of their ability to tolerate exposure to antimicrobial compounds. Persisters, due to their ability to survive an antimicrobial challenge, are often implicated in the re-growth and re-establishment of bacterial populations; however this has not been studied in the context of the surface associated microbial biofilms and the role of persisters in determining the spatial and temporal pattern of biofilm formation following antibiotic treatment has not been studied. A key feature of biofilms thought to play a role in antimicrobial tolerance is their ability to develop discrete, differentiated microcolony structures during colonization of a surface. These foci within biofilms are highly recalcitrant towards antimicrobials yet little is known of the factors that determine their differentiation and growth. This chapter aimed to investigate whether persisters play a role in the initiation of microcolony foci and in mediating regrowth of biofilms. In this work, biofilm initiation was studied under a variety of conditions including with or without exposure to lethal or sub-lethal antibiotic challenge, and using inocula comprising persister cell or non-persister populations, or a 50:50 mix of the two populations. As expected, following antibiotic treatment with ofloxacin, biofilms that were inoculated with persister cell populations generated significantly more biomass than those formed from non-persister populations. However this effect was only apparent for the first 6 days of biofilm development after which time similar levels of biomass were obtained in both experiments. To investigate the role of persisters in microcolony formation, dual labelling experiments were also carried out, where mixed persister and non-persister populations were tagged with either red or green fluorescent proteins. These experiments demonstrated that persister-mediated regrowth predominantly occurs as discrete microcolony foci that do not mix with the non-persister cell population, suggesting that persisters are the progenitors of clonal microcolony foci within biofilms that are structurally distinct from the rest of the population of cells within the biofilm.

Introduction

Bacterial biofilms are extremely recalcitrant to antibiotic stress. They are typically over 1000-fold more tolerant of extrinsic mortality factors than their counterpart planktonic cells, and exhibit a generalised tolerance to almost all known antimicrobial compounds (Brooun *et al.*, 2000; Gilbert *et al.*, 1997; Gilbert and McBain 2001; Hoyle *et al.*, 1992; Suci *et al.*, 1994). As a result of this tolerance biofilms are linked to chronic infections such as that seen in the CF lung, infections of prosthetic implants, and chronic wound infections.

While most acute infections can be overcome by a combination of appropriate antimicrobial therapy and the host immune system, once a biofilm has been established the infection becomes much more recalcitrant (Costerton *et al.*, 1999). Antibiotic treatment may suppress bacterial populations in these conditions, but removal of the antibiotic leads to re-seeding of the biofilm, and re-growth of the bacterial population, resulting in exacerbation of the infection.

Persister cells are important contributors to bacterial tolerance both in biofilms and to a lesser extent in planktonic populations (Keren *et al.*, 2004a; Mulcahy *et al.*, 2010). Recent studies have shown that persister levels increase in populations during chronic infections, which may explain much about the recalcitrant nature of biofilm infections (Lafleur *et al.*, 2010; Mulcahy *et al.*, 2010). No published studies have explicitly recorded the role of persisters in the re-seeding of bacterial biofilm infections. It is generally agreed, nevertheless, that bacteria surviving antimicrobial attack re-establish the bacterial biofilm, and cause resurgence in the disease state (Lewis 2005).

Microcolony structures are differentiated, discrete structures that form from single foci in the bacterial biofilm. They are very important in the context of antimicrobial tolerance, because it is inside these structures that the tolerant cells reside. Bacterial microcolonies differentiate from the mono-layer at specific points, where the first cells are thought to initiate the clonal lineage that forms the microcolony, rather than it forming from an aggregation of cells to these focal points (Conibear *et al.*, 2009). However, the mechanism of differentiation by which only small subpopulations of cells within the biofilm proliferate as microcolonies is as yet unexplored. A role is suspected

for persisters, not only in biofilm tolerance from antimicrobials, but also in re-seeding biofilms after antibiotic treatment, and in particular contributing to the differentiation process that creates new microcolony structures. By seeding flow cells with either early log phase or antibiotic-isolated persister cells, the resultant biofilms were examined for total biomass and biofilm thickness, to discover which type of cells best initiated biofilm development. In addition to this, the ability of persister cells and non-persister cells to initiate growth into microcolony structures was assessed.

Experimental Procedures

Bacterial strains, media and growth conditions

Pseudomonas aeruginosa PA01 (Stover *et al.*, 2000) was used in all experiments. Unless otherwise stated, all planktonic cultures of bacteria were grown either in Luria-Bertani (LB) broth, or LB agar. Liquid bacterial culture was carried out at 22°C with 150 rpm orbital shaking, and plates were incubated at 37°C unless otherwise stated. Fluorescent plasmids pMF230 (Nivens *et al.*, 2001) and TdTomato (Shaner *et al.*, 2004) were used to tag *P. aeruginosa* PA01 with fluorescent green and red tags respectively. Plasmids were maintained in biofilm culture by the addition of 40 µg·ml⁻¹ carbenicillin.

Preparation of flow cell inoculum

P. aeruginosa PA01 with green fluorescent plasmid pMF230 was recovered from frozen samples by overnight growth in LB. This culture was used to inoculate 100 ml LB at a dilution factor of 10⁻³, and cells were grown to 0.6 OD. Persister cells were isolated by incubating with 3 µg·ml⁻¹ ofloxacin for 3 h. This resulted in the 'persister' inoculum, containing approximately 10⁶ live cells. Simultaneously, a fresh culture of green fluorescent *P. aeruginosa* was inoculated into 100 ml LB at a dilution factor of 10⁻³, and grown to an OD of 0.07. This culture also contained 10⁶ live cells, in early log phase. The third inoculum used was a 50:50 mix of the above two samples.

Biofilm growth in the absence of antibiotics

Biofilms were grown in rectangular three-channel flow cells with individual channel dimensions of 1×4×40 mm and flow rate of 270 µl min⁻¹. Biofilm cultures were maintained on M9 medium containing 0.1 mM CaCl₂, 1 mM MgSO₄, 6.78 g·l⁻¹ Na₂HPO₄, 3 g·l⁻¹ KH₂PO₄, 1 g·l⁻¹ NH₄Cl, and 0.5 g·l⁻¹ NaCl, with 1 g·l⁻¹ glucose as the sole carbon source. The technique used for culturing biofilms in flow cells is as previously described (Moller *et al.*, 1998; Webb *et al.*, 2003). After inoculation of flow cell channels, bacteria were allowed to attach to the surfaces of the flow cell reactor for 30 min before flow was started. For each flow cell experiment, 3 separate flow cells were used, with each inoculum growing in 3 replicate channels, spread across the 3 flow cells, to allow for variation between flow cells. Biofilms were imaged using confocal laser scanning microscopy on days 4 and 6 of growth.

Biofilm growth in the presence of antibiotics

Antibiotic was introduced to two flow cell experiments, in two different ways. The first was a single lethal antibiotic treatment immediately after attachment. A final concentration of $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin was added to flow cell medium, and flow of medium with antibiotic challenge continued for 3 h. After this, fresh medium was substituted, and biofilm growth was continued for 6 days with no more antibiotic challenge. In all other points, the experiment was identical to the growth without antibiotics.

The second antibiotic challenge was in the form of a constant sub-lethal presence of the lethal antibiotic ofloxacin. Medium was prepared with a final concentration of $1.5 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin, and this was used throughout the whole experiment, lasting 6 days. All other aspects of this experiment were kept as the control flow cells.

Analysis of biofilm total biomass using COMSTAT

Confocal laser scanning images of 6-day old continuous culture biofilms were obtained using a Leica (TCS SP2 MP FCS) upright microscope and x 20 objective. Images had 512×512 -pixel resolution and used identical gain, offset and pinhole settings for each data collection point. A single argon laser line was used with excitation wavelength of 488 nm and using an emission filter with a bandpass of 500–520 nm in the case of single colour microscopy, and an additional (Melles Griot) laser with excitation wavelength of 561 nm and using an emission filter with a bandpass of 620–730 nm was used for the two colour biofilm images. Three-dimensional rendering and analysis was performed using COMSTAT software (Heydorn *et al.*, 2000) on the Matlab platform. COMSTAT analysed each stack for total biomass ($\mu\text{m}^3/\mu\text{m}^2$), average thickness (μm), and number of microcolonies. Values are calculated means of data from several image stacks (an average of 5 image stacks from three different channels in three separate experiments).

Transforming *P. aeruginosa* with green and red fluorescent plasmids

While stocks of *P. aeruginosa* PA01 transformed with either the pMF230 or the TdTomato plasmid already exist in our lab, these strains had significant differences in growth rate and persister levels. In order to create isogenic fluorescent strains, plasmids were extracted from previously transformed bacteria using the Peqlab peqGOLD plasmid miniprep kit I, and transformed into a fresh culture of *P. aeruginosa* divided into two, to create two identical populations. The cell and DNA mix were suspended in

a 300 mM sucrose solution, transformed by electroporation (Choi *et al.*, 2006), and plated onto LB agar containing 40 $\mu\text{g ml}^{-1}$ carbenicillin. Colonies picked from these plates were grown in LB broth containing 40 $\mu\text{g ml}^{-1}$ carbenicillin and were frozen in 13% (v/v) glycerol, prior to examination for variation in growth rate and persister level.

Growth rates and persister levels of transformant strains

Each transformed colony isolate was recovered from frozen stock by growing in LB overnight at 37°C with 250 rpm shaking. Overnight cultures were diluted 10^{-1} and growth curves were measured in replicate in a 96 well plate (Sunrise remote plate reader, Tecan UK Ltd, Theale, Reading). Optical density (OD) measurements (620 nm) were taken every 10 min. Growth rates were calculated by using the following formula $\text{Growth Rate} = (\text{OD } 0.6 - \text{OD } 0.2) / (\text{Time } 0.6 - \text{Time } 0.2)$.

Transformed colony isolates were recovered from frozen samples by growth in LB overnight at 37°C with 250 rpm shaking. This culture was used to inoculate 200 μl aliquots at a dilution factor of 10^{-1} in a 96 well plate TDK assay. Antibiotic killing of planktonic cultures of *P. aeruginosa* was carried out with ofloxacin (3 $\mu\text{g}\cdot\text{ml}^{-1}$) as previously reported (Keren *et al.*, 2004a). Aliquots were incubated for 3 hours with antibiotic at room temperature. Control samples were incubated with the equivalent volume of PBS instead of antibiotic. Persister cells were identified as described previously by CFU count after serial dilution in LB broth, and plating on LB agar plates using a drop plate method (Reed and Reed 1948).

2-colour biofilm growth with single antibiotic challenge

The flow cell inoculum for this experiment comprised solely the 50:50 mix of persister and early log phase cells. This was made as described above, with the exception that the culture used to form the persister cells contained the green fluorescent plasmid, and the culture used to form the early log cells contained the red fluorescent plasmid. Once inoculated and allowed to attach to surfaces for 30 min, medium containing 3 $\mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin was run through the flow cells for 3 h, before substituting with antibiotic-free medium. Again, confocal imaging occurred at days 4 and 6.

Statistical analysis

After normalisation of residuals with square root transformations of total biomass ($\mu\text{m}^3/\mu\text{m}^2$), average thickness (μm), and number of microcolonies, one-way ANOVA

tested for differences between groups; orthogonal contrasts further tested for an overall difference between two pooled channels and a control, and for a difference between the two channels. Levene's test was used to assess homogeneity of variances. The threshold of acceptable Type-I error was taken as $\alpha = 0.05$ throughout. For the 2-colour flow cell experiment only, two-way ANOVA tested for differences between the colour of the transformant, as well between the persister and early log phase cells.

Results

Biofilm growth with no antibiotic stress

In order to determine the effect of persister cells on biofilm regrowth and microcolony development, flow cell channels were inoculated with 10^6 cells of persisters, non-persister early log phase cells, or a 50:50 mix of persisters and non-persisters. Persister cells were seen to have no advantage to early log phase cells during biofilm growth under conditions without antibiotics. After 4 days of antibiotic-free growth, orthogonal contrasts for one-way ANOVA showed that there was no difference between persister and non-persister biofilm growth (Figure 17, Figure 19, Table 2 and Table 3). After 6 days of biofilm growth under antibiotic free conditions, the early log phase inoculated channels showed increased total biomass, and average thickness of the biofilm (Figure 18, Figure 20, Table 2 and Table 3), indicating that the non-persister inoculum have a growth advantage within biofilms in the absence of antibiotic stress.

Biofilm growth with single 3-hour treatment of ofloxacin

Experiments were carried out to determine the effect of antibiotic treatment on biofilm growth of attached persister and non-persister populations. Upon the addition of a single lethal treatment of ofloxacin to the flow cells immediately following attachment of the inocula, the persister inoculated channels showed increased total biomass and average biofilm thickness compared to the early log phase channels by day 4 of biofilm growth, indicating that persister cells were better able to survive lethal attack and to re-seed biofilm growth (Figure 17, Figure 19, Table 2 and Table 3). However, by day 6 of biofilm growth, with no further antibiotic stress this effect had been lost, and both the persister and non-persister channels had similar levels of biomass and microcolony thickness (Figure 18, Figure 20, Table 2 and Table 3).

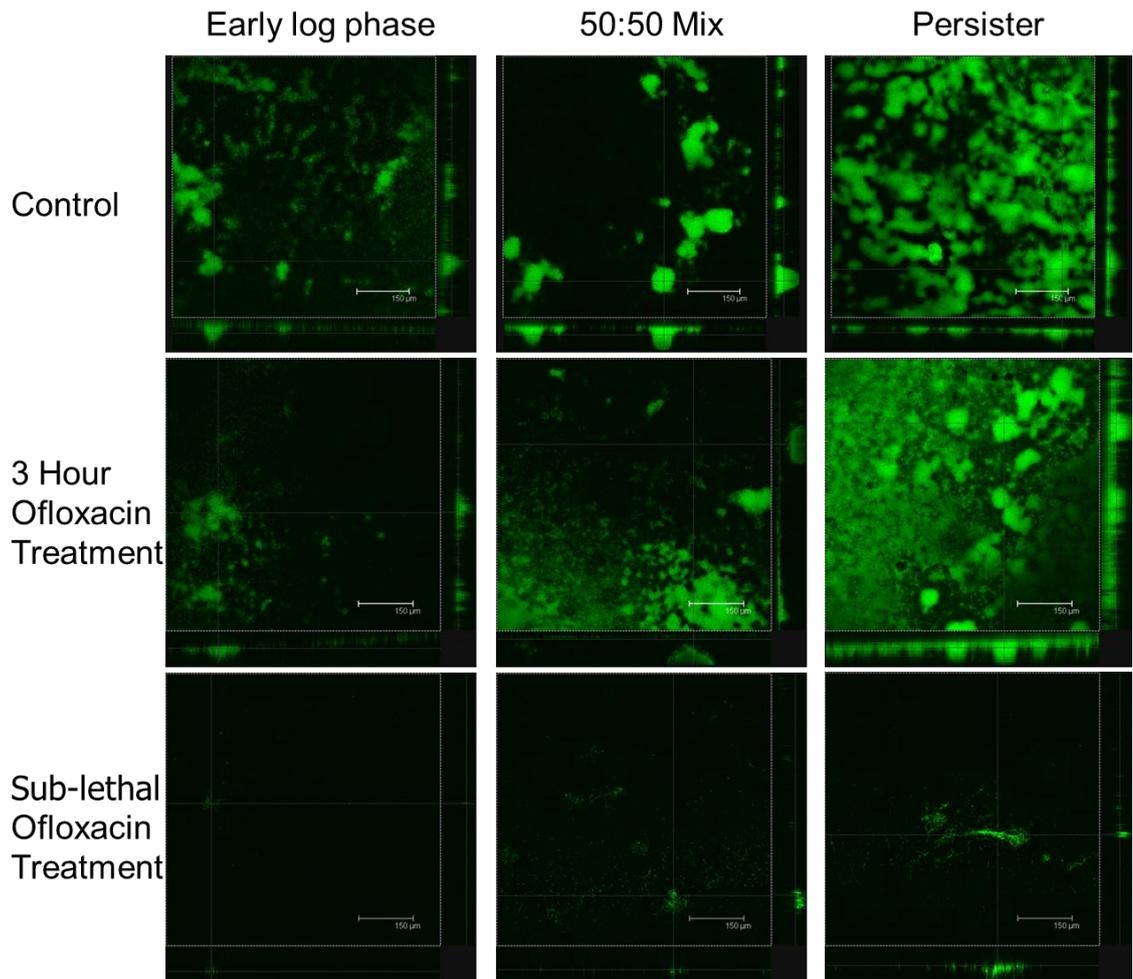


Figure 17 Confocal imaging from day 4 of biofilm growth. Four day- old biofilms treated with no antibiotic (top row); a single 3 h treatment of $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (middle row); or a continual sub-lethal concentration of $1.5 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (bottom row) were examined using confocal microscopy. Flow cell channels contained either persister derived biofilm (right column), biofilm from early log phase cells (middle column), or an equal mixture of the two inocula (left column). Images taken using 200x magnification.

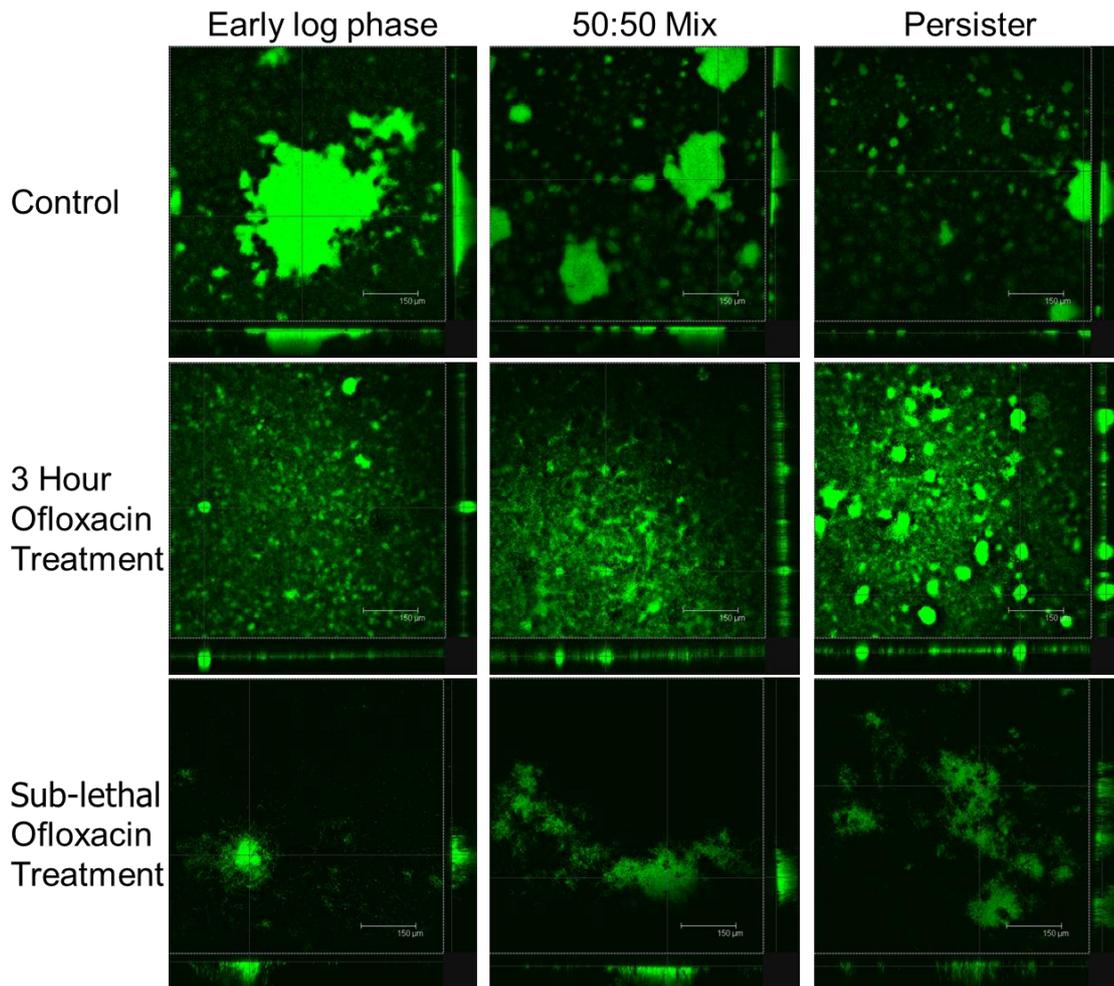


Figure 18 Confocal imaging from day 6 of biofilm growth. Six day- old biofilms treated with no antibiotic (top row); a single 3 h treatment of $3 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (middle row); or a continual sub-lethal concentration of $1.5 \mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin (bottom row) were examined using confocal microscopy. Flow cell channels contained either persister derived biofilm (right column), biofilm from early log phase cells (middle column), or an equal mixture of the two inocula (left column). Images taken using 200x magnification.

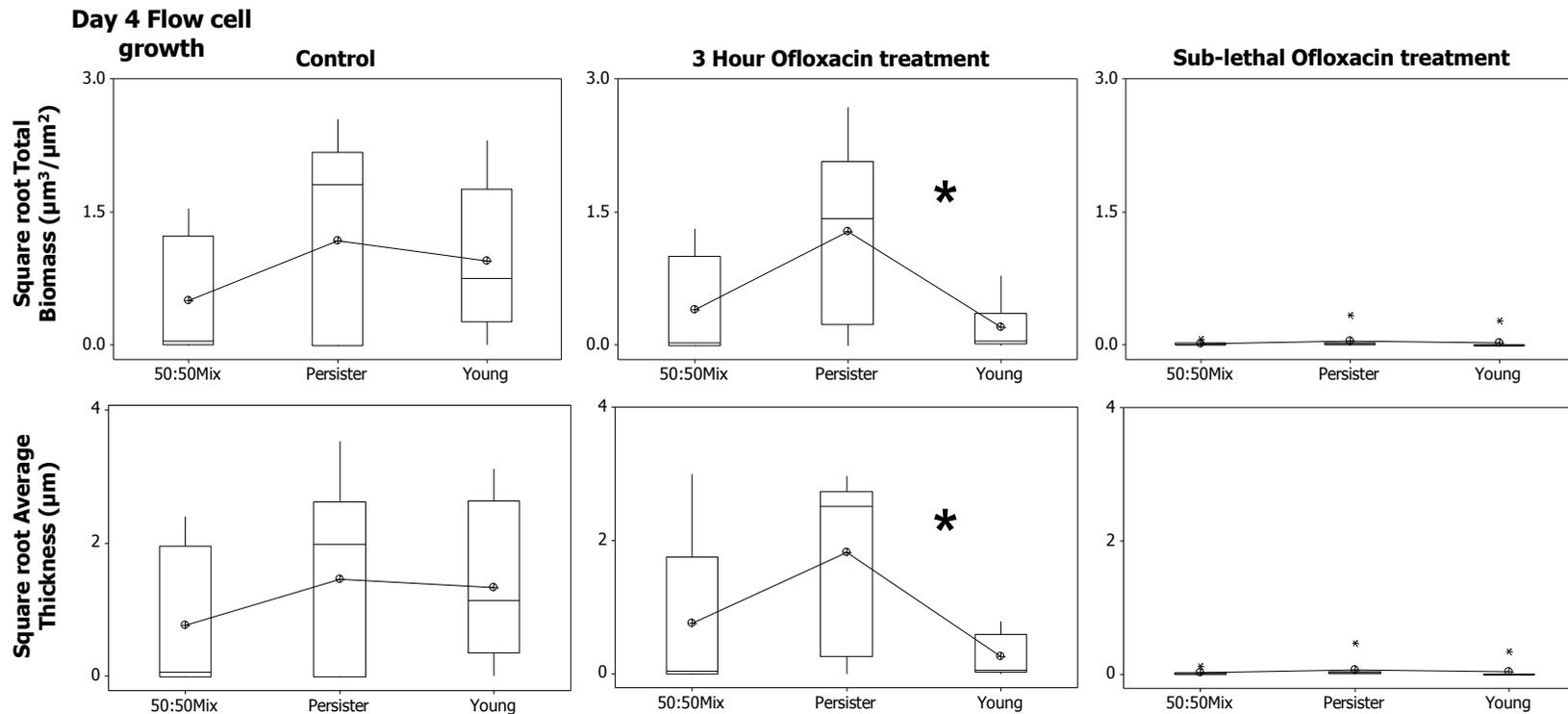


Figure 19 By day 4 of biofilm growth, persister-derived biofilm showed increased development compared to early log phase-derived biofilm during the 3 h ofloxacin treatment. No difference was detected between the mixed channel and the combined persister and early log phase channels. In all 3 hour ofloxacin treatment biofilms, increased growth was seen in the persister channels for both total biomass and average biofilm thickness, while both the control biofilms and the sub-lethal ofloxacin treatment biofilms showed no difference between persister and early log phase channels. Significant differences are marked with a large asterisk. All P and F values are shown in Table 2 and Table 3. Box plots show median and inter-quartile ranges; means are shown as an encircled cross. Outliers are shown as small asterisks.

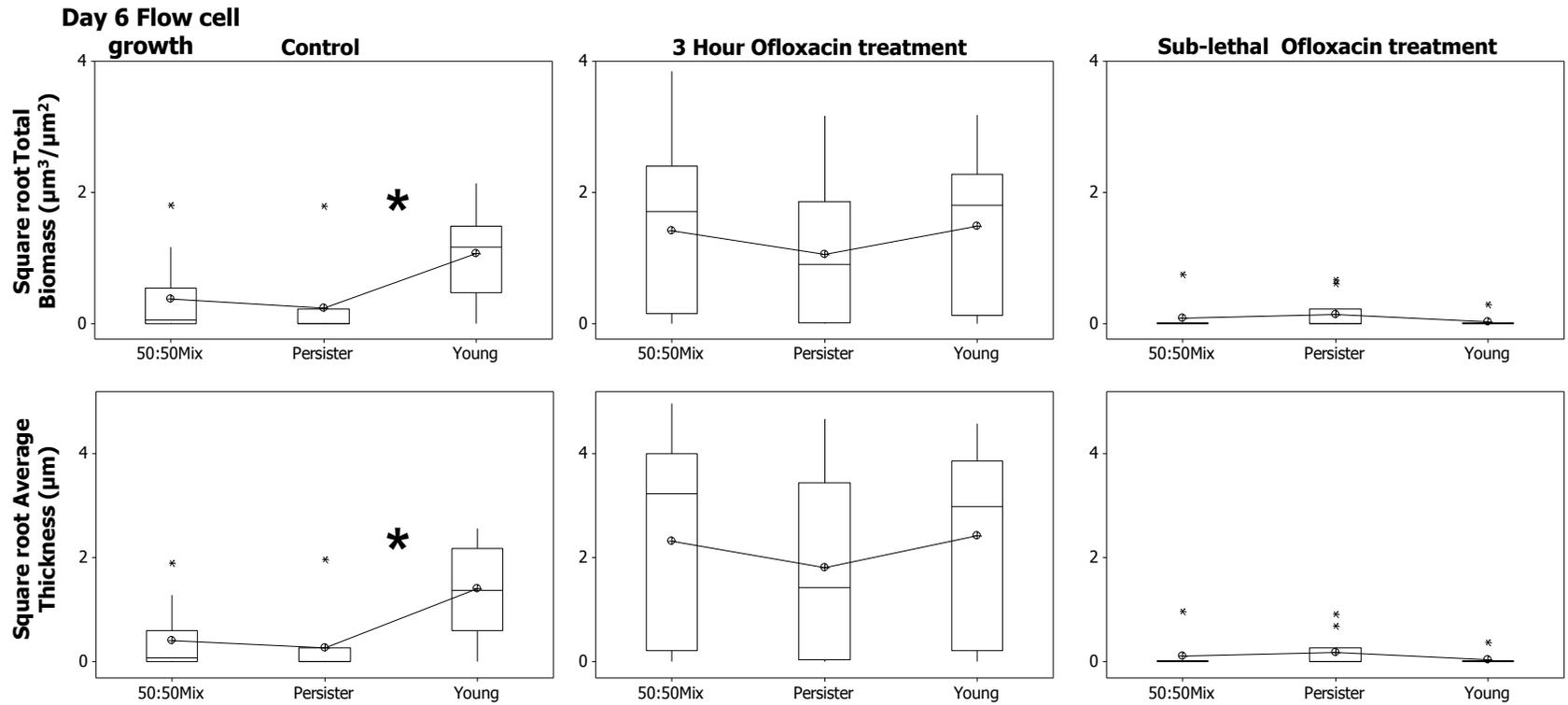


Figure 20 By day 6 of biofilm growth, early log phase-derived biofilm showed increased development compared to persister-derived biofilm during the control treatment. No difference was detected between the mixed channel and the combined persister and early log phase channels. In all control treatment biofilms, significant growth was seen in the non-persister channels for both total biomass and average thickness of biofilms, while both the 3 h ofloxacin biofilms and the sub-lethal ofloxacin treatment biofilms showed no difference between persister and early log phase channels. Significant differences are marked with a large asterisk. All P and F values are shown in Table 2 and Table 3. Box plots show median and inter-quartile ranges; means are shown as an encircled cross. Outliers are shown as small asterisks.

Sub-lethal ofloxacin treatment during biofilm growth

After treatment with $\frac{1}{2}$ MIC ofloxacin (1.5 μg / ml) throughout the time course, confocal microscopy was used once again to image stacks of the biomass produced. The levels of biomass were greatly reduced throughout, and significant elongation of cells was seen (Figure 17, Figure 18). Orthogonal contrasts for one-way ANOVA were carried out for both day 4 and day 6 of this study, but levels of bacterial growth equally low in all channels so no significant differences were able to be determined (Figure 20, Figure 19, Table 2, and Table 3).

Table 2 One-way ANOVAs on days 4 and 6 comparing total biomass of the 50:50 mix control channel with the two treatment channels (persisters or non-persisters). Here, orthogonal contrasts of the pooled groups of early log phase and persisters are shown versus the 50:50 mix, and the early log phase versus the persisters. Significant effects ($P < 0.05$) in bold.

Orthogonal contrasts SQRT Total Biomass	Control		3 Hour Ofloxacin Treatment		Sub-lethal Ofloxacin Treatment	
	$F_{(1, 23)}$	P	$F_{(1, 24)}$	P	$F_{(1, 24)}$	P
DAY 4						
50:50mix compared with [early log phase and Persisters]	2.36	0.138	1.67	0.209	0.56	0.463
Early log phase compared with Persisters	0.28	0.603	12.04	0.002	0.23	0.636
DAY 6	$F_{(1, 27)}$	P	$F_{(1, 24)}$	P	$F_{(1, 25)}$	P
50:50mix compared with [early log phase and Persisters]	1.46	0.238	0.09	0.768	0.00	0.985
Early log phase compared with Persisters	9.22	0.005	0.57	0.457	1.12	0.300

Table 3 One-way ANOVAs on days 4 and 6 comparing average thickness of the 50:50 mix control channel with the two treatment channels (persisters or non-persisters). Here, orthogonal contrasts of the pooled groups of early log phase and persisters are shown versus the 50:50 mix, and the early log phase versus the persisters. Significant effects ($P < 0.05$) in bold.

Orthogonal contrasts SQRT Average Thickness	Control		3 Hour Ofloxacin Treatment		Sub-lethal Ofloxacin Treatment	
	$F_{(1, 23)}$	P	$F_{(1, 24)}$	P	$F_{(1, 24)}$	P
DAY 4						
50:50mix compared with [early log phase and Persisters]	1.55	0.226	0.46	0.503	0.46	0.505
Early log phase compared with Persisters	0.04	0.844	10.85	0.003	0.30	0.589
DAY 6	$F_{(1, 27)}$	P	$F_{(1, 24)}$	P	$F_{(1, 25)}$	P
50:50mix compared with [early log phase and Persisters]	2.36	0.136	0.06	0.808	0.00	0.997
Early log phase compared with Persisters	12.01	0.002	0.46	0.502	1.10	0.303

Fluorescent protein colour tagging of *P. aeruginosa*

Growth rates and persister formation were compared for *P. aeruginosa* transformants expressing either GFP or RFP (Figure 21). Two transformants were selected that were identical that had the closest phenotypes. Once this had been done, effects of biofilm growth and formation are expected to be due to the treatment of the cells pre-inoculation, rather to differences in strain growth rates. The red and green transformants were used as both persisters and early log phase cells, in order to further control for any differences being due to the fluorescent tagging.

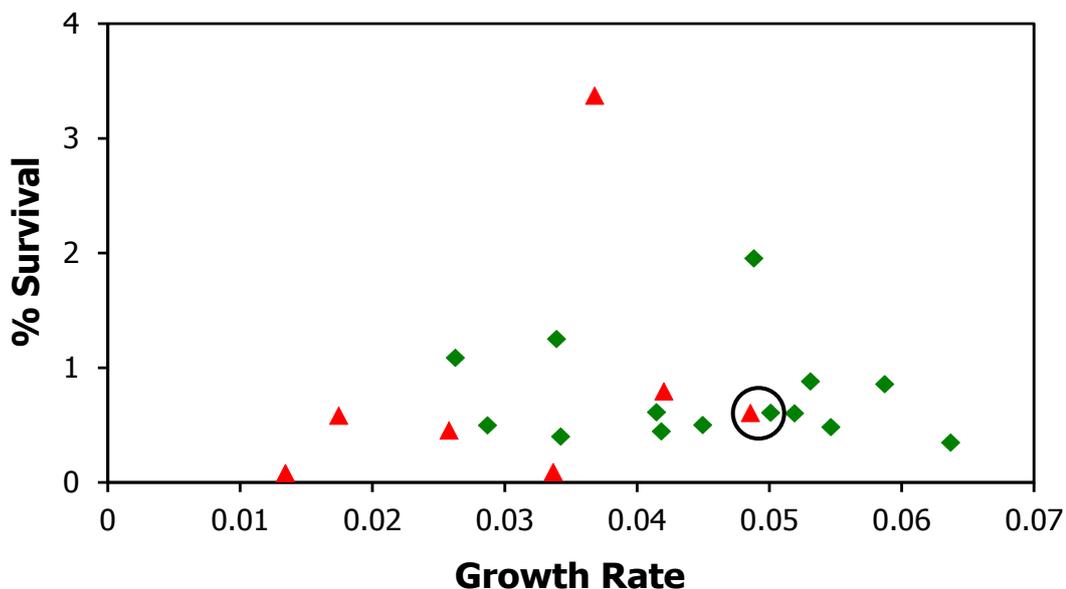


Figure 21 Comparison of growth rate and survival to ofloxacin with different isolates from transformation of *P. aeruginosa*. *P. aeruginosa* PA01 wild-type isolates were examined for similarities of growth rate and persister allocation, to allow equality in flow cell growth. Two transformants (circle), one expressing GFP and one expressing the tomato fluorescent protein, were selected for further experimentation because they exhibited both the same growth rate and percentage of persisters.

It was decided to study in more detail the interactions between *P. aeruginosa* cells that originated as persisters or early log phase cells under single, lethal antibiotic stress, and to determine the effect on microcolony differentiation. By day 4, biofilm growth was considerably reduced compared to the same experiment using only one fluorescent tag (Figure 23A). In fact, the level of bacterial growth at day 4 was too low to carry out statistical analysis (data not shown). By day 6, the level of biofilm growth from the persister derived cells had exceeded that of the growth from the early log phase derived cells (Figure 23B). Microcolonies were very distinctly formed by one fluorescently tagged strain, indicating that microcolony growth is clonal, that is, from one original cell. This experiment was repeated with the fluorescent tags used in the opposite configuration - red cells for persister derived biofilm, and green cells for non-persister-derived biofilm (data not shown).

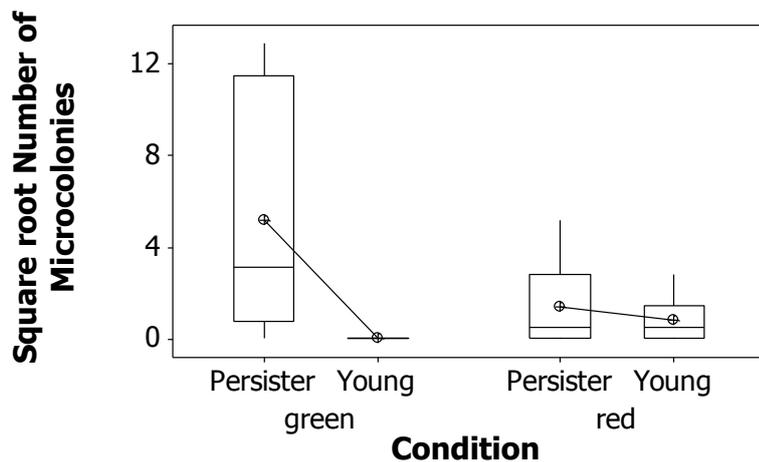


Figure 22 Persister-derived cells form more microcolonies than non-persister derived cells. Two-way ANOVA showed that persister-derived biofilms had increased numbers of microcolonies compared to non-persister early log phase-derived biofilms. The coloured tag had no significant effect on the biofilms, and no interaction between the two variables was seen. Box plots show median and inter-quartile ranges; means are shown as an encircled cross.

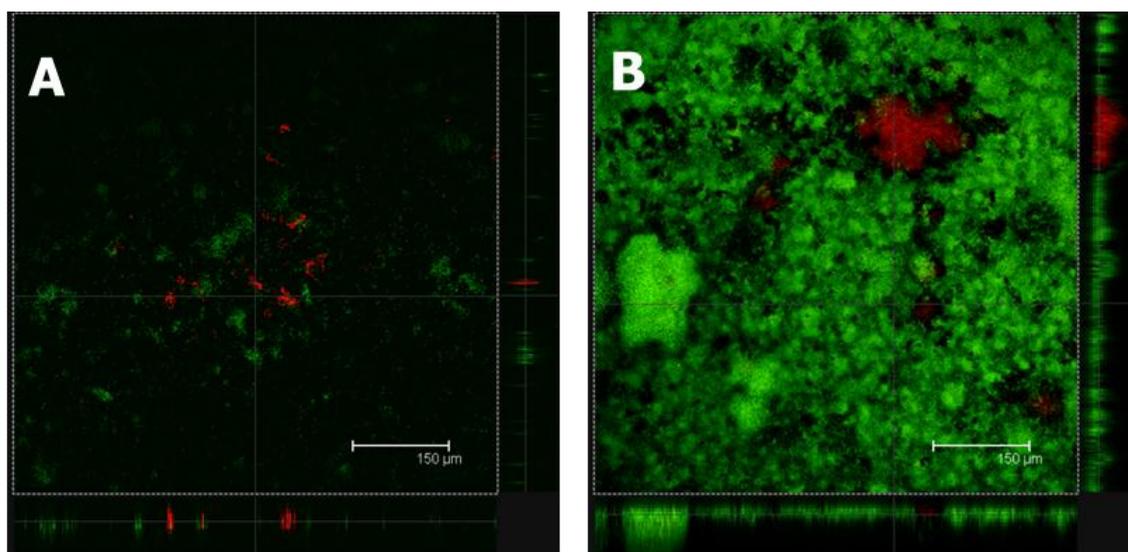


Figure 23 Microcolonies are formed by unmixed clonal populations of cells. Biofilms were treated with one single lethal 3 h treatment of $3 \mu\text{g}\cdot\text{mL}^{-1}$ ofloxacin. (A) Growth after 4 days. (B) Growth after 6 days. Flow cell channels contained either persister derived biofilm (green cells), or early log phase-derived biofilm (red cells). Images taken using 200x magnification.

Statistical analysis using 2-way ANOVA showed that the persister derived biofilm had increased numbers of microcolonies compared to the early log phase derived biofilm ($F_{1, 20} = 5.59$, $P = 0.028$) (Figure 22). The analysis also showed that the fluorescent tag had no effect on the growth of the bacteria ($F_{1, 20} = 1.54$, $P = 0.229$), with no interaction between the colour of the bacteria and the bacterial condition ($F_{1, 20} = 3.62$, $P = 0.072$).

Discussion

Persister cells play an important role in the survival of bacterial populations and are commonly the only bacteria to survive following a lethal antimicrobial stress (Balaban *et al.*, 2004; Keren *et al.*, 2004a; Kim *et al.*, 2011; Shah *et al.*, 2006; Sufya *et al.*, 2003). As such, surviving persister cell subpopulations are considered to be the cause of bacterial biofilm recurrence following antibiotic treatment but this process has not been studied in any detail. This chapter therefore has examined the spatial and temporal pattern of persister-mediated regrowth of biofilms following antibiotic challenge.

Under conditions of no antibiotic stress, persister cells that had been pre-exposed to the antibiotic ofloxacin had no advantage over non-persister cells in biofilm initiation. No difference was seen between early stage (4-day) biofilms formed from persister and non-persister early log phase, and as the biofilms continued to grow the non-persister-derived biofilms grew faster, resulting in channels with increased total biomass and biofilm thickness compared to the persister-derived biofilms. As the non-persister inoculum comprised an early log phase inoculum consisting of healthy cells entering exponential growth, these cells might be expected to colonise surfaces and initiate biofilms faster than the persister cells, which had been isolated by exposure to DNA damaging antibiotic stress. In addition, before biofilm growth can occur in the persister cell channel, cells must first revert to normal replication, which they do at different speeds (Balaban *et al.*, 2004). This may help to explain the decreased biofilm growth by persister-derived cells, compared to early log phase-derived biofilms.

After a single 3h period of lethal antibiotic stress, biofilms inoculated with a higher proportion of persister cells were more effective at biofilm initiation (Figure 17). Ofloxacin was delivered to the flow-cell system immediately after attachment, and after 4 days of biofilm growth, persister-derived bacteria had a significantly enhanced total biomass and average thickness of biofilm than the non-persister, early log phase-derived cells (Figure 19). These observations presumably result from a higher number of viable cells being present in persister derived biofilms following ofloxacin treatment, and therefore allowing for a faster re-colonisation. To my knowledge there are few studies that have examined the dynamics of surface re-colonisation following antibiotic treatment. Attachment of cells to a surface is known to rapidly (within hours) enhance

the tolerance of those cells to antimicrobial agents (Cochran *et al.*, 2000; Das *et al.*, 1998), and many studies have examined the now well established phenomenon of enhanced antimicrobial resistance in biofilms. However, given that biofilms in medical settings are often exposed to many cycles of therapeutic treatment, there is a dearth of studies that have investigated the regeneration of biofilm biomass following exposure to lethal factors. Clearly, persisters can facilitate the rapid re-growth of biofilms but the characteristics of these biofilms for example their generalised tolerance to antimicrobials or their physical or genetic properties should be studied in further detail.

Biofilm grown during the constant stress of sub-lethal levels of ofloxacin had no advantages for either persister- or non-persister- derived cells. There was no difference in total biomass or biofilm thickness seen between persister and non-persister channels during constant sub-lethal antibiotic exposure (Figure 19, Figure 20). In these conditions, replicating cells were subjected to a non-lethal but DNA damaging agent throughout biofilm growth (Figure 17, Figure 18). Previous studies have shown that sub-inhibitory levels of aminoglycosides can induce *P. aeruginosa* biofilm growth (Hoffman *et al.*, 2005). Another study, using *S. epidermidis* discovered that ½ MIC concentrations of ofloxacin had no effect on biofilm growth (Rupp and Hamer 1998). Ofloxacin, however, had the effect of reducing biofilm growth in this study, even at ½ MIC concentrations, resulting in a attenuated, biofilm with elongated cells, which may indicate the induction of stress responses (Figure 17, Figure 18). Persister cells exhibited little or no advantage over non-persisters in these experiments possibly because they remain in a dormant physiology due to the continued presence of the antimicrobial stress.

The hypothesis that persister cells may play an important role as progenitors of microcolony structures within biofilms, thereby contributing to processes of structural differentiation in biofilms was also investigated. This may help to explain why only a subpopulation of cells in bacterial biofilms form microcolonies. A dual-colour labelling system was used to examine the interactions between persister- and non-persister-derived cells, and to observe the effect that persister cells have on microcolony formation. Using strains with similar growth characteristics and tagged with green and red fluorescent proteins (Figure 21), it was found that microcolonies formed principally from persister cells and that microcolonies appeared as unmixed single colour structures

despite close proximity of cells with the opposite tag (Figure 23). These data may have similarities to observations by Klayman *et al.* (2008) which showed, using isogenic *P. aeruginosa* strains tagged with two colours, that single colour clonal microcolonies only arose within the biofilm. Conibear *et al.* (2009) also showed that microcolonies can originate from a single bacterial cell, and that microcolonies can have increased mutation frequencies compared to the non-microcolony biofilm. The study carried out in the previous chapter of this thesis showed that persister cells have increased mutation frequencies compared to normally replicating cells, especially following ofloxacin treatment. Taken together, these latter studies and the present work in this thesis may suggest that persister cells act as foci that trigger microcolony growth from the biofilm lawn.

In conclusion, it has been shown in this chapter that in the absence of antibiotic stress, persister cells have no advantage, but once an antibiotic stress has affected the bacterial population, persister cells become integral to the role of biofilm re-colonisation. Persister cells were also shown to play a role in microcolony differentiation, by promoting the formation of clonal structures arising within the biofilm. Due to the two-fold role of persister cells, as a means of survival from antibiotic attack, and the mechanism for biofilm initiation after antibiotic attack, these cells play an integral part in chronic infection, necessitating focused research to combat persister cell formation.

GENERAL CONCLUSIONS AND FUTURE WORK

Much remains to be learned about the phenomenon of persister cells. Molecular biologists have discovered many genes involved in persister phenotypes, such as cell-replication halting toxin-antitoxin genes in *E. coli* and *Mycobacterium tuberculosis* (Brown and Shaw 2003; Pandey and Gerdes 2005). Microarray and insertion mutation studies have uncovered a diverse set of genes that are differentially expressed in the persister state or that can impact directly on persister cell allocation (De Groote *et al.*, 2009; Shah *et al.*, 2006). Despite this research interest, no all-encompassing persister mechanism has been uncovered, indicating the complexity of the persister phenotype.

This study investigated the role of persister cells within biofilms from ecological and evolutionary perspectives in order to gain an insight into the role of persisters in bacterial populations. The model by Seymour and Doncaster (2007) was used as a basis to examine the relationship between growth rate mutation and persister levels, and to determine the effects of antibiotic pressure on the persister levels in bacterial populations (Chapter 2). A possible link was examined between mutation and persister cells in bacterial populations (Chapter 3), and the role of persister cells in seeding new biofilms was examined, with particular reference to the initiation of microcolony structures (Chapter 4). The principle conclusions from each of these chapters, as well as the opportunities for future research that have arisen, are summarised below.

Chapter 2 - Ecological and Evolutionary Influences on Persister Cell-Mediated Antimicrobial Tolerance in P. aeruginosa Biofilms

The model proposed by Seymour and Doncaster (2007) was the basis for the hypothesis that persisters arise as an adaptation to conditions of dense cellular crowding that can occur in biofilms. This model proposed that selection will favour lineages with deferred replication and increased longevity (i.e. slow growth and reduced senescence) within bacterial populations that are too crowded to sustain fast recruitment. This response to crowding stimulates selection on ever-slower growth and reduced senescence as organisms decrease their intrinsic rate of production of new biomass in favour of a longer lifespan potential (Doncaster 2003; Seymour and Doncaster 2007). As persisters are dormant cells, with resulting decreased fecundity and reduced senescence, mutations

will also be favoured that result in increased allocation of persister cells within a lineage population. The fitness advantage of increased lifespan then exacerbates crowding, further favouring the long-slow strategy with increased persister allocation.

In examining this model, this study has shown that the biofilm environment produces bacterial isolates with a wide range of growth rates compared to stationary phase cultures, with slow-growth isolates demonstrating heritability of growth over several passages *in vitro*. Slow-growth isolates had higher persister fractions compared to wild-type and fast-growth isolates. Slow-growth isolates showed decreased biomass when subsequently grown as biofilms, and were incapable of reverting to faster-growth phenotypes, but the susceptibility of the slow-growth biofilm to ofloxacin was decreased compared to the wild-type biofilm. These results support the hypothesis, indicating that slower growth may be linked to increased persistence, as predicted. This link between biofilm-derived mutation in growth rate and persister level has not been described before, and greater understanding of this phenomenon may become clear after sequencing of the slow-growth mutants have been completed.

By treating mature wild-type biofilms with frequent bactericidal and bacteriostatic antibiotic stresses, it was shown that frequent antibiotic stress on biofilm populations resulted in enhanced persister allocation among biofilm isolates, regardless of whether the antibiotic used is lethal or non-lethal in its mode of action. The results from this study did not support the Seymour and Doncaster hypothesis, suggesting that stress-induced persister response is a more complex process than predicted, and requires a model which emulates this. These observations provide an insight into the importance of ecological influences on persister formation. The environment in which the bacterial population grows has important effects on persister formation, whether by nutrient starvation on planktonic populations (Sufya *et al.*, 2003), or growth within a host as biofilms (Mulcahy *et al.*, 2010).

Further research into the role of environmental selection forces on the levels of persisters within a bacterial population is required to gain further understanding of the conditions that result in increased persister allocation. For example, the oxygen is a limited commodity in the biofilm. Does persister allocation increase as oxygen decreases? Logically, one would suppose that it does, as replication rates in *P. aeruginosa* biofilms decrease as oxygen becomes more limited. Another example of

environmental stress arises by competition with multiple species in the biofilm environment. To my knowledge, no work has been done to examine persister allocation in biofilm isolates from multi-species biofilms. Examining the environmental pressures that affect persister allocation will increase knowledge of the persister response, and will ultimately advance scientific understanding of antimicrobial tolerance, and its control in clinical, environmental and industrial settings.

Chapter 3 - Role of Mutation Frequency and SOS Response on Persistence in P. aeruginosa Populations

Bacteria within biofilms exhibit many mechanisms by which they can adapt to changing environments. Two ways in which this can occur are firstly, mutation in response to selective pressures within the microcosm, and secondly, the formation of a persister subpopulation, which are able to tolerate lethal bacterial stresses. This study makes an important association between mutation and persister cells, demonstrating that the two processes may be more closely linked than previously considered.

Examination of the frequency of rifampicin mutation allowed study of the levels of mutation in differing sub-populations of bacteria. Examination of the ofloxacin-isolated persister fraction in both the wild-type and $\Delta mutS$ strain showed a significant increase in levels of mutation compared to early log phase populations. Repeating the experiment using ethanol as a mechanism for persister isolation also resulted in increased mutation frequencies in the wild-type strain, although not in the $\Delta mutS$ strain.

By isolating persister RNA, this study was able to determine the effect that ofloxacin and ethanol have on the expression of DNA damage repair systems such as the SOS response system, and mismatch repair. While SOS response genes *recA* and *dinB* were only up-regulated in ofloxacin treated cells, the mismatch repair gene *mutS* showed up-regulation in both ofloxacin and ethanol treatments. While the up-regulation of *mutS* can be explained in the ofloxacin-treated persister cells as a means of correcting mismatched base-pairs from the SOS response system polymerases, it was unclear why *mutS* was up-regulated in non-DNA damaging ethanol treatment. Increased understanding of the relationship between the *mutS* gene and the persister phenotype

would prove beneficial, as *mutS* is already known to play an important role in the development of antibiotic resistance. Describing a tangible inter-connection between these two bacterial processes may result in new approaches to the problem of antimicrobial tolerance.

In order to more fully understand the role that ethanol plays on persister isolation, the examination of intracellular levels of hydroxyl radicals and other reactive oxygen species should be carried out, by using glutathione redox buffering with an appropriate indicator such as Ellman's reagent. By carrying this study out, it can be ascertained whether the addition of ethanol results in the release of hydroxyl radicals (potentially by uncoupling ATP production through increased membrane permeability, resulting in increased concentrations of reactive oxygen species), which would explain the up-regulation of DNA repair genes in ethanol-isolated persisters, or, if hydroxyl radicals are not present, this would suggest that *mutS* plays an as yet unknown role in persister formation.

After carrying out RNA gene expression on three DNA repair genes, it became apparent that these genes in themselves cannot explain the effects that were seen in the persister isolated cells, as potentially different pathways were used to form the persister cells in each isolation method. A more global assay such as the RNA microarray should therefore be carried out, examining the total genes up- and down- regulated in a range of persister isolating treatments, such as the two methods used in this thesis, and also after isolating naïve persister cells from a population by cell sorting. Analysis of the expression of persisters isolated by different lethal treatment will provide details as to whether the mechanism for producing the persister phenotype is the same, or, as I suspect, differs according to the stress under which the persister has been formed. Once genes have been identified as part of the 'persister regulon' from a range of isolation methods, then directed therapy can be used to block the formation of persister cells, which could potentially revolutionise antimicrobial therapies in persistent infections.

Chapter 4 - Role of Persisters in Microcolony Initiation and Seeding of New Biofilm Growth Following Antibiotic Treatment

Persister cells are known to play an important role in population survival and this study was devised to explore the role that persister cells have in biofilm initiation following antibiotic challenge. By seeding biofilms with persister and non-persister cells, environments where persister cells give an advantage over normally replicating cells in biofilm initiation were able to be determined.

Biofilm regrowth with persister or non-persister inocula was examined in a number of different environments. In conditions of no antibiotic stress, persister cells previously exposed to ofloxacin had no advantage over non-persister cells in biofilm initiation. No difference was seen between early stage (4-day) biofilms, and as the biofilms continued to grow, the early log phase non-persister derived biofilm showed increased total biomass and biofilm thickness compared to the persister-derived biofilm, indicating the speed with which cells entering exponential growth can colonise surfaces and initiate biofilms, in conditions exclusive of antibiotic stress.

Once lethal antibiotic stress was added to the flow cell environment however, the population with a higher proportion of persister cells showed most effective biofilm initiation, with persister-derived biofilm having increased total biomass and biofilm thickness at 4 days growth compared to early log phase-derived biofilms. Given that biofilms in medical settings are often exposed to many cycles of therapeutic treatment, there is a dearth of studies that have investigated the regeneration of biofilm biomass following exposure to lethal factors. Clearly, persisters can facilitate the rapid re-growth of biofilms but the characteristics of these biofilms for example their generalised tolerance to antimicrobials or their physical or genetic properties should be studied in further detail.

By using a dual-colour labelling system of isogenic *P. aeruginosa* strains, it was shown that after lethal antibiotic stress, persister-derived cells had increased microcolony formation to non-persister cells. It was also observed that persister-derived and non-persister-derived cells formed unmixed single colour microcolonies despite growing in close proximity with cells of the opposite tag. Conibear *et al.* (2009) showed that microcolonies can originate from a single bacterial cell, and that microcolonies can have increased mutation frequencies compared to the non-microcolony biofilm. The study carried out in the previous chapter of this thesis showed that persister cells have

increased mutation frequencies compared to normally replicating cells, especially following ofloxacin treatment. Taken together, these latter studies and the present work in this thesis may suggest that persister cells act as foci that trigger microcolony growth from the biofilm lawn. Due to the two- fold role of persister cells, as a means of survival from antibiotic attack, and the mechanism for biofilm initiation after antibiotic attack, these cells play an integral part in chronic infection, necessitating focused research to combat persister cell formation.

In order to understand the role persisters play in *in situ* biofilm development, a means of tagging persister cells must be developed. In an attempt to create a persister specific tag, proteomic studies need to be performed to ascertain whether the persister phenotype displays a unique protein that is down- regulated in non-persister but otherwise identical cells. If a persister tag could be developed, then the role of persisters in biofilm development could be observed. For example, is the cell that initiates microcolony clonal growth a persister cell, or has it previously been a persister cell? Where in the *P. aeruginosa* microcolony are persister cells situated? Do all cells in the biofilm or planktonic growth at some point switch to persister growth for a time (for example, as part of cell replication), or do only specific cells act as persisters (for example aged cells in the population)? By determining the physiology of the persister cell, how it forms, and why and where it forms, the role the persister plays in persistent infection can be understood to a greater depth, and specific targets for persister cells can be devised.

In summary, this thesis provides new information on the biology of persister cells in *P. aeruginosa* biofilms. It has shown how persister cell levels within bacterial biofilms increase with biofilm stress, how that the persister phenotype is linked to mutation in a manner not linked to SOS response pathways, and has shown the importance of the persister cell in re-growth of the bacterial biofilm after antibiotic treatment. These novel data contribute to current understanding of the emergence of antibiotic tolerance in bacterial biofilm populations.

APPENDICES

Appendix 1 - Characterization of *P. aeruginosa* Biofilm Colony Variants (Chapter 2)

Flow cell biofilm reactors were inoculated with *Pseudomonas aeruginosa* PAO1, and cell attachment to the glass substratum was allowed to take place. The biofilm was fed with a constant supply of M9 medium containing 1 g·l⁻¹ glucose (see methods section, chapter 1). Photographs were taken on days two, three, six, and ten of biofilm development, using bright field microscopy. The figure below (Figure 24) shows the progression of the biofilm over the time course. On day two, small clumps of bacteria were seen attached to the surface of the glass cover slip prior to biofilm development. By day three, microcolonies were observed to be forming at edges of the flow cell, where shear forces are lowest, and by day six, mature microcolonies can be seen over the whole surface of the flow cell. Day ten showed further enlargement and maturation of microcolonies. After biofilms have been established for several days, bacteria that have detached from their microcolonies were collected from the effluent tubing of the flow cell, and plated out onto LB agar. Several mutants were picked from the plates and frozen in glycerol. Mutants were all confirmed to be *P. aeruginosa* by multiplex PCR (data not shown).

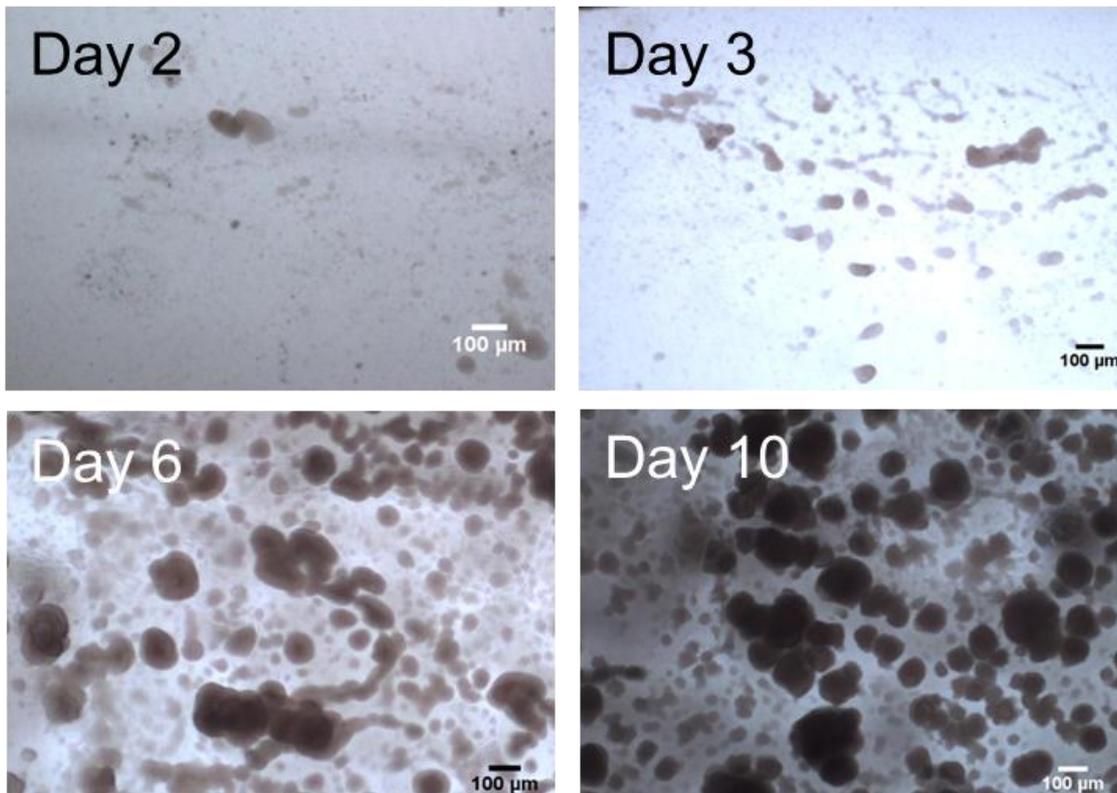


Figure 24 Progression of a Biofilm over time. Pictures taken on days 2, 3, 6 and 10, using light microscopy (40 x magnification). On day two, small clumping of bacteria can be seen; on day three, small microcolonies are beginning to form at the edge of the flow cell where shear forces are lowest; day six shows mature microcolonies covering the entire surface of the flow cell; day ten shows further enlargement and maturation of the microcolonies.

Mutant colony morphology

Upon examination, all mutants collected from the *P. aeruginosa* PA01 flow cell were classified into phenotypic groups based on colony morphology by eye, and 40x phase contrast microscopy. All mutants fell under one of two colony morphology categories: They either showed wild type morphology, with flattened, spherical colonies showing evidence of growth out from the edges of the colony boundary; or small colony variant (SCV) morphology, with smaller, spherical colonies with increased surface tension, and no evidence of growth from the edges of the colony boundary (Figure 25). While most mutants showed varying shades of green/blue, a few mutants presented with a deep red pigment.

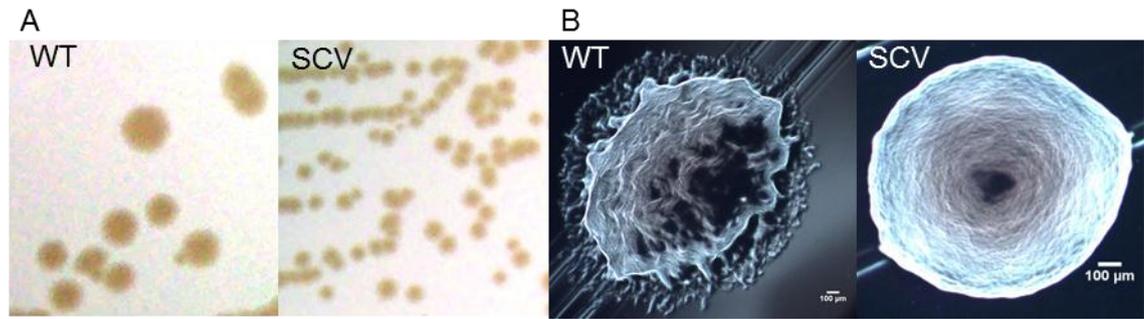


Figure 25 Colony morphologies of growth variants, showing either wild type (WT) or small colony variant (SCV) morphologies. A Wild-type (WT) and small colony variant (SCV) colony variants on LB agar photographed using a light box. SCV colonies are smaller than WT colonies due to increased surface tension. B WT and SCV colony variants viewed under phase contrast light microscopy (40x magnification). WT colony shows evidence of growth out from the edges of the colony boundary, which is not observed in the SCV colony.

Motility of biofilm isolate mutants MT24 and MT30

In addition to colony morphology, biofilm isolates MT30 and MT24 are lacking in both swimming and swarming motility, but showed similar levels of twitching motility compared to wild-type *P. aeruginosa* (Figure 26, Figure 27, and data not shown). Twitching motility requires the presence of type IV pili, and the ability of both these biofilm isolates to spread on twitching agar under a glass cover-slip indicated that loss of function mutations had not occurred in this mechanism (data not shown). Both swimming and swarming morphologies require active flagella, which suggest that mutations have arisen in these two biofilm isolates that has affected the flagella operation.

Initial attachment requires flagellum-associated hook protein 1 coded for by a *flgK*-like gene, to be present in *P. aeruginosa* PA14 (O'Toole and Kolter 1998). This is a crucial part of the flagella from which the filament of the flagellum extends, and removal of which would lead to no external flagellum presence. As both MT30 and MT24 are able to attach to surfaces, but are lacking in flagella motility, this suggests that attachment requires only the presence of flagella on the surface of the bacterium, but does not require this structure to be active.

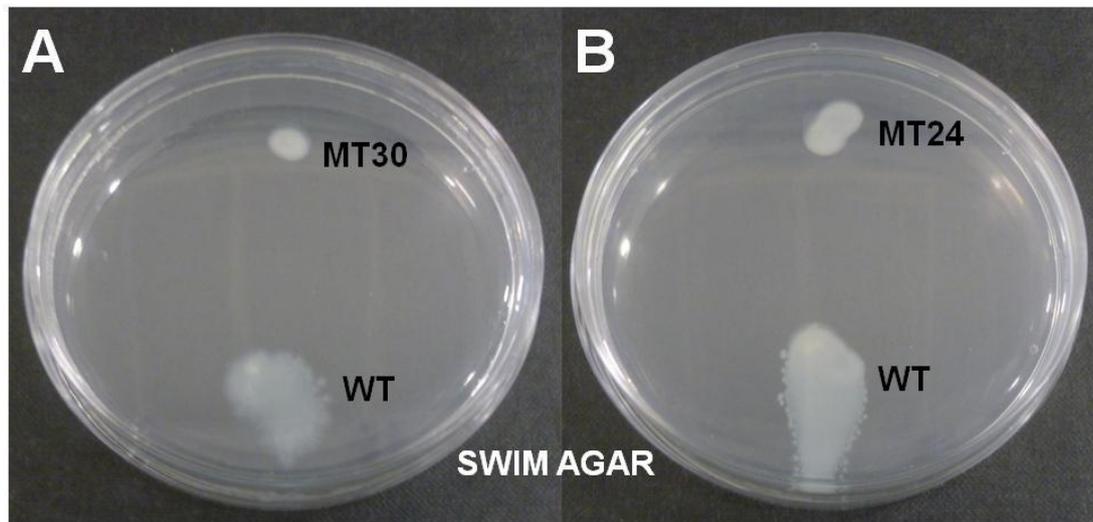


Figure 26 Biofilm isolated mutants show decreased swim motility. Both A. MT30 and B. MT24 show decreased swimming motility compared to the wild-type *P. aeruginosa* PA01 after overnight growth on specialist 'swim' agar. Each picture is a representative of 6 replicates.

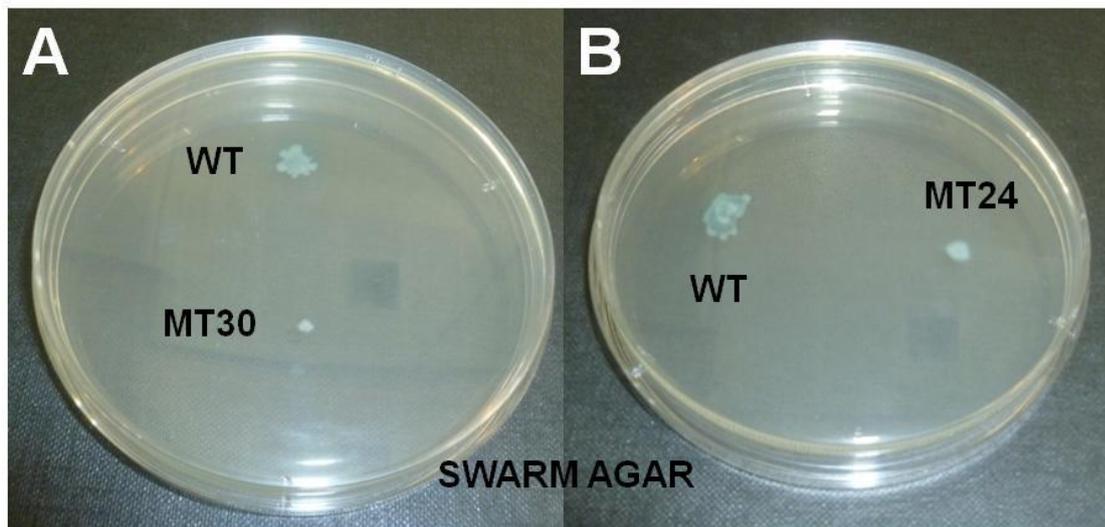


Figure 27 Biofilm isolated mutants show decreased swarming motility. Both A. MT30 and B. MT24 show decreased swimming motility compared to the wild-type *P. aeruginosa* PA01 after overnight growth on specialist 'swarm' agar. Each picture is a representative of 6 replicates.

When grown in biofilms, both mutants showed decreased biomass compared to the wild-type (Figure 11 chapter 2), but they each had slightly different biofilm morphology. While microcolony 'stalk' morphology requires type IV pili in *in vitro* flow cell conditions (Klausen *et al.*, 2003), the more motile 'cap' populations need working flagella to form (Yang *et al.*, 2009). As both of these biofilm isolates showed biofilm presence, but no 'cap' formation, this would agree with the conclusion that while twitching motility is present in both isolates, flagella operation has been affected in both

MT30 and MT24. In order to analyse the differences between the biofilm isolate and the wild-type in this study, both the *P. aeruginosa* lab wild-type and MT30 have been sent off for sequencing.

Appendix 2 - Determination of Optimum Rifampicin Concentration for Mutation Frequency Assays (Chapter 3)

Although the standard agar concentration for mutation frequency studies is 300 $\mu\text{g}\cdot\text{ml}^{-1}$ rifampicin, it was necessary to determine whether this was an appropriate concentration to use for this particular lab strain of *P. aeruginosa*. Lethal killing with various rifampicin concentrations was carried out on late log phase growth of *P. aeruginosa* PA01 wild-type, and the isogenic ΔmutS strain (Figure 28). This confirmed that a concentration of 300 $\mu\text{g}\cdot\text{ml}^{-1}$ rifampicin was sufficient to kill the majority of cells, and that the level of killing in the two strains was similar.

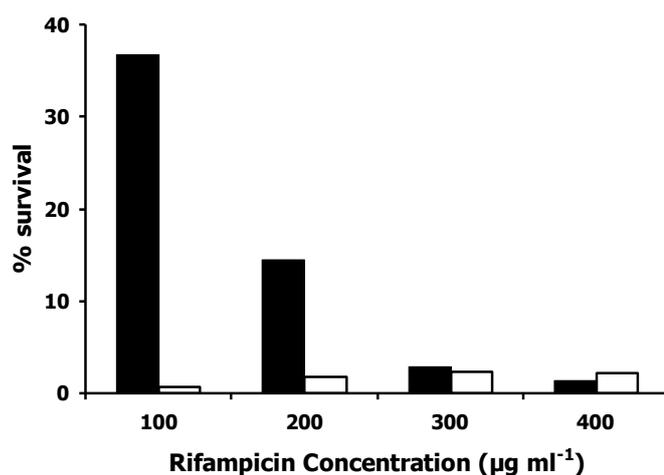


Figure 28 Rifampicin concentration study. 300 $\mu\text{g}\cdot\text{ml}^{-1}$ Rifampicin was considered the best concentration to use in the mutation frequency assay, as it resulted in similar killing in both the wild-type and ΔmutS strains. Each bar is an average of three replicates. ■ = ΔmutS , □ = wild-type.

Appendix 3 - Isolating Persister RNA (Chapter 3)

As persister cells are transient, and indistinguishable from normal cells in planktonic growth except through killing studies, it was necessary to validate a process which

would remove RNA from dead cells, allowing reverse transcription of only persister cell RNA. It was decided to use propidium monoazide (PMA) as an RNA chelator. It has previously been used as a chelator of dead bacterial DNA, because, like the very similar molecule propidium iodide, it cannot cross intact cell membranes, but is able to bind to DNA in cells with disrupted membranes, resulting in the bound DNA being washed away in the DNA extraction step. Although not currently in use to chelate RNA, except with a study involving RNA viruses (Parshionikar *et al.*, 2010), it was decided that it would be a valid mechanism for binding RNA, as it binds to the GC bases in nucleic acid, which are found in both DNA and RNA.

Treatment of *P. aeruginosa* wild-type cells showed that PMA is able to chelate bacterial RNA in live: dead cell mixes (Table 4). After RNA extractions of identical samples, the control samples had universally high levels of RNA extracted, while the samples with PMA showed decreases in RNA corresponding to the percentage of live cells in the samples. This method was used in the gene expression study in order to ensure that only persister cell RNA was examined for relative expression.

Table 4 PMA chelates bacterial RNA from cells with comprised membranes. PMA treatment of *P. aeruginosa* PA01 wild-type showed decreased concentrations of RNA after extraction than the control samples, indicating that PMA successfully chelates RNA as well as DNA.

Treatment	Live cells (%)	RNA Concentration (ng / μ l)
Control (no PMA)	100	755.63
	50	934.49
	0	930.61
PMA	100	995.80
	50	648.76
	0	177.48

PMA should not be used with proprietary solution RNALater

As the experiment was time-consuming, and RNA is time-sensitive, it was proposed that an RNA preserving substrate such as RNALater may be of use. RNALater is a proprietary solution used for ensuring RNA is kept in an un-degraded and unaltered

state from experiment termination until extraction. However, when RNALater was added to a sample, it bound all present RNA, both in live and dead cells. This meant that PMA was unable to bind to RNA in damaged cells, and consequently, dead cell RNA was not removed from the sample (

). As a result, RNALater was omitted from the protocol, and the gene expression study was carried out as fast as possible, keeping the samples on ice to minimise RNA degradation.

Table 5 Samples treated with a combination of RNALater and PMA showed increased concentrations of RNA compared to those treated with PMA only. This suggests that RNALater affects the binding of PMA, and stops it from chelating RNA from dead cells.

Treatment	Live cells (%)	RNA Concentration (ng /μl)
PMA only	100	653.44
	50	637.12
	0	115.42
PMA + RNALater	100	966.43
	50	990.29
	0	865.17

Appendix 4 - Relative Expression of Housekeeping Gene *rpoD* (Chapter 3)

All genes examined for relative expression in the persister cell gene expression study were compared to the housekeeping gene *rpoD*, which encodes the sigma 70 subunit in RNA polymerase. This was chosen due to its stability in a series of real time reverse transcription PCR studies using *P. aeruginosa* (Savli *et al.*, 2003). However, it was not an ideal housekeeping gene for this study, as it showed up-regulation itself, to a small extent at 3 hours of the ofloxacin treatment, but to a much larger extent throughout the ethanol treatment (Figure 29).

This meant that when the genes of interest *recA*, *mutS* and *dinB* were normalised to the housekeeping gene, some up-regulation of genes may have been masked by the up-regulation of the housekeeping gene. While this may be true for the SOS response genes during ethanol treatment, because the expression levels of *recA* and *dinB* were low, levels of the *mutS* gene during ethanol treatment were extremely high, meaning that despite the possible masking effect of the housekeeping gene, it was still observed to be up-regulated. This suggests that the up-regulation of *mutS* here is not a false report.

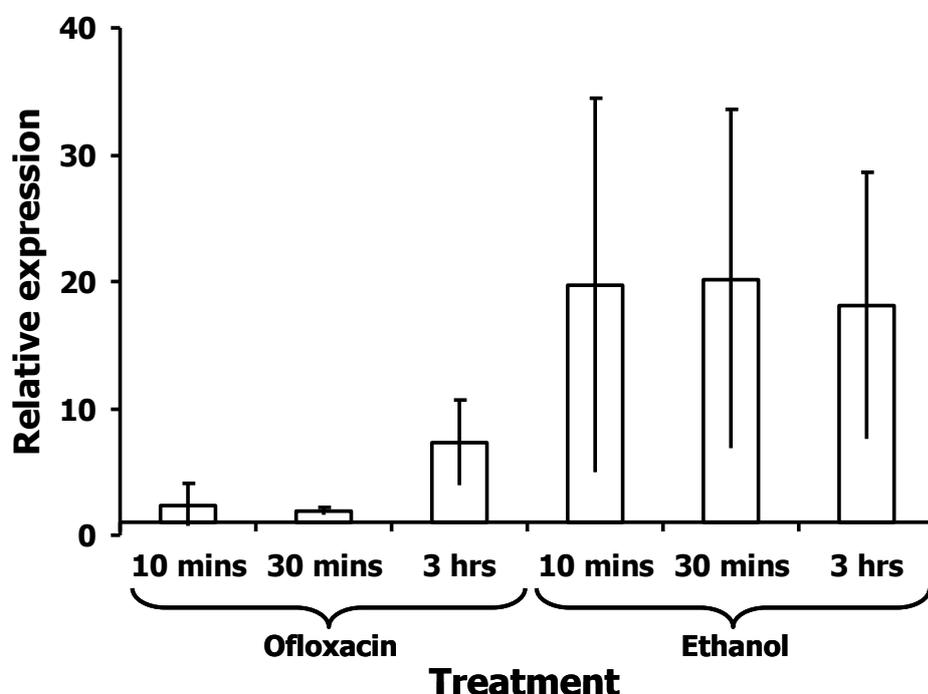


Figure 29 Relative expression of housekeeping gene *rpoD*. Live persister cell RNA was collected during treatment with 3 $\mu\text{g}\cdot\text{ml}^{-1}$ ofloxacin or 12.5% ethanol, and gene expression was

determined by RT-PCR. Gene expression was observed for the housekeeping gene *rpoD*. Relative expression of the housekeeping gene was calculated as follows:

$$2^{-([\text{mean } rpoD C_T \text{ treatment samples}] - [\text{mean } rpoD C_T \text{ control samples}])}$$

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