Investigation of the effects of *Pseudomonas aeruginosa* cell signalling on host-pathogen interactions during infection

Yam, Joey Kuok Hoong

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**INVESTIGATION OF THE EFFECTS OF
PSEUDOMONAS AERUGINOSA CELL SIGNALLING
ON HOST-PATHOGEN INTERACTIONS DURING
INFECTION**

YAM KUOK HOONG JOEY

**INTERDISCIPLINARY GRADUATE SCHOOL
SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE
SCIENCES ENGINEERING**

2017

**Investigation of the effects of *Pseudomonas aeruginosa* cell
signalling on host-pathogen interactions during infection**

Yam Kuok Hoong Joey

Supervisor: Professor Michael Givskov

Co-supervisor: Associate Professor Scott A. Rice

Mentor: Assistant Professor Yang Liang

Interdisciplinary Graduate School
Singapore Centre for Environmental Life Sciences Engineering

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ABBREVIATION

°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromole
µm	Micrometer
µm ³	Cubic Micrometer
ANOVA	Analysis of Variance
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
CFU	Colony Forming Unit
CLSM	Confocal Laser Scanning Microscope
d	Day
DNA	Deoxyribonucleic Acid
dpi	Day Post-infection
EPS	Extracellular Polymeric Substances
g	Gram
GFP	Green Fluorescence Protein
h	Hour
hpi	Hour Post-infection
L	Liter
LB	Luria Bertani
MIC	Minimum Inhibitory Concentration
min	Minutes

mg	Milligram
ml	Milliliter
mm	Millimeter
NCBI	National Center for Biotechnology Information
nl	Nanoliter
nm	Nanometer
OD	Optical Density
PBS	Phosphate Buffer Saline
QS	Quorum Sensing
RNA	Ribonucleic Acid
RNA-Seq	Ribonucleic Acid Sequencing
rpm	Revolution Per Minute
s	Seconds
w/v	Weight/Volume

ABSTRACT

Multicellular bacterial biofilms are highly tolerant and resistant to harsh conditions including antimicrobial agents and host immunity. Biofilms have been associated with chronic and persistent infections, which cause severe threat to the public health. Hence, there is an urgent need to develop new therapies to eliminate this threat. *Pseudomonas aeruginosa* utilizes quorum-sensing systems and c-di-GMP signalling to regulate a wide range of cellular functions for bacterial survival. Understanding the mechanisms that led to this tolerant or resistant behaviour allows targeted-approaches to be developed to halt these threats. The development of colistin-tolerant subpopulations in *P. aeruginosa* biofilms might severely impair the antimicrobial efficacy of colistin, which serves as one of the “last-resort” antibiotic against infections caused by Gram-negative bacteria.

Here, we monitored the real-time development of colistin-tolerant subpopulations and observed the coordinated social behaviour of these viable colistin-tolerant cell aggregates in seeking for iron during the development process. The colistin-tolerant phenotype is reversible and DNA sequencing showed no convergent non-synonymous mutation. Through pulsed-SILAC proteomics-approach, proteins involved in quorum-sensing systems and type IV pili were upregulated in these colistin-tolerant subpopulations. While gene-mutation approach and bioassays showed the importance of both quorum sensing (QS) activity and type IV pili-mediated motility in contributing to the development of colistin-tolerant phenotype, type IV pili-mediated motility preceded the QS activity during the development process. Interference to the QS activity and type IV pili-mediated motility with erythromycin halted development of colistin-tolerant subpopulations in both in flow

chamber and animal implant model experiments. Hence, erythromycin and colistin combinatorial treatment is a potential therapy to treat chronic infections.

P. aeruginosa ocular infection is a sight-threatening process and is possible to lead to loss of vision in worst case scenario. In this study, the transcriptomics analysis of *P. aeruginosa* from *P. aeruginosa*-infected mouse ocular infections showed an increased activity of iron uptake and type III secretion system in *in vivo* PAO1, while a decreased in QS activity comparing to *in vitro* PAO1 biofilms. The upregulation of pel exopolysaccharide and cup fimbriae during the ocular infections suggested the presence of biofilms. The biofilm physiology was verified using PAO1/*p_{cdrA}-gfp* c-di-GMP reporter strain, showing an enhancement of intracellular c-di-GMP contents in *P. aeruginosa* after 2 hour of ocular infection. The increased intracellular c-di-GMP concentration is likely caused by the reduced activity of a range of phosphodiesterases. PAO1 Δ *wspF*; PAO1 mutant with high concentration of intracellular c-di-GMP, formed microcolonies in the infected corneas and were more tolerant to clearance by host immunity as compared to PAO1/*p_{lac}-yhjH*; PAO1 carrying plasmid that constitutively express *yhjH* PDE, resulting in low level of intracellular c-di-GMP. Addition of arabinose (biofilm-dispersal agent) to the PAO1/*p_{BAD}-yhjH* biofilms; PAO1 carrying arabinose-inducible *yhjH* plasmid, lowered the intracellular c-di-GMP concentration in PAO1/*p_{BAD}-yhjH*, and improved the effectiveness of colistin treatment against *P. aeruginosa* biofilms in flow chamber and *P. aeruginosa*-induced mouse ocular experiment. Hence, the combination use of biofilm-dispersal agent and colistin is a potential therapy in treating *P. aeruginosa*-infected ocular infections.

CHAPTER 1. INTRODUCTION

1.1 Background

Bacteria are either in planktonic or biofilm mode is depended on the environmental and physiological conditions. Biofilm is defined as a surface-associated group of microorganism that are surrounded by self-secreted extracellular-polymeric substances (EPS), which includes exopolysaccharides, adhesion proteins, lipids and extracellular DNA (eDNA) (Czaczyk and Myszka 2007, O'Toole et al 2000a).

There are five stages in the biofilm lifestyle (Fig 1a). Firstly, planktonic bacteria attach reversibility to the surface via their flagella, before binding irreversibly to the surface. After which, the development of small microcolonies were initiated and further developed into large and mature microcolonies. Dispersion stage is the final stage of the biofilm lifestyle where the single bacteria disperses from the biofilm and eventually revert back to planktonic form. Bacteria biofilms can be found in various environment including industrial and clinical settings ranging from water distribution pipelines to contact lenses and medical devices (Costerton and Stewart 2001). Biofilm cells are surrounded in the self-synthesized EPS, which confers enhanced resilience and protection against stresses such as biocide (Costerton et al 1999a, Stewart and William Costerton 2001), antibiotics (Gordon et al 1988, Haagensen et al 2007) and cell-mediated host defenses (Bjarnsholt et al 2005, Jensen et al 2007).

Biofilms are a major problem in medical and industrial settings. Biofilms are often associated to infections such lung of cystic fibrosis patients (Høiby 2002), chronic wound infection (Høiby et al 2011), urinary tract infection (Anderson et al 2003) and

implanted medical devices such as stents and catheters (Cross et al 1983, Ganderton et al 1992). Biofilm-associated infections are notoriously difficult to be eradicated by antimicrobial agents and they constitute a wide range of clinical challenges including impaired wound healing, chronic inflammation and rapidly acquired antibiotic resistance (Bryers 2008). Also, biofilms are a major problem in industrial water distribution where it contribute to biofouling, which leads to damaging of water pipeline, affecting water quality as well as incurring financial burden for maintaining and repairing biofouling-related damaged pipeline (Murthy and Venkatesan 2008).

Figure 1a. Five stages of biofilm development. Initial stage where the bacteria reversibly attached to the surface (a); then irreversibly attached to the surface (b); formation of microcolonies (c); development of mature biofilms (d); and biofilm dispersal (e). The microscopy images of *P. aeruginosa* of each development stage are displayed in the bottom panel. Reproduced with permission from (Davies 2003).

Biofilms are responsible for a large percentage of treatment failures for infections, placing a huge economic burden for health care systems (Donlan and Costerton 2002, Stewart and William Costerton 2001). The US National Institutes of Health (NIH) has estimated about 65 - 80 % of all microbial infection involved bacterial biofilms, due to their persistence and chronic nature (Davies 2003, United States. National Intelligence 2000). Many factors contribute to the antimicrobial resistance of bacterial biofilms. Densely-packed EPS serves as a protective shield that reduces the penetration of antimicrobial agents through the biofilm matrix, thus protecting the cells against these stresses (Chiang et al 2013b, Flemming et al 2007b). The highly active efflux pumps and quorum sensing-mediated gene products also contribute to biofilm resistance against the antimicrobial agents (Barken et al 2008, Hentzer et al 2003, Liu et al 2010).

Pseudomonas aeruginosa, motile Gram-negative bacterium, can be found in widespread environments, which include soil, water and plant. *P. aeruginosa* is also prevalent in hospital and clinical environments, where it can cause opportunistic infections in immunocompromised patients (Mesaros et al 2007), keratitis (Elder et al 1995), urinary tract infection (UTI)(Anderson et al 2003) and respiratory infection (Høiby 2002). *P. aeruginosa* respiratory infections are largely responsible for much of the mortality and morbidity in cystic fibrosis patients (Govan and Deretic 1996). The ability of *P. aeruginosa* cause infections lies in its ability to form biofilms. The two key cell signalling systems namely; c-di-GMP signalling pathways and quorum sensing systems, are crucial in regulating a wide range of cellular activities including

formation of biofilm and production of virulence factors (De Kievit 2009, Hengge 2009, Miller and Bassler 2001, Römling et al 2013).

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (, an intracellular secondary messenger, can be found in both Gram-negative and Gram-positive bacteria. Elevated level of intracellular c-di-GMP content switches the bacterial from planktonic lifestyle to biofilm lifestyle by triggering the synthesis of EPS matrix substances in biofilms and reducing the motility of the cells, (Jenal 2004, Römling, Gomelsky et al. 2005, Jenal and Malone 2006, Römling and Amikam 2006, Ryan, Fouhy et al. 2006, Wolfe and Visick 2008) Other cellular functions via the increased in intracellular c-di-GMP contents include antibiotic production (Fineran, Williamson et al. 2007), progression in cell cycle (Duerig, Abel et al. 2009) and virulence factors production, which can cause chronic infections to the host (Dow, Fouhy et al. 2006, Tamayo, Pratt et al. 2006, Cotter and Stibitz 2007, Ryan, Fouhy et al. 2007).

Quorum sensing (QS) is defined as the communication between bacterial cells whereby small diffusible chemical signal molecule known as autoinducers, are synthesized and released by the bacterial (Henke and Bassler 2004, Miller and Bassler 2001, Waters and Bassler 2005, Williams et al 2007). Accumulation of these autoinducers upon certain concentration in the environment due to the increased bacterial population density, will trigger the activation of quorum sensing-mediated gene products such as rhamnolipids (Soberón-Chávez et al 2005, Zulianello et al 2006), *Pseudomonas* quinolone signal (PQS) (Mashburn and Whiteley 2005), siderophores (Michel et al 2005) and pyocyanin (Hasset et al 1992), which contribute to both acute and chronic infections (van Delden 2004).

Figure 1b. Role of c-di-GMP and quorum sensing signalings in *P. aeruginosa*.

1.2 Research gaps and Challenges

Bacterial biofilms are difficult to be eradicated in both *in vitro* and *in vivo* conditions due to the different strategies used by biofilms to resist killing by antimicrobial agents. Colistin antibiotics have been a focus in recent years due to its effectiveness in managing multi-drug resistant Gram-negative bacterium in clinical settings (Falagas and Kasiakou 2006, Li et al 2005, Li et al 2006). However, *P. aeruginosa* was capable to develop tolerance to colistin as colistin-tolerant subpopulations developed and survived in *P. aeruginosa* biofilms after challenged with colistin in continuous-culture flow system experiments (Chiang et al 2012, Pamp et al 2008). Although the same studies have demonstrated that the colistin-insensitive cells were metabolically-active and the efflux pump-encoding genes and lipopolysaccharide modification-encoding genes were upregulated, the exact mechanism and physiology of the development of colistin-tolerant subpopulations remains unclear. Thus, understanding the mechanism of colistin-tolerant subpopulations development

will enable better understanding of the keys proteins involved in the development process. With this knowledge, better treatment therapy in eradicating the *P. aeruginosa* biofilm-associated infections in public health can be potentially developed.

Pseudomonas aeruginosa causes chronic or acute infections depends on the genes being expressed during the infections (Broder et al 2016, Li et al 2013). Chronic infections are often positively correlated with the presence of biofilms in the infection (Høiby et al 2010a), while the expression of cell motility and type III secretion system (T3SS) and flagella are often associated with acute infections (Goodman et al 2004). There are numerous published studies that focus on *P. aeruginosa*-induced keratitis. These studies have shown that virulence factors including *ExoS*, *ExoT* and quorum sensing-regulated virulence factors are crucial for establishment of infection on the cornea (Finck - Barbançon et al 1997, Fleiszig et al 1997, Kessler and Blumberg 1987, Zhu et al 2004). There are also many studies that focus on the host immunity response towards the *P. aeruginosa* keratitis (Cole et al 2005, Hazlett 2004, Wu et al 2005, Xue et al 2002). *P. aeruginosa* biofilm-like matrix were observed on the mouse keratitis (Saraswathi and Beuerman 2015). However, the physiological status of the *P. aeruginosa* biofilm-like matrix on the keratitis remains unclear. There is no published study that investigate the effect of c-di-GMP signalling on host-pathogen interactions on *P. aeruginosa* keratitis though one study recently demonstrated *P. aeruginosa* c-di-GMP signalling mediate catheter-associated urinary tract infections (Cole and Lee 2016). By characterizing the role of c-di-GMP signalling on *P. aeruginosa* keratitis, new therapeutic treatment can be designed to effectively eradicate the *P. aeruginosa* on the infected cornea.

1.3 Objectives and Scope

In this thesis, the objectives are;

1. **Chapter 3:** To elucidate the mechanism of colistin-tolerant subpopulations development in the flow chamber experiment. These include the real-time monitoring of colistin-tolerant subpopulations development in *P. aeruginosa* biofilms in flow chamber experiment and utilizing pulsed-SILAC, a powerful proteomics-approach, which uses light C12 L-Lysine and heavy C13 L-Lysine to selectively label both the colistin-sensitive and colistin-insensitive populations respectively in *P. aeruginosa* biofilm flow chamber experiment. Gene mutation-approach will be used to validate findings from pulsed-SILAC.
2. **Chapter 4:** To determine the physiological status of the *P. aeruginosa* biofilm-like structures on the mouse keratitis animal model via transcriptomics-approach and cell imaging-approach with a range of *P. aeruginosa* mutant strains as well as c-di-GMP reporter strain. To characterize the role of c-di-GMP signalling in the Host-*P. aeruginosa* interactions during the mouse ocular infections.
3. **Chapter 5:** To design new potential therapies for eradication of *P. aeruginosa* biofilms in *in vitro* and *in vivo* experiments based on the results and knowledge gain from objective 1 and 2.

CHAPTER 2. LITERATURE REVIEW

2.1 Bacterial biofilm

2.1.1 Bacterial biofilm: A major problem in healthcare and industrial settings

Bacterial biofilm has been a major problem in healthcare and industrial settings including food and water industry (Costerton et al 1999b, Murthy and Venkatesan 2008, Schwartz et al 1998, Van Houdt and Michiels 2010). In drinking water distribution systems, biofilms can be found attached to the pipe wall (Flemming et al 2002). These biofilms can harbor pathogenic bacteria (Flemming et al 2002, Ingerson-Mahar and Reid 2013), which can cause disease upon consumption by elderly, young children or immunocompromised personnel (Ashbolt 2004). Waterborne infections are responsible for over 40 000 hospitalizations, costing about \$970 million every year in United States (Collier et al 2012). Infections caused by bacterial biofilms are a significant global health problem, and contributing to the economic burden of infectious disease (Blackledge et al 2013). Hospital-acquired infections caused by bacteria are costing the United States health care system more than \$20 billion every year (Roberts et al 2009). 80% of these hospital-acquired infections are caused by bacterial infections (Davies 2003).

2.1.2 Biofilm-associated infections

Bacterial cells are surrounded in the self-synthesized EPS and the structural and functional properties of the EPS matrix confers enhanced resilience and protection against stresses such as biocide (Costerton et al 1999a, Stewart and William

Costerton 2001), antibiotics (Gordon et al 1988, Haagensen et al 2007) and cell-mediated host defenses (Bjarnsholt et al 2005, Jensen et al 2007). Studies have shown that the minimal inhibition concentration (MIC) of the antibiotics is 100 to 1000 fold higher in biofilms than in planktonic mode (Aaron et al 2002, Ceri et al 1999, Hoyle and Costerton 1991, Nickel et al 1985). The biofilm caused chronic infections, which is defined as the persistence infections despite the action of antimicrobial agent and host immune system, and this can lead to the ongoing inflammation and severe damages to the tissue (Bjarnsholt et al 2009, Costerton et al 2003, Høiby et al 2010a). Many chronic infectious diseases are associated to bacterial biofilm (fig 2d) (Costerton et al 1999a, Hall-Stoodley et al 2006, Høiby et al 2001, Parsek and Singh 2003). Bacterial biofilm can be found in many biotic surfaces such as chronic wounds, cystic fibrosis patients' lungs (Bjarnsholt et al 2009, Høiby et al 2010b), chronic obstructive pulmonary diseases (Martínez-Solano et al 2008), dental plaques (Li et al 2004, Marsh 2006), endocarditis (Hoiby et al 1986), kidney stone (Potera 1999), urinary tract infections (UTIs) (Nickel et al 1994) as well as medical devices or prothese such as stents and catheters (Cross et al 1983, Ganderton et al 1992, Tacconelli et al 2009), contact lens (Abidi et al 2013), cerebrospinal fluid shunts (Fux et al 2006), breast implants (Rieger et al 2013), intrauterine devices (Auler et al 2010) and prosthetic heart valves (Donlan 2001). Different biofilm-associated infections can be caused by different bacterial species. For example, *P. aeruginosa* and *Burkholderia cepacia* biofilms are commonly involved in CF pneumonia, while Group A streptococci biofilms are commonly involved in necrotizing fasciitis (Costerton et al 1999b).

Figure 2. Clinical biofilm-associated infections. Reproduced with permission from (Pozo and Patel 2007).

2.1.2.1 *Pseudomonas aeruginosa* biofilm-associated infections

The model organism *Pseudomonas aeruginosa* was used for biofilm-associated infection studies in this thesis. *P. aeruginosa* is commonly used as model organism for studies because its genome completely sequenced as the genes are well-annotated in the *Pseudomonas* Genome Database (www.pseudomonas.com) (Winsor et al 2016). More importantly, *P. aeruginosa*, an opportunistic Gram-negative bacterium, is one of the major cause of nosocomial infections in the clinical settings where more than two millions patients are affected and contributed to approximately 90, 000 deaths every year (Cross et al 1983). In a recent report “Global priority list

of antibiotics resistant bacteria to guide research, discovery, and development of new antibiotics” by World Health Organization (WHO) (<http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/>), *Pseudomonas aeruginosa* is classified under the “Priority 1: Critical” under WHO priority pathogens list for R&D of new antibiotics. This stressed the importance of managing infections caused by *P. aeruginosa*. *Pseudomonas aeruginosa* nosocomial infection have been linked with medical devices such as the implants, catheters and tubings and was found that *P. aeruginosa* is capable to form biofilms on these medical devices (Cross et al 1983, Ganderton et al 1992, Peters et al 1980, Raad and Hanna 1999). Cystic fibrosis (CF) is a genetic disease whereby there is a mutation in cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to building up and thickening of mucus in the lung, pancrea and other organs (Cohen and Prince 2012). CF patients are susceptible to chronic lung infections and in most cases, the CF patients are infected mainly by the common pathogen, *P. aeruginosa*. Chronic lung infections caused by *P. aeruginosa* is also one of the leading cause of mortality and morbidity in CF patients (Burns et al 1998, Burns et al 2001, Saiman 2004). The mucoidal morphology of the *P. aeruginosa* was observed from clinical isolates obtained from the lung of CF patients (Koch and Hoiby 1993, Pedersen 1991). It has been showed that it is due to the overexpression of alginate, one of the EPS of the biofilm matrix, which give rise to the mucoidal phenotype (Govan and Deretic 1996, Pedersen 1991). *P. aeruginosa* biofilms in the lungs of CF patients leads to high tolerance to antibiotics treatments and prevent clearance by host immune systems (Davies and Bilton 2009, Döring et al 2000, Geller 2009, Rosenfeld et al 2003). Apart of CF lung infection, *P. aeruginosa* biofilms

were also associated to non-CF bronchiectasis, chronic obstructive pulmonary disease (COPD) and other chronic respiratory diseases (Davies et al 2006, Lieberman and Lieberman 2003, Valderrey et al 2010).

2.1.2.2 *Pseudomonas aeruginosa* keratitis

Bacterial keratitis is the infection of cornea (the outer most transparent membrane of the eye) that is caused by bacteria. It is characterized by growing of bacteria and the recruitment of polymorphonuclear leukocytes (PMNs) in the cornea tissue, which can lead to damage of cornea or blindness in worst cases. Bacterial keratitis has an annual incidence rate of approximately 19 / 10, 000 people per year during contact lens wear (Stapleton et al 2007). *Pseudomonas aeruginosa* is the most common organism isolated in bacterial keratitis, accounting for approximately 40 – 70 % of clinical isolates (Green et al 2008, Schornack et al 2008, Willcox et al 2004). Several predisposing factors are identified that include wearing of contact lens, cornea trauma, ocular surface diseases or ocular surgery (Green et al 2008). It has been reported that the wearing of contact lenses was the most common risk factor for keratitis (Green et al 2008, Mah-Sadorra et al 2005). Szczotka-Flynn and co-workers have demonstrated that clinical and laboratory reference *P. aeruginosa* strains have the capability to form biofilms on contact lens as evidence from confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) showed the presence of matrix in the aggregate of cells on the contact lens, but not in planktonic cells on the contact lens (Szczotka-Flynn et al 2009). In recent study by Beuerman and colleague, aggregates of *P. aeruginosa* cells can be observed in the *Pseudomonas aeruginosa*-induced keratitis in mouse cornea (Saraswathi and

Beuerman 2015). However, the physiology of *P. aeruginosa* aggregates or biofilms on the mouse cornea remains unknown.

2.2 Characteristics of a biofilm

Bacteria adopt one of two types of mode: in planktonic mode, which is free-living, singly bacterial cell, or in biofilm mode, a multicellular sessile complex. Whether the bacteria are in a planktonic-mode or biofilm-mode depends on the environmental and physiological conditions such as oxygen tension, nutrient availability, pH, osmolarity and temperature (Jackson et al 2002a, Jackson et al 2002b, O'Toole and Kolter 1998b, O'Toole et al 2000b, Prigent-Combaret et al 1999, Sauer et al 2004, Singh et al 2002, Thormann et al 2006). Biofilm is defined as a surface-associated group of microorganism that is surrounded by self-produced hydrated extracellular-polymeric substances (EPS) matrix (Davey and O'toole 2000). This matrix consist of extracellular-polymeric substances (EPS), which includes exopolysaccharides, adhesion proteins, lipids and extracellular DNA (eDNA) (Czarczyk and Myszka 2007, O'Toole et al 2000a). As described by Hans-Curt Flemming, the biofilm EPS matrix is also known to be the "House of biofilm cells" (Flemming et al 2007a). These EPS are recognized to maintain the biofilm structural integrity as well as protection of the biofilm against the harsh environmental conditions (Häussler and Parsek 2010, Parsek and Singh 2003, Ryder et al 2007).

The common exopolysaccharides identified in *P. aeruginosa* biofilms are pel, psl and alginate. These exopolysaccharides are vital in the formation of *P. aeruginosa* biofilm. *P. aeruginosa* PAO1 genetically encode for pel, psl and alginate exopolysaccharides, while PA14 only encode for pel and alginate exopolysaccharides (Colvin et al 2011,

Ghafoor et al 2011, Yang et al 2011a). *P. aeruginosa* PAO1 lacking of either functional pel or psl is deficient in forming a mature biofilm. In contrast, findings showed that alginate is not vital in the development of biofilm in laboratory condition (Wozniak et al 2003).

Studies have found that alginate is more important in clinically settings than laboratory settings such as *P. aeruginosa* PDO300, an over-producing alginate strain that gives rise to the mucoidal phenotype, is commonly found in the lung of cystic fibrosis patients (Govan and Deretic 1996, Mathee et al 1999). The same studies demonstrated that alginate provided the integrity of the biofilm structure and protects the biofilms. It also has the properties of nutrient and water retention to keep the biofilm in the hydrated state (Sutherland 2001). The enhanced secretion of alginate by *P. aeruginosa* protects the cells from host immunity as well as antibiotics (Leid et al 2005b, Simpson et al 1993, SIMPSON et al 1988).

Psl exopolysaccharide contains a repeating pentasaccharide consisting of L-rhamnose, D-glucose and D-mannose (Byrd et al 2009). Overexpression of psl enhanced the surface adhesion of *P. aeruginosa*, thus indicating the crucial role of psl in initiating formation and maturation of biofilm (Byrd et al 2009, Ma et al 2006). The presence of psl in the matrix decrease the killing-efficacy of antibiotic on *P. aeruginosa* biofilm (Yang et al 2011a).

Pel exopolysaccharide matrix is rich in glucose moiety (Friedman and Kolter 2004a). Recent studies showed that pel exopolysaccharide is involved in the bacterial communication in *P. aeruginosa* PA14 and serves as a architecture structure for the formation of biofilm (Colvin et al 2011). *P. aeruginosa* pel-deficient cells are deficient

in forming pellicle on the air-liquid interphase and have poor initial surface attachment, thus suggesting its importance in the formation of biofilm (Friedman and Kolter 2004b).

Table 2-1. Functions of *Pseudomonas aeruginosa* exopolymeric substances (EPS)

EPS	Function(s)	Reference
Pel exopolysaccharide	Biofilm structure	(Friedman and Kolter 2004a) (Jennings et al 2015)
	Structural and protective role in biofilm matrix	(Colvin et al 2011)
	Pellicle formation at air-water interphase	(Friedman and Kolter 2004a)
Psl exopolysaccharide	Initiating biofilm formation	(Byrd et al 2009)
	Maturation of biofilm	(Ma et al 2006)
	Protective function against antibiotics	(Mishra et al 2012, Yang et al 2011a)
Alginate	Integrity of biofilm structure	(Govan and Deretic 1996)
	Keep biofilm in hydrated state	(Sutherland 2001)
	Protect biofilms from antibiotics	(Hentzer et al 2001)
	Protect biofilms from host immune system	(Leid et al 2005a)

Adhesion proteins, which include proteinaceous components and extracellular proteins, are critical for the formation of biofilm. CdrA, an adhesion protein, is involved in biofilm formation by interacting with psl exopolysaccharide to stabilize the structure of biofilm (Borlee et al 2010).

Flagellar, a proteinaceous component, is required for the swimming and swarming motility (Kearns 2010). Swimming is the movement of individual bacteria in liquid, powered by rotating flagella, while swarming is the multicellular movement of bacteria across a surface and is also powered by rotating helical flagella (Kearns 2010). Flagella is important for the initial attachment of bacterial cells to the biotic or abiotic surface (O'Toole and Kolter 1998a). Pili are vital for twitching motility and twitching is surface movement of bacteria that is powered by the extension of pili, which then attach to the surface and subsequently retract, pulling the cell closer to the site of attachment (Burrows 2012). Pili are responsible for forming the 'Cap' of the mushroom-shape mature biofilm. Type IV pili-null *P. aeruginosa* is unable to form proper 'mushroom-shape' biofilm as type IV pili are necessary for the cells to move from the stalk to the cap (Barken et al 2008, O'Toole and Kolter 1998a, Skerker and Berg 2001).

Extracellular DNA (eDNA) is an crucial component of the biofilm matrix (Whitchurch et al 2002). Research have shown that there is high amount of eDNA at the stalk of the "mushroom-shaped" biofilm compared to the cap of the "mushroom-shaped" biofilm (Allesen - Holm et al 2006). eDNA facilitates macrocolonies formation from microcolonies. Addition of DNase I into the EPS disrupted the architecture of the "young" biofilm, but not the mature "old" biofilm (Whitchurch et al 2002). eDNA also confers resistance to antimicrobial agent, a gene pool for horizontal gene transfer,

maintain the integrity of the biofilm as well as serve as a nutrient source for the biofilm (Chiang et al 2013a, Dominiak et al 2011, Molin and Tolker-Nielsen 2003).

2.2.1 Bacterial biofilm life-cycle

The bacterial biofilm development life-cycle involved five stages (Fig 1a). As depicted in the figure 1a, A) Initial attachment to the surface, the free-living planktonic bacteria first bind to the surface via reversible attachment through Van der Waals forces; B) Irreversible attachment to the surface, when the cells bind or attach irreversibly, a process mainly due to the synthesis of exopolymeric substance and the loss of flagellar-mediated motility; C) Formation of the microcolonies; D) Mature biofilm development and E) Dispersion of bacteria from the mature multicellular complex and reversion back to planktonic form. More details will be discussed in the following subsections.

2.2.1.1 Reversible and irreversible attachment

The initial stage of biofilm development is the reversible attachment to the surface. The bacterial is required to move or swim near to the surface by cross the hydrodynamic boundary layer, to enter the near surface bulk-area (O'Toole and Kolter 1998a, Pratt and Kolter 1998). At this stage, the surface-attached bacterial cells are capable to swim away as only a small amount of EPS is presence to surround the cells. A huge portion of the bacterial cells leave the surface eventually, while leaving minute number of cells still attached to the surface, which would then proceed to the next stage of the biofilm development.

To transit into the irreversible stage, the bacterial cells have to overcome the Gibbs energy barrier, which are the electrostatic forces and Van der Waals forces (Palmer et al 2007). Upon crossing the Gibbs energy barrier, the bacterial cells come in contact with the surface. In the presence of cell surface structures including pili, flagellar, cell surface proteins and polysaccharides, the cells are able to attach strongly to the surface (Flint et al 1997, Vesper and Bauer 1986, Vesper 1987).

2.2.1.2 Maturation of biofilm

After the irreversible attachment to the surface, the next phase is the formation of the microcolonies and biofilm maturation. Structurally, single-layer of irreversible-attached cells divides and grow in number, causing the increase in the overall size of the population, leading to the formation of microcolonies and followed by macrocolonies, which typically give rise to a “mushroom-like” shape structure. There is a huge difference in physiology between biofilm cells and planktonic cells. Sauer and colleagues showed that there is a huge difference in protein expression between biofilm cells and planktonic cells (Sauer et al 2002). There is an increased production of exopolysaccharide such as pel, psl, and alginate, which are known for their role in the stability and integrity of the “mushroom-like” structure (Colvin et al 2011, Ghafoor et al 2011, Yang et al 2011a). *P. aeruginosa* type IV pili-driven motility is crucial for the “cap” formation at mushroom-shape structure (Barken et al 2008, Klausen et al 2003). Nuclei acids are important for both structural integrity and a nutrient source in biofilm maturation (Whitchurch et al 2002). EPS enhanced the biofilm cells survivability as it form a protective barrier against hostile environment (Häussler and Parsek 2010).

2.2.1.3 Dispersion of biofilm

In the final stage of the bacterial biofilm life-cycle, the biofilm cells disperse from the mature biofilm and revert back to free-living planktonic form to colonize new environment for further growth and development of new biofilm life-cycle. This process of colonization of new environment is crucial for the survival of the bacterial cells.

The biofilm dispersal can be categorized into passive dispersal or active dispersal. The passive dispersal is defined as detachment of the cells mediated by external factors such as sloughing and erosion. Sloughing is the process of detachment of huge portion of biomass from the surface, leading to largely amount of biomass loss. Erosion is the process of detachment of gradual and constant loss of small portion of biomass from the surface. Both sloughing and erosion are mediated by shear-forces (Choi and Morgenroth 2003, Stoodley et al 1999, Stoodley et al 2001). Active dispersal is defined as well-regulated transition of the sessile cells to the planktonic state (Kaplan 2010). Seeding dispersal is an active process, in which the single biofilm cells leave the central region biofilm, resulting in the hollow colonies (Boles et al 2005, Ma et al 2009, Webb et al 2003). Studies have shown that the seeding dispersal is mediated by quorum sensing system, while several enzymes involved in degradation of EPS and extracellular surfactant are upregulated (Boles et al 2005, Boyd and Chakrabarty 1994, Harmsen et al 2010, Ott et al 2001, Purevdorj-Gage et al 2005). Kaplan and colleagues demonstrated that dispersin B, an EPS-degrading enzyme, was upregulated and was able to degrade the biofilm-matrix poly-*N*-acetylglucosamine (PNAG) during the biofilm-dispersal phase (Kaplan et al 2003).

Sauer and co-worker demonstrated that addition of new carbon sources to *P. aeruginosa* biofilms caused nutrient-induced dispersal. The sudden introduction of succinate, citrate, glutamate or glucose to mature PAO1 biofilms is able to cause about 80 % loss of the biofilm biomass (Sauer et al 2004). From the same study, *P. aeruginosa* type IV pili-driven twitching motility was downregulated and flagellar-driven motility was upregulated, implying the dispersed bacterial cells have enhanced motility to swim away from the biofilms. Furthermore, limiting the nutrient supply to the biofilm also led to the active dispersal of biofilm. The 4-day-old mature *in vitro* *P. putida* biofilm was dispersed when the nutrient supply was ceased, indicating that lack of nutrient triggered the biofilm active dispersal (Gjermansen et al 2005). The biofilm, being a multicellular complex with the abundance of EPS, has limited amount of oxygen being able to diffuse into the core of the biofilm, thus creating an oxygen concentration gradient with the core of the biofilm having least amount of oxygen compared to the biofilm closest to the environmental interface (Anderl et al 2003, Walters et al 2003). The sudden halted of oxygen supply to the *Shewanella oneidensis* biofilm caused the biofilm to have massive loss of biomass in a short time (Stoodley et al 2001, Walters et al 2003). The mature *P. aeruginosa* biofilms also faced nitrosative stress. These nitrosative stresses are caused by the reactive nitrogen intermediates (RNIs) including nitrogen trioxide (N_2O_3), nitric oxide (NO), nitrous acid (HNO_2) and peroxynitrite (ONOO^-). These RNIs are often the by-products in the anaerobic respiration metabolism occur in the core of the mature biofilm (Hassett et al 2002). Kjelleberg and colleagues have demonstrated that the introduction of effective concentration of 500 nM of sodium nitroprusside (SNP), a NO-donor, to the *in vitro* *P. aeruginosa* biofilms led to the biofilm dispersal and the loss of approximately 80 % of the biomass from the surface of the glass-slide

(Barraud et al 2006). In the same study, the low dose of NO enhanced the swimming and swarming motility of *P. aeruginosa*, supporting the notion of the sessile cells leaving the biofilm. Also, nitric oxide and tobramycin have synergistic biofilm-killing effective than tobramycin treatment alone. Furthermore, the same group of researchers have demonstrated that single and multi-species biofilms in the clinical and industrial settings were able to be dispersed by the addition of NO, hence enhancing the efficacy of the antimicrobial agents in eliminating the biofilms (Barraud et al 2009b). In the presence of NO, the activity of c-di-GMP-specific phosphodiesterases was enhanced, resulting in reduced *P. aeruginosa* intracellular c-di-GMP concentration. It was found that *BdlA* (biofilm dispersion locus) gene was involved in the NO-induced dispersal and enhanced PDEs activities (Barraud et al 2009a).

2.3 Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP)

Bis-(3'-5')-cyclic-dimeric guanosine monophosphate (c-di-GMP) is a water-soluble molecule and is broadly conserved bacterial intracellular secondary messenger. Beiziman and colleagues demonstrated c-di-GMP as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (Ross et al 1987). In recent decade, research have focused on c-di-GMP as it is recognised to be an important universal secondary messenger, which is found in a broad range of bacteria. More importantly, c-di-GMP controls many cellular functions, which includes regulation of cell cycle, cell differentiation, cell motility, cell adhesion, virulence of bacterial pathogen, biofilm formation and biofilm dispersion (D'Argenio and Miller 2004, Hengge 2009, Jenal

2004, Jenal and Malone 2006, Römling and Amikam 2006, Römling et al 2013, Ryan et al 2007, Tamayo et al 2006).

2.3.1 C-di-GMP regulation

The intracellular concentration of c-di-GMP is well-regulated and fine-tuned by two classes of enzymes, which are the diguanlylate cyclases (DGCs) and phosphodiesterases (PDEs) (fig 2a). C-di-GMP is synthesized by DGCs, from two molecules of Guanosine-5'-triphosphate (GTP), while 5'-pppGpG as the reaction intermediate (Ross et al 1987). DGCs contains GGDEF domain, a set of amino acids that are highly conserved, which is the crucial in the active site of the DGCs (Christen et al 2005, Paul et al 2004, Ryjenkov et al 2005) PDE (Christen et al 2005, Schmidt et al 2005, Tischler and Camilli 2004). The c-di-GMP-specific PDEs catalyze the c-di-GMP degradation by breaking the water-soluble molecule into 5'-phosphoguanylyl-(3',5')-guanosine (pGpG), which pGpG is then separated into two guanosine 5'-monophosphate (GMP). There are two kinds of c-di-GMP-specific PDEs, which either consists of an EAL domain or a HD-GYP domain. EAL-domain-containing PDE has "Glu-Ala-Leu" motif that is highly conserved, hence named as EAL domain. The c-di-GMP-specific EAL-domain PDEs, which requires magnesium (2+) or manganese (2+), to catalyze c-di-GMP into 5'-pGpG and further degradation by non-c-di-GMP-specific PDEs (Christen et al 2005, Schmidt et al 2005, Tamayo et al 2005). HD-GYP domain PDEs are proteins that are classified under HD superfamily of metal-dependent phosphohydrolases. Similarly, they catalyze c-di-GMP degradation into 5'-pGPG and further degradation by non-c-di-GMP-specific PDEs into GMP (Ryan et al 2009).

From *Pseudomonas* Genome Database (www.pseudomonas.com), *P. aeruginosa* PAO1 annotated genome encodes for 5 proteins with PDE domain, 17 proteins with DGC domain and 16 proteins with DGC-PDE dual domains. *Pseudomonas aeruginosa* PA14 annotated genome (<http://ausubellab.mgh.harvard.edu/pa14sequencing>) encodes for similar DGC- and PDE-encoding genes as PAO1. However, there is a lack of PA2818 PDE-encoding gene and PA21771 DGC-encoding gene, while an addition of *pvrR* PDE-encoding gene in PA14 genome as compared to PAO1 genome (Drenkard and Ausubel 2002). Kulesekara and colleagues showed that 41 PAO1 proteins and 40 PA14 proteins are likely to be involved in c-di-GMP metabolism (Kulesekara et al 2006, Ryan et al 2009).

Figure 2a. Regulation of c-di-GMP and its cellular functions. Intracellular c-di-GMP concentration is regulated by DGCs and PDEs. High intracellular c-di-GMP content activates cellular functions including upregulation of adhesion proteins and

exopolysaccharides, while a downregulation of cell motility and acute virulence gene expressions. Reproduced with permission from (Hengge 2009).

2.3.2 Cellular functions regulated by c-di-GMP

2.3.2.1 Biofilm formation and dispersion

High level of intracellular c-di-GMP promotes the formation of biofilm and low level of intracellular c-di-GMP is associated with planktonic mode (Kirillina et al 2004, Kulesekara et al 2006, Merritt et al 2007, Nakhamchik et al 2008, Simm et al 2004, Weber et al 2006). As demonstrated by Hickman et al, a mutation in *P. aeruginosa* *wspF* gene, which is a chemosensory signal-transduction operon, led to constitutive expression of intracellular c-di-GMP. This high intracellular c-di-GMP content *wspF* mutant has increased expression of *pel* and *psl* exopolysaccharides, resulting in enhanced formation of biofilm (Hickman et al 2005). Christensen and colleagues used silicone implant coated with *P. aeruginosa*, carrying *p_{BAD}-yhjH* plasmid, which contains an arabinose-inducible *Escherichia coli* PDE, for mouse implant model. Upon addition of 0.25 % arabinose into the mouse peritoneal cavity, the expression of *yhjH* led to decrease level of c-di-GMP and caused biofilm dispersion (Christensen et al 2013). Moreover, quantification by HPLC showed c-di-GMP content was lowered in dispersal cells compared to biofilm cells and planktonic cells (Chua et al 2014, Chua et al 2015a)

2.3.2.2 Motility

The intracellular c-di-GMP level affects the bacterial pilus-based twitching motility as well as flagella-based motility (Choy et al 2004, Fang and Gomelsky 2010, Hengge 2009, Hickman et al 2005, Jones et al 2015, Paul et al 2010, Pratt et al 2007, Ryjenkov et al 2006, Wolfe and Visick 2008). Urs Jenal and colleagues have demonstrated that in *Escherichia coli*, at high level of intracellular c-di-GMP, c-di-GMP binds to *YcgR*, a molecular “brake” protein, and affects the flagellar motor protein, *MotA*, resulting in less motile bacterial cells (Boehm et al 2010). At high c-di-GMP level, *Vibrio cholerae* transcriptional regulator, *VspT*, binds to c-di-GMP, which inversely control the production of EPS and motility (Krasteva et al 2010). A mutation in *P. aeruginosa* *wspF* gene, led to the constitutive production of *wspR* DGC, which subsequently elevated the c-di-GMP concentration and inhibit bacterial motility (D'Argenio et al 2002, Hickman et al 2005). (Simm et al 2004) showed *AdrA*, a protein containing GGDEF domain, is necessary to elevate intracellular c-di-GMP content, which results in activation of the cellulose production in *Salmonella typhimurium*. In contrast, *yhjH*, an EAL domain-containing PDE, is required for the degradation of c-di-GMP, which blocks the cellulose synthesis in *S. typhimurium*. Furthermore, increased expression of *ArdA* results in repressed the swarming and swimming motility. In contrast, these motility were enhanced when *yhjH* was expressed. Thus, demonstrating that DGCs and PDEs have opposing effect on the production of c-di-GMP that affects the bacterial motility as well as the transition between biofilm sessile state and planktonic state.

2.3.2.3 Virulence

Studies have associated the virulence expression to c-di-GMP signalling pathways (Dow et al 2006, Hengge 2009, Kulesekara et al 2006, Ryan et al 2009). HD-GYP domain is responsible for the breakdown of c-di-GMP into 5'-phosphoguanylyl-(3',5')-guanosine (pGpG), which pGpG is then separated into two guanosine 5'-monophosphate (GMP). Ryan and colleagues showed that mutation in PA4108 and PA4781, encoding HD-GYP domain, lead to increased level of intracellular c-di-GMP content. Furthermore, they also showed that the mutants have reduced bacterial virulence factors to the larva of *Galleria mellonella* (Ryan et al 2009). *Vibrio cholerae*, a Gram-negative bacterium, is able to cause severe acute diarrhoeal infection by producing and secreting cholera toxin (CT). A mutation in *VieA*, a c-di-GMP phosphodiesterase in *V. cholerae*, led to high concentration of c-di-GMP and enhanced biofilm formation in *in vitro* experiments (Tischler and Camilli 2004). The same research group also demonstrated that *VieA* modulate the cholera toxin expression via the control of c-di-GMP (Tischler and Camilli 2005). A comprehensive study by Kulesekara and colleagues showed the contribution of virulence by *P. aeruginosa* diguanlyate cyclase (DGC) or phosphodiesterase (PDE). In the study, defective single DGC domain, single PDE domain and DGE-PDE dual domain in *P. aeruginosa* PA14 strains were used to test for their virulence contribution in murine thermal injury models. None of the mice that were infected by PA3947 (*rocR*) and *pvrR* mutant strains were killed. Half of the mice in each group infected with DGCs PA4332, PA3311, and PA5017 mutant strains were killed, thus demonstrating the contribution of each DGC and PDE towards the virulence during infections (Kulesekara et al 2006).

2.3.2.4 Cell cycle

C-di-GMP has been associated with cell cycle progression. While *Caulobacter crescentus* transits from free-swimming state to sessile biofilm state, the morphological state changes during the transition process (Curtis and Brun 2010). Progression in cell cycle progression in *Caulobacter crescentus* is controlled via proteins degradation (Grünenfelder et al 2001, Jenal and Fuchs 1998). (Duerig et al 2009) demonstrated that binding of c-di-GMP to I-site of PopA, a c-di-GMP effector protein, degrade the cell cycle regulator, CtrA, which led to the G1-to-S cell cycle progression. This timely-manner and localized c-di-GMP-dependent process is vital for *C. crescentus* cell cycle progression.

2.4 Quorum Sensing signalling in bacteria

2.4.1 Concept of quorum sensing signalling

Quorum sensing (QS) is defined as the communication between bacterial cells whereby small diffusible chemical signal molecule known as autoinducers, are synthesized and released by the bacteria (Henke and Bassler 2004, Miller and Bassler 2001, Waters and Bassler 2005, Williams et al 2007). The increased bacterial population density led to the accumulation of autoinducers in the environment. Upon a certain concentration threshold sensed by the bacteria, the entire bacterial population will alter the gene expressions in a synchronized manner. Through QS signalling, the behavior of the population is synchronized and assumed a coordinated social behavior at the population level (Henke and Bassler 2004,

Shapiro 1998, Waters and Bassler 2005). The QS signalling regulates a range of functions such as biofilm formation, expression of virulence factors, antibiotic productions, bioluminescence and sporulation (Ng and Bassler 2009, Novick and Geisinger 2008, Williams and Cámara 2009). These QS-controlled expressed proteins or biomolecules are termed as “public” good as they are beneficial to the bacteria population for they increase the survivability of the bacteria population by adapting and responding to the environment (Lazdunski et al 2004)

2.4.2 Quorum sensing system

The concept of quorum sensing was first discovered on two bioluminescent marine bacterial, *Vibrio fischeri* and *Vibrio harveyi* (Eberhard 1972, Nealson et al 1970). *V. fischeri* has a symbiont relationship with marine animal such as the squid *Euprymna scolopes* and the fish *Monocentris japonicas* (Nealson and Hastings 1979, Ruby and McFall-Ngai 1992, Visick and McFall-Ngai 2000). The role of *V. fischeri* in these symbiotic relationships is to provide light for the host such that the host like *Euprymna scolopes* can avoid predators or *Monocentris japonicas* can attract mates. In turn, *Euprymna scolopes* provide nutrients and protection for *V. fischeri*. *V. fischeri* is able to provide light due to the LuxI/LuxR QS system. The light production is due to the activation of luciferase proteins, which is encoded in *luxICDABE* operon (Engebrecht et al 1983, Lee et al 1993). In this system, *LuxI* proteins are required to synthesize N-(3-oxohexanoyl)-homoserine lactone, an autoinducer, which binds to the *luxR* receptor and activates the expression of *luxICDABE* operon, resulting in production of light (Eberhard et al 1981, Engebrecht and Silverman 1984). After the discovery of *LuxI/LuxR* QS system, many other QS systems have been described and have linked to functions such as biofilm formation and expression or virulence

factors (Deng et al 2010, Ng and Bassler 2009, Whitehead et al 2001). Both Gram-negative and Gram-positive bacterial were found using QS signalling to regulate their gene expression though they use different QS systems. For example, Gram-negative bacterial such as *P. aeruginosa* uses *lasI/lasR-RhlI/rhlR* and *Pseudomonas* *quinolone signal (PQS)* system (Brint and Ohman 1995, Passador et al 1993, Pesci et al 1999) and *Agrobacterium tumefaciens* use *Tral/TraR* system (Fuqua and Winans 1994, Hwang et al 1994, Zhang et al 1993). Gram-positive bacterial such as *Staphylococcus aureus* uses peptide quorum sensing *AgrC/AgrA* system (Bassler 1999, Novick 1999, Yarwood et al 2004) and *Bacillus subtilis* uses *comP/comA* system (Magnuson et al 1994).

Figure 2b. The regulation of *P. aeruginosa* quorum sensing systems. Reproduced with permission from (Lee and Zhang 2015).

Pseudomonas aeruginosa quorum sensing systems will be focused as *P. aeruginosa* is the model organism used in this thesis. Four quorum sensing systems have been described in *P. aeruginosa*, namely the LasI/LasR, RhII/RhIR, PQS and recently discovered, IQS systems (fig 2b) (Brint and Ohman 1995, Lee et al 2013, Passador et al 1993, Pesci et al 1999). LasI/LasR and RhII/RhIR systems are *N*-acylated homoserine lactones signalling systems and they function in a hierarchical manner where LasI/LasR system activates RhII/RhIR system (Latifi et al 1996, Pesci et al 1997). In the LasI/LasR system, LasI, an autoinducer synthase, synthesizes N-3-oxo-dodecanoyl-homoserine lactone (OdDHL) that binds signal receptor LasR. The LasR-OdDHL complex would immediately activate the target genes, which expressed a range of virulence factors such as *LasB*, a metalloprotease elastase; *arpA*, an alkaline phosphatase; *lasA*, a protease; *toxA*, Exotoxin A (Davies et al 1998, De Kievit and Iglewski 2000, Gambello et al 1993, Jones et al 1993, Passador et al 1993, Toder et al 1991). In addition, LasR-OdDHL complex also induces the expression of *lasI*, thus creating a positive feedback loop regulation (Seed et al 1995).

The LasR-OdDHL complex also activates the RhII/RhIR system. In the RhII/RhIR system, RhII synthesizes a second autoinducer, N-butanoyl-homoserine lactone (C4-HSL) and RhIR is the signal receptor for C4-HSL (Brint and Ohman 1995, Ochsner and Reiser 1995, Pearson et al 1995). At high concentration of C4-HSL, the C4-HSL autoinducer will bind to RhIR leads to RhIR-C4-HSL complex, which then triggers a

range of gene expressions including virulence factors production such as pyocyanin, proteases, siderophores and rhamnolipid (Déziel et al 2004, Latifi et al 1996, Schuster et al 2003, Schuster and Greenberg 2007). Similar to the LasI/LasR system, the binding of C4-HSL to RhIR activates the RhII expression, leading to a positive feedback loop. Wu and colleagues have demonstrated that *P. aeruginosa* LasI and rhII Qs mutants caused mild lung infections in rat than PAO1 wild-type strains (Wu et al 2001)

Pesci and colleagues discovered *Pseudomonas quinolone signal* (PQS) system as the third QS system in *P. aeruginosa* (Pesci et al 1999). PQS system differs from LasI-LasR and RhII-RhIR system, which are homologous to *V. fischeri* LuxI/LuxR system that use N-acylated homoserine lactone as autoinducers. In PQS system, 2-heptyl-3-hydroxy-4-quinolone is the diffusible signalling molecule. *PqsA*, *PqsB*, *PqsC*, *PqsD*, *PqsH* and the regulator, *PqsR* are required for the synthesis of 2-heptyl-3-hydroxy-4-quinolone (Gallagher et al 2002). It has been demonstrated that Las system regulates the PQS system as LasR-OdDHL complex is required to activate *pqsH* and *pqsR* (Camilli and Bassler 2006, Pesci et al 1999, Schertzer et al 2009). The binding of PQS to *pqsR* leads to *pqsR*-PQS complex, which activates the expression and biosynthesis of PQS pathway (Xiao et al 2006). *PqsR*-PQS complex regulate the expression of RhII and RhIR (Diggle et al 2007). Hence, PQS system is interlinked between the LasI/LasR and RhII/RhIR QS systems. PQS system regulates biofilm formation and expression of virulence factors including rhamnolipid, elastase and pyocyanin (Allesen - Holm et al 2006, Cao et al 2001, Diggle et al 2003, Rahme et al 2000, Yang et al 2009). *P. aeruginosa* PQS is highly expressed in lungs of cystic fibrosis patients (Collier et al 2002, Guina et al 2003) and PQS-mediated

virulence is required to kill mice, *Caenorhabditis elegans* and plants (Gallagher and Manoil 2001, Gallagher et al 2002, Lau et al 2004).

A fourth QS system, *IQS*, was recently discovered by Lee and colleagues (Lee et al 2013). The study by (Lee et al 2013) found that signalling chemical molecule in *IQS* system is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde and this molecule is synthesized with genes in *ambABCDE* gene cluster. Functional *ambABCDE* gene cluster is necessary for the regulation of virulence in animal models such as the mouse, nematode, zebrafish and fruit fly. It was demonstrated that the mutations in these genes led to the null-virulence phenotype whereby PQS, C4-HSL, pyocyanin and rhamnolipid are significantly reduced in expression as compared to wild-type strain. This also showed that *IQS* system is interlinked with PQS and Rhl system and was suggested to have overlapping function with *las* system.

2.5 Biofilm tolerance to antibiotics

2.5.1 Physical properties

The biofilm consists of bacterial cells and a huge portion of matrix, which includes exopolysaccharides, extracellular DNA and proteins. A biofilm three-dimensional structure and matrix provides tolerance towards antibiotics, thus less susceptible to antibiotics. Several studies have shown that alginate enhanced the biofilm tolerance towards aminoglycosides (Hentzer et al 2001, Mah et al 2003, Sadovskaya et al 2010). As demonstrated by Hentzer, the overproduction of alginate in clinical *P. aeruginosa* mucoidal isolate, PDO300, affects the overall structure biofilm and

enhanced tolerance to tobramycin in *in vitro* condition (Hentzer et al 2001). Study by Colvin showed that the overexpression of pel polysaccharide enhanced the *P. aeruginosa* biofilm tolerance towards tobramycin and ciprofloxacin, hence suggesting the protective role of pel in the biofilm matrix (Colvin et al 2011). There was less killing of *P. aeruginosa* in the presence of psl polysaccharide via the oxidative response by limiting complement-mediated opsonisation and phagocytosis by neutrophils (Mishra et al 2012).

Quorum sensing plays a role in contributing to the tolerance and resistance to antimicrobial agents (Bjarnsholt and Givskov 2007, Parsek and Greenberg 2000, Whitehead et al 2001). The release of eDNA in *P. aeruginosa* is mediated by both quorum-sensing-dependent and quorum-sensing-independent mechanism (Allesen - Holm et al 2006, De Kievit 2009). The QS-dependent molecules; *N*-acyl-L-homoserine lactones (AHL) and PQS molecule, induced expression of prophage and phenazine to increase the lysis of *P. aeruginosa*, which led to the release of eDNA (Allesen - Holm et al 2006, Webb et al 2003). Mulcahy and colleagues have demonstrated that eDNA chelate divalent metal cations such as Mg²⁺, Mn²⁺, Zn²⁺ and Ca²⁺ (Mulcahy et al 2008). The same study by Mulcahy also showed that addition of exogenous DNA to the *P. aeruginosa* biofilm increased the resistance to tobramycin, gentamycin and colistin compared to control without exogenous DNA addition. Bjarnsholt demonstrated that there were more killing of *P. aeruginosa* Δ lasRrhIR, QS-mutant strain, in *in vitro* biofilm by tobramycin comparing to wild-type *P. aeruginosa*, strongly suggested QS role in antibiotic resistance (Bjarnsholt et al 2005).

2.5.2 Physiological properties

Pseudomonas aeruginosa wild-type is able to develop into a three-dimensional architecture biofilm in *in vitro* experiment. In this three-dimensional architecture biofilm, cells within biofilms exhibit physiological heterogeneity, due to chemical gradients existing within these spatially structured communities (Wessel et al 2014). Studies have found that region in the centre of the biofilm is largely anoxic as oxygen penetrate up to 50 μm into the biofilm, while the average biofilm thickness is about 210 μm (Borriello et al 2004). With abundant of matrices in the biofilm, fluoroquinolones antibiotics, such as ofloxacin and ciprofloxacin are still able to penetrate into the biofilm (Suci et al 1994, Vrany et al 1997, Yasuda et al 1993). However, it was also demonstrated aminoglycoside antibiotics such as tobramycin and gentamycin have slow penetration or diffusion into the biofilms (Gordon et al 1988, Walters et al 2003). Walters et al found that though tobramycin has retarded diffusion into the biofilm as compared to ciprofloxacin due to the interaction with the matrix, it is able to penetrate into the biofilm given longer period of time (Walters et al 2003). The high tolerance towards tobramycin in the interior of the biofilm was due to the low oxygen availability and low metabolic rate (Walters et al 2003). The presence of active respiratory cells that are nearest to the biofilm surface, consumes oxygen faster than the rate of oxygen diffusion into the biofilm, resulting in an oxygen gradient and anaerobic condition in the biofilm interior (Costerton et al 1995, Flemming et al 2016, Kragh et al 2016, von Ohle et al 2010). The oxygen limitation in the interior biofilm leads to low metabolic activity of the cells, which affects the killing of biofilm by different antibiotics. Tobramycin kills the highly active cells located at the biofilm surface, whereas colistin kills the lowly active cells in the interior of the

biofilm (Chiang et al 2012, Herrmann et al 2010, Pamp et al 2008, Walters et al 2003). Hence, cell metabolic activity and oxygen limitation contribute to the tolerance of antibiotics.

Persister cells are defined as a small population that typically compromised of around 1 % bacterial cells in the biofilm are phenotypically in dormancy state, which are metabolically inactive and do not grow or react to aggressive antibiotic therapy (Lewis 2007, Lewis 2008, Lewis 2010). These persister cells in the dormant state do not undergo genetic modifications as they are phenotypic variants of the normal cells (Lewis 2010). When the condition is deemed suitable for growth such as the removal of antibiotics, persister cells are able to return to its normal state and repopulate the biofilm for survival (Lewis 2010). Hence, the presence of persister cells in the biofilm contributes to the high tolerance to antibiotics.

2.5.3 Adaptive resistance mechanism properties

The adaptive resistance of the bacterial cells is defined as the capability of bacterial cells in adapting and surviving in presence of antibiotics or antimicrobial agents. Colistin, a polymyxin B antibiotic, kills the lowly active cells at the core of the *P. aeruginosa* biofilm, but not the highly active cells located near the biofilm surface. These active cells were found to upregulate *pmrA-pmrB* two-component regulatory system, which synthesized 4-amino-4-deoxyarabinose that bound Lipid A of lipopolysaccharide found at the outer membrane of *P. aeruginosa*, resulting in inefficient binding of colistin to the LPS (fig 2c) (Haagensen et al 2007, Pamp et al 2008). On the other hand, it was showed by Bjarnsholt *et al* that aminoglycoside such as tobramycin was found less efficient in killing the lowly metabolic active cells

in the biofilm and was also found that quorum-sensing contributed to this adaptive tolerance mechanism (Bjarnsholt et al 2005). Another example of adaptive resistance mechanism is the expression of β -lactamase in *P. aeruginosa* clinical isolates from CF patient lung to resist killing by β -lactam antibiotics (Giwerzman et al 1990). β -lactam antibiotics are commonly used by cystic fibrosis patients to clear the bacterial lung infections. The mode of action of β -lactam antibiotics such as penicillin and cephalosporin is that they inhibit enzymes required for bacterial cell wall synthesis, hence causing bacterial cell death (Benton et al 2007). However, *P. aeruginosa* clinical isolates are often to resist β -lactam antibiotics. It was showed that a mutation of *ampD* gene led to the constitutive expression of β -lactamase in *P. aeruginosa* (Bagge et al 2002). In *in vitro* *P. aeruginosa* biofilms, addition of β -lactam antibiotics resulted in the expression of β -lactamase (Bagge et al 2004, Giwerzman et al 1991, Høiby et al 2010a).

Figure 2c. *In vitro* *Pseudomonas aeruginosa* biofilms treated with colistin. Green and red fluorescence represent live and dead cells respectively. Reproduced with permission from (Pamp et al 2008).

2.1 Treatment strategies against biofilms

2.1.1 Colistin antibiotic

Colistin (fig 2e), a polymyxin E, is a bactericidal drug and its mode of action is the binding of colistin to the phospholipids in the outer membrane of the Gram-negative bacterial, causing membrane disruption and leading to cell death (Evans et al 1999, Li et al 2001). However, the use of colistin was restricted to topical administration in mid-1980s due to the high incidence of nephrotoxicity and neurotoxicity were reported (Brown et al 1970, Landman et al 2008, Ryan et al 1969). In recent years, there were emerging multi-drug resistant Gram-negative strains and the commercially available antibiotics were not effective in killing the bacterial. Moreover, the slow development of new classes of antibiotics further aggravated the problem. The serious threat of these multi-drug resistant (MDR) Gram-negative strains caused in the public health has led to the reintroduction of colistin back to the clinical settings though the side effects caused by colistin were already recognised (Biswas et al 2012, Li et al 2005, Michalopoulos and Falagas 2011). The use of colistin is considered one of the “last-resort” antibiotics in treating MDR Gram-negative bacterium such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Hachem et al 2007). Colistin, as monotherapy or combination therapy with other antibiotics, is considered effective in treating MDR Gram-negative bacterial infections such as urinary tract infections, bacteremia and pneumonia as is less toxic to renal system than previously reported in other studies (Falagas et al 2005a, Falagas et al 2005b, Markou et al 2003, Sobieszczyk et al 2004).

2.1.2 Quorum Sensing Inhibitor (QSI)

As mentioned in the regulation of quorum sensing section above, quorum sensing systems in *P. aeruginosa* regulate the biofilm formation and a range of virulence factors. Decreased expression of virulence factors have been showed in the QS-mutant strains. *P. aeruginosa* biofilms were presented in the CF patients' lungs (Bjarnsholt 2013, Høiby 2002, Høiby et al 2010b). Moreover, there are evidence that QS systems are involved the QS autoinducers and QS-mediated gene products were found in the sputum of CF patients (Erickson et al 2002, Geisenberger et al 2000, Storey et al 1998). Therefore, many studies have suggested QS system as a therapeutic target to preventing cell-to-cell communication (Hentzer et al 2003, Rasmussen and Givskov 2006, Wu et al 2000). Unlike antibiotics whose modes of action are either bactericidal or bacteriostatic, quorum-sensing inhibitors (QSI) are either natural or synthesized molecules that are able to block the cell-to-cell communication, but are neither bactericidal nor bacteriostatic. Givskov and colleagues were first to discover QSI in the seaweed *Delisea pulchra* as it produced halogenated furanones, which have similar chemical structure as AHL, that acts as antimicrobial agent that interfere AHL-based quorum sensing in bacterial (Givskov et al 1996). It has been demonstrated that the motility of the *Serratia liquefaciens* was affected and the bacterial surface colonization was affected in the presence of the furanones (Givskov et al 1996, Kjelleberg et al 1997). Since then, studies have developed QSI screening platforms to screen for more compounds with QSI activity such as the furanone C-30 compound against *P. aeruginosa* (Manefield et al 2002, Rasmussen et al 2005). The feasibility of using Furanone-C30 in combination with an antibiotic in killing of *in vitro* *P. aeruginosa* biofilm has been demonstrated

(Hentzer et al 2003). The efficacy of Furanone C-30 in inhibiting the QS signalling in mouse infection lung model was demonstrated in the same study (Hentzer et al 2003). This feasibility test greatly boosted the research into discovery of more QSI compounds. Recently, quorum-sensing inhibitors were also discovered in food sources. For example, iberin from horseradish, ajoene from garlic and Panax ginseng were found to be quorum-sensing inhibitors of *P. aeruginosa* (Jakobsen et al 2012a, Jakobsen et al 2012b, Song et al 2010). In *in vivo* experiment, the combination used of QSI such as furanone C-30, ajoene or Iberin, together with tobramycin were demonstrated to have synergistic antimicrobial activity in controlling *P. aeruginosa* biofilm in mouse implant model compared to QSI or tobramycin single-treatment (Christensen et al 2012). Some antibiotics were also found to have QSI activity. For example, ciprofloxacin, ceftazidime and macrolides antibiotics such as erythromycin, azithromycin, clarithromycin were found to have QSI activity against *P. aeruginosa* (Høiby et al 2011, Skindersoe et al 2008). The use of azithromycin in controlling *P. aeruginosa* in cystic fibrosis patients' lungs, was found to be effective (Equi et al 2002, Wolter et al 2002). Therefore, using of QSI to target QS system to prevent bacterial cell-to-cell communication proved to be a potential therapeutic drug.

2.1.3 C-di-GMP as therapeutic target

Many studies have concluded that targeting bacterial intracellular c-di-GMP as one of the potential therapeutics to control the biofilm (Barraud et al 2006, Christensen et al 2013, Cole and Lee 2016, Römling et al 2013). As described in the c-di-GMP section above, increased level of c-di-GMP is involved the production of EPS, leading to the formation of biofilm and regulate cellular functions such as cell motility,

virulence and cell cycles. Unlike acute infections, chronic infections have been associated with the presence of biofilm. Bacteria in chronic infections are often difficult to eradicate due to the presence of biofilm. For example, *P. aeruginosa* biofilm found in the lungs of cystic fibrosis patients and these patients required aggressive antibiotic therapy to manage the infections. Decreasing the level of intracellular c-di-GMP in *P. aeruginosa* biofilms has been suggested since this approach will cause biofilm dispersion, resulting in biofilm cells transitioning back to planktonic state, which is more susceptible to antibiotic treatment. It is crucial that this approach has to be coupled with antibiotics or other antimicrobial agents as lowering intracellular c-di-GMP concentration caused biofilm dispersion as well as resulting in dispersed cells to colonize new surface, not killing of bacterial cells.

Figure 2e. Chemical structure of colistin. Reproduced with permission from National Center for Biotechnology Information. PubChem Compound Database; CID=5311054, (<https://pubchem.ncbi.nlm.nih.gov/compound/5311054>)

A recent study by Jain has shown that though not all patients' visions were fully recovered, using of topical colistin application was deemed safe and effective in controlling the multi-drug resistant *P. aeruginosa* bacterial keratitis by applying topical colistin 0.19 % as a single-drug therapy on patients' eyes (Jain et al 2014). Although low dose colistin antibiotic is effective in eliminating planktonic *P. aeruginosa* cells, similar concentration of colistin used is not effective in killing *P. aeruginosa* biofilms. Haagensen and colleagues found that a population of colistin-tolerant cells developed when *P. aeruginosa* flow-chamber biofilm was treated with up to 100 $\mu\text{g ml}^{-1}$ of colistin for 3 days, a 100 times Minimum Inhibitory Concentration (MIC) for planktonic *P. aeruginosa* (Haagensen et al 2007). This population of tolerant cells were typically found at the "cap" of the mature mushroom-shape biofilm. The same study also showed that all cells in *P. aeruginosa pmr* mutant biofilm were killed when given 25 $\mu\text{g ml}^{-1}$ of colistin, hence suggesting *pmr* operon is required for the tolerance of colistin. Pamp and co-workers demonstrated that the colistin-tolerant population are metabolically active by using to *P. aeruginosa rrnBP1-gfp[AGA]*, a growth rate-dependent fluorescent reporter fusion with a short half-life of green fluorescence, to grow and treat with colistin in the flow-chamber (Pamp et al 2008). The same study also showed that these metabolically active cells expressed high level of *pmrHFIJKLME* upon colistin treatment and *MexAB-OprM* efflux pumps were crucial to the tolerance of colistin. Furthermore, it was showed that this colistin-

tolerance phenomenon was mediated by two-component regulatory system, ParR-ParS (Fernández et al 2010).

As colistin monotherapy are deemed not effective in eradicating *P. aeruginosa* biofilm, there were studies that focus on the use of combination therapy to eliminate the biofilm. Antibiotics with different mode of actions are used such as the penicillin-aminoglycoside combinations. Soothill and colleagues showed that using combination of ceftazidime, gentamicin and rifampicin effective eradicate the *in vitro* *P. aeruginosa* biofilm (Ghani and Soothill 1997). Combination of colistin and ciprofloxacin, or colistin and tetracycline are more effective in eradicating *P. aeruginosa* biofilm than single-treatment in flow-chamber experiment (Pamp et al 2008). Colistin and tobramycin combination therapy was also more effective than colistin or tobramycin monotherapy in killing the *P. aeruginosa* biofilm in *in vitro* and *in vivo* experiments (Herrmann et al 2010). Also in the same study, it was showed that the colistin killed the metabolically inactive biofilm cells that are often found at the bottom of the *P. aeruginosa* biofilm, whereas tobramycin killed the metabolically active biofilm cells at the cap of the mushroom-shape-like biofilm. Hence, by combining colistin and tobramycin, of different mode of actions in killing biofilm, it is more effective in controlling the biofilm than colistin-alone or tobramycin-alone treatment.

CHAPTER 3. SELECTIVE LABELLING OF COLISTIN-TOLERANT BACTERIAL POPULATIONS IN *PSEUDOMONAS AERUGINOSA* BIOFILM

3.1 Introduction

The bacterial defence strategies can reduce the bacterial-killing efficacy of antibiotic. The two major bacterial defence strategies are the resistance and the tolerance strategies. Resistance strategy is defined as the change in genome due to genetic mutation or mobile genetic elements that occurred under the exposure of antibiotics (Neu 1992), which leads to the bacterial survival in the harsh antibiotic conditions. The second strategy is the tolerance strategy, whereby the bacteria alter the proteins expression, presenting a phenotypic variation instead of a change in genome (Balaban et al 2004, Lewis 2007). The presence of EPS matrix in the biofilms confers protection to the biofilm cells against harsh conditions such as biocides, antibiotics and immune defences, but not present in planktonic cells (Bjarnsholt et al 2005, Costerton et al 1999a, Costerton and Stewart 2001, Gordon et al 1988, Jensen et al 2007, Stewart and William Costerton 2001). Bacterial pathogens are known to contribute to the nosocomial infections in clinical settings and presence of bacterial biofilms in these infections have caused severe threat to the public health (Costerton et al 1995).

Bacterial biofilm has been described to form certain pattern, in which one or more subpopulations within the biofilm and are able to survive the harsh conditions (Parsek and Tolker-Nielsen 2008). Treatment of bacterial biofilm with antibiotic leads to elimination of antibiotic-sensitive populations, but not the antibiotic-insensitive

subpopulations. Thus, this could result in recurring infections when the antibiotic treatment is ceased and the antibiotic-insensitive subpopulations are able to repopulate and cause infections again (Dawson et al 2011, Parsek and Tolker-Nielsen 2008). The study of the physiology of the antibiotic-insensitive subpopulations is often difficult as these subpopulations are low in abundance within the biofilm though few studies have attempted (Allison et al 2011, Conlon et al 2013, Shah et al 2006, Vega et al 2012). However, the current approaches to study these antibiotic-insensitive subpopulations required the disruption of the biofilm in order to isolate these cells for study. These disruptive approaches may alter the physiology of these cells, thus likely to lead to inaccurate results and the mechanism of the development of these antibiotic-insensitive subpopulations still remains unclear.

Pseudomonas aeruginosa is able to form biofilm and is a common bacterial pathogen that causes nosocomial infections. According to the report “Global priority list of antibiotic-resistant bacterial to guide research, discovery, and development of new antibiotics” published by World Health Organization (WHO) in 2017 (<http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/>), *P. aeruginosa* is classified under “CRITICAL” category of WHO PRIORITY PATHOGENS LIST FOR R&D OF NEW ANTIBIOTICS. Thus, this emphasizes the urgency for new antibiotics or treatment strategies to be developed to control infections caused by *P. aeruginosa*.

Colistin is often the last-resort antibiotic when other antibiotics are deemed ineffective killing the multidrug-resistant Gram-negative bacteria (Falagas et al 2005a, Li et al 2006). Studies have shown colistin-tolerant cells developed in the *P. aeruginosa*

biofilm in flow chamber and *pmr* operon are highly expressed in these cells (Chiang et al 2012, Pamp et al 2008).

In this chapter, we monitored the real-time colistin-tolerant subpopulations development in *P. aeruginosa* biofilms in flow chamber upon exposure to colistin. We deployed the use of pulsed stable isotope labelling with amino acids (Pulsed-SILAC), a powerful proteomic-approach, to study the physiology and mechanism of the development of these colistin-tolerant cells without causing any disruption or disturbance to the biofilm. This method uses heavy C13 L-lysine to selectively label the newly expressed proteins in colistin-insensitive cells, while the proteins of colistin-sensitive cells will be selectively labelled with C12 L-lysine. Here, the development of the colistin-tolerant subpopulations is driven by type IV pili-mediated motility and quorum sensing-mediated activities.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions.

Pseudomonas aeruginosa strains and plasmids used in this chapter are listed in Table 3-1. *P. aeruginosa* strains were grown in ABT minimal medium (Chua et al 2013) supplemented with 5 g l⁻¹ glucose (ABTG) or 2 g l⁻¹ glucose and 2 g l⁻¹ casamino acids (ABTGC) at 37°C. For selection of plasmids in *P. aeruginosa*, 30 µg ml⁻¹ gentamicin and 200 µg ml⁻¹ carbenicillin were used as appropriate.

Table 3-1. List of *P. aeruginosa* strains used in chapter 3.

Strains	Characteristic(s)	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototypic nonmucoid wild-type strain	(Holloway and Morgan 1986)
PAO1 ^a	Prototypic nonmucoid wild-type strain	(Jacobs et al 2003)
PAO1Δ <i>lysA</i>	Tc ^r ; L-lysine synthesis defective <i>lysA</i> mutant in PAO1 ^a	(Jacobs et al 2003)
PAO1/ <i>pmr-gfp</i>	Gm ^r ; PAO1 containing the <i>pmr-gfp</i> translational fusion	(Haagensen et al 2007)
PAO1/ <i>lasB-gfp</i>	Gm ^r ; PAO1 containing the <i>lasB-gfp</i> translational fusion	(Hentzer et al 2002)
PAO1Δ <i>pilA</i>	Tel ^r ; <i>pilA</i> mutant of PAO1	(Barken et al 2008)
PAO1Δ <i>lasI</i> Δ <i>rhII</i>	Gm ^r ; Tc ^r ; <i>lasI</i> and <i>rhII</i> double mutant of PAO1	(Allesen - Holm et al 2006)
PAO1Δ <i>lasI</i> Δ <i>rhII</i> / <i>lasB-gfp</i>	Gm ^r ; Tc ^r ; <i>lasI</i> and <i>rhII</i> double mutant of PAO1 containing the <i>lasB-gfp</i> translational fusion	(Hansen et al 2012)
PAO1Δ <i>pilA</i> Δ <i>lasR</i> Δ <i>rhIR</i>	Tel ^r ; Gm ^r ; Tc ^r ; <i>pilA</i> , <i>lasI</i> and <i>rhII</i> double mutant of PAO1	(Barken et al 2008)
PAO1Δ <i>pilA</i> /pDA2	Tel ^r ; Carb ^r ; <i>pilA</i> mutant of PAO1 carrying the pDA2 plasmid (pUCP22/ <i>pilA</i>)	(Yang et al 2011b)

Tc^r, Tetracycline resistance. Gm^r, Gentamicin resistance. Tel^r, Tellurite resistance.

Carb^r, Carbenicillin resistance

3.2.2 Growing of *Pseudomonas aeruginosa* biofilms in flow chambers.

P. aeruginosa PAO1-tn7-*gfp* biofilms were cultivated with ABTG medium at 37°C using 40 mm x 4 mm x 1 mm three-channel flow chambers. The flow chamber was setup as previously described (Sternberg and Tolker - Nielsen 2006). Briefly, the overnight PAO1-tn7-*gfp* culture was diluted 1:1, 000 and 300 μ l of the diluted culture was injected into each channel using needle and syringe. The flow cell was placed in inverted position for 1 h and was reverted back to upright position prior to the flow of nutrient medium. The flow was maintained at velocity of 0.2 mms^{-1} using Cole-Palmer Masterflex peristaltic pump. The biofilms were developed for 3 days prior to 2 day treatment with 10 $\mu\text{g ml}^{-1}$ of colistin and followed by staining with 300 μ l of 20 μM of propidium iodide (PI) (LIVE/DEAD BacLight Bacterial Viability Kit, ThermoFisher) through injection into each channel. The results were presented as mean $\pm$ s.d from 3 individual experiments.

3.2.3 Reversion of colistin-tolerant phenotype in biofilms.

P. aeruginosa PAO1-tn7-*gfp* and PAO-1/*pmr-gfp* biofilms were grown in the flow chamber and were treated with colistin as described in the section mentioned above. The medium of the colistin-treated biofilms was switched to colistin-free medium for 48 h to allow the colistin-tolerant phenotype to revert back to the wild-type phenotype. To determine if these cells are able to physiologically revert to colistin-tolerant phenotype when colistin is introduced again, the colistin-free medium was switched back to the colistin-containing medium. At 0 h and 4 h of the challenged by second

round of colistin, the biofilms were then visualized under CLSM after injection of PI stain into each channel to stain the dead cells.

3.2.4 Reversion of colistin-tolerant cells in planktonic cultures.

P. aeruginosa PAO1-tn7-*gfp* and PAO1/*pmr-gfp* biofilms were grown with ABTG medium in flow chamber and treated with colistin in the colistin-treatment group as mentioned above. The colistin-treated biofilm consists of colistin-tolerant subpopulation as well as colistin-sensitive cell were removed from flow chamber using syringe with 5 ml of 0.9 % NaCl. The washed-out biofilms were then homogenised for 30 s to disrupt the biofilm followed by washing with 1 ml of 0.9 % NaCl twice. The colistin-tolerant cells were then cultured in either 2 ml of ABTGC medium or ABTGC medium containing 10 $\mu\text{g ml}^{-1}$ colistin, for 16 h at 37°C, 200 rpm shaking incubator. After which, the cells were serially diluted, plated onto either LB agar or LB agar containing 10 $\mu\text{g ml}^{-1}$ colistin and incubated overnight in 37°C for Colony Forming Unit (CFU) count. The fluorescence expression by PAO1/*pmr-gfp* were quantified by microplate reader (Tecan Infinite 2000) in 96-well microplate and readings were normalized by cell density (OD_{600}). The results were presented as mean $\pm$ s.d from 3 individual experiments.

3.2.5 Viable cells in colistin-treated biofilms.

PAO1 biofilms were grown in flow chamber for 72 h supplied with ABTG medium as mention above. At 72 h, the biofilms were treated with ABTG medium containing 10 $\mu\text{g ml}^{-1}$ of colistin. At time-point 0 h, 6 h, 24 h and 48 h after treatment with colistin,

the biofilms were washed out of the flow chamber with using syringe with 5 ml of 0.9 % NaCl. The washed-out biofilms were then homogenised for 30 s to disrupt the biofilm followed by washing with 1 ml of 0.9 % NaCl twice. The cells were then serially diluted and plated for CFU count. The results were presented as mean $\pm$ s.d from 3 individual experiments.

3.2.6 Microscopy and imaging of biofilms.

The flow chamber *P. aeruginosa* biofilms microscopy images were taken with Zeiss LSM 780 confocal laser scanning microscope (CLSM) using 20x objective lens (Carl Zeiss, Germany). The green fluorescence (GFP) and red fluorescence (PI) were observed by excitation with wavelength 488 nm and 561 nm respectively. The acquired confocal images were further processed with IMARIS software (Bitplane AG, Switzerland). The results were presented as representative CLSM image of each condition from 3 individual experiments.

3.2.7 Video and particle tracking of tolerant cell migration in biofilms.

PAO1-*gfp* biofilms were cultivated for 72 h in flow chamber, followed by treatment with 10 $\mu\text{g ml}^{-1}$ colistin and 4 $\mu\text{g ml}^{-1}$ PI for 48 h. The green fluorescence (GFP) and red fluorescence (PI) were viewed by excitation with wavelength 488 nm and 561 nm respectively. Videos of biofilms treated with colistin for 24 h to 32 h were acquired with Zeiss CLSM using 40x objective lens. The videos and the particle tracking of colistin-tolerant cells were processed with IMARIS software. The results were

presented as representative CLSM video of each condition from 3 individual experiments.

3.2.8 DNA sequencing analysis of biofilm cells.

P. aeruginosa PAO1 biofilms were cultivated for 72 h in flow chamber. The biofilms were treated with or without colistin for 48 h as described above. The biofilms were washed out with 0.9 % NaCl as described in the above. The genomic DNA of the biofilm cells was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) and were sequenced with Illumina MiSeq V3 platform generating 300 bp long paired-end reads. The experiments were performed with three biological replicates for both colistin-treated biofilms and control biofilms groups. The average genomic coverage depth range from 63 to 167 folds and the average insert sizes range from 490 to 544 nucleotides. CLC Genomics Workbench 8.0 software (CLC Bio, Aarhus, Denmark) was used to generate nucleotide differences and the parameters used are shown in supplementary method A1.

3.2.9 Pulsed-SILAC

P. aeruginosa PAO1 Δ lysA biofilm were grown in the flow tubes as described in (Sternberg and Tolker - Nielsen 2006). The overnight PAO1 Δ lysA culture was diluted 1: 1,000 and 1 ml of the diluted culture was injected into the flow tubes. The cells were incubated for 1 h before the supply of ABTG medium, containing 500 μ M C12 L-lysine and 192 mg l⁻¹ Amino acid Drop-out Mix Minus Lysine without Yeast Nitrogen Base (United States Biological, MA) at the flow rate velocity of 0.2 mm s⁻¹

using Cole-Palmer Masterflex peristaltic pump at 37°C. The 72 h-old biofilms were exposed to 10 µg ml⁻¹ colistin for 8 h to eliminate the colistin-sensitive subpopulations before switching to new ABTG medium, containing with 500 µM C13 L-lysine and 192 mg l⁻¹ Amino acid Drop-out Mix minus Lysine without Yeast Nitrogen Base and 10 µg ml⁻¹ colistin for 48 h in order for the newly expressed proteins in colistin-tolerant cells to tag with C13 L-lysine. After 48 h, the biofilms were washed out from the flow tubes with 5 ml of PBS before subjected sonication using probe sonicator for the lysis of biofilm cells.

The proteins were obtained and were subjected to SDS-PAGE for protein separation. The proteins in the SDS-PAGE were washed with ddH₂O mixed with 50 % acetonitrile (ACN)/50 % 25 mM NH₄HCO₃ by vortexing for 30 min, followed by dehydration with 100 % ACN. The proteins undergo reduction via 10 mM dithiothreitol at 56°C, followed by alkylation with 55 mM iodoacetamide (IAA) for 45 min in the dark. After which, the protein were again washed with ddH₂O mixed with 50 % acetonitrile (ACN)/50 % 25 mM NH₄HCO₃ and the gel undergo dehydration with 100 % ACN and dried using vacuum. To digest the proteins into peptides, trypsin (V5111, Promega, United States) was added to the gel and 25 mM NH₄HCO₃ was added to cover the gel for incubation at 37°C for overnight. Two 20-min sonications with 50 % ACN containing 0.1 % Trifluoroacetic acid (TFA) were deployed to extract peptides from the gel. These extracts were then dried under vacuum and resuspended in 0.1 % TFA prior to liquid chromatography (LC)–MS/MS analysis.

Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive (Thermo Fisher, MA) were used to separated peptides as previously described (Hao et al 2014).

Briefly, 1 μg of the peptides was inserted into the Acclaim peptide trap column (Thermo Fisher, MA, USA) using the Dionex RSLCnano auto-sampler and was then separated using Dionex EASY-Spray 75 μm x 10 cm column, packed with PepMap C18 3 μm , 100 $\text{\AA}$ (Thermo-Scientific, MA, USA). The column was subjected with mobile phase A (0.1 % formic acid in 5 % ACN) and mobile phase B (0.1 % formic acid in 90 % ACN) for a ACN gradient concentration at a rate of 300 nl min^{-1} . Peptides were then analysed on Q Exactive with an EASY nanospray source (Thermo Fisher, MA) at an electrospray potential of 1.5 kV. A full MS scan (350–1,600 m/z range) was acquired at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 15 s. The resolution of the higher energy collisional dissociation (HCD) spectra was set to 17,500 at m/z 200. The automatic gain control (AGC) settings of the full MS scan and the MS2 scan were 3E6 and 2E5, respectively. The 10 most intense ions above the 2,000 count threshold were selected for fragmentation in HCD, with a maximum ion accumulation time of 100 ms. An isolation width of 2 was used for MS2. Single and unassigned charged ions were excluded from MS/MS. For HCD, the normalized collision energy was set to 28%. The underfill ratio was defined as 0.2%.

Two injections have been performed for the first biological replicates and three injections have been performed for the second and third biological replicates, respectively, to evaluate the technical reproducibility of the instrument and workflow. Raw data files of the eight technical replicates were processed and searched as eight experiments using MaxQuant (v1.5.2.8) (Cox et al 2011, Tyanova et al 2014) and the Genbank *P. aeruginosa* protein database (downloaded on 21 May 2015, 55,063 sequences, 17,906,244 residues) together with the common contaminant

proteins. Standard search type with 2 multiplicity, 3 maximum labelled AAs and heavy labelled Lys6 were used for the pulsed-SILAC quantitation. The database search was performed using the Andromeda search engine bundled with MaxQuant using the MaxQuant default parameters for Q Exactive Orbitrap mass spectrometer. The first and main searches peptide mass tolerance for were 20 and 4.5 parts per million (p.p.m.) respectively, while the MS/MS match tolerance was 20 p.p.m. with FTMS de-isotoping enabled. The parameters include setting of Carbamidomethylation as a fixed modification, Oxidation and deamination as variable modifications as well as allowance of absence of two trypsin cleavage sites per protein. Revert decoy mode with PSM FDR, protein FDR and site decoy fraction set to 0.01 settings were used during the search.

Using Andromeda in MaxQuant with FDR <1 %, 4,382 proteins, including proteins from *P. aeruginosa* were identified (supplementary table A7). To validate the reproducibility of the technical replicates in each biological replicate, scatter plots were plotted (supplementary data A3 and A4). Similarly, to validate the reproducibility of the biological replicates, raw data files of each biological replicates were grouped together and searched using MaxQuant in order to get the pulsed-SILAC protein abundance levels in the three biological replicates. The scatter plots of inter-biological replicates were shown in (supplementary data A2). The abundance of proteins in colistin-tolerant cell were normalised by dividing C13 L-lysine-tagged proteins against C12 L-lysine-tagged proteins.

3.2.10 Colistin and DIPY treatment in *P. aeruginosa* flow chamber biofilm

GFP-tagged *P. aeruginosa* PAO1 biofilms were cultivated in flow chamber as described above. The 72 h old biofilms were treated with; 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ DIPY; or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ DIPY for 48 h. After which, 300 μl of 20 μM of PI was injected into each flow channel for observation of the dead cells. The biofilms were viewed under CLSM as mentioned above. The results were presented as representative CLSM image of each condition from 3 individual experiments.

3.2.11 Statistical analysis

To determine the difference between the treatment groups in; the reversion of colistin-tolerant cells in planktonic cultures experiment; the viable cells in colistin-treated biofilms across 0 h, 6 h, 24 h and 48h time-point experiment; Live/dead ratio of biofilms of *P. aeruginosa* wild type and mutant strains after treatment with colistin experiment; and virulence factors production in control cells and colistin-treated cells experiment, P-values were calculated using Student's T-test.

3.3 Results

3.3.1 Colistin-tolerant subpopulations development in *P.*

***aeruginosa* biofilms**

The 72 h old flow chamber *P. aeruginosa* PAO1 biofilms were monitored real-time under CLSM from 2 h to 32 h after treatment with 10 $\mu\text{g ml}^{-1}$ colistin for the dynamic development of colistin-tolerant subpopulations and the dead populations that stained red with PI stain. The top-view and side-view videos of the killing of biofilms by colistin from 0 h to 20 h were shown in supplementary video A1 and A2 respectively. The killing of biofilms was observed at the 2 h time-point as there were presence of PI-stained cells (dead cells) and a majority of the cells depicted red at 20 h time-point (fig 3-1a). Even treatment with colistin for 24 h, viable cells were still observed at the substratum layer of the biofilm. Thus, this could likely be either due to the physical properties of EPS matrix that prevents colistin from penetrating into the biofilms (Hengzhuang et al 2011) or the colistin kills only the metabolically inactive cells in the biofilms (Pamp et al 2008). The biofilm cells viability during the colistin treatment was determined through CFU plating. There was approximately 200-fold reduction in viable cells from $9 \times 10^8 \text{ CFU ml}^{-1}$ to $4 \times 10^6 \text{ CFU ml}^{-1}$ after 12 h of exposure to colistin (fig 3-1b). Interestingly, there was an increased in number of viable cells from 12 h onwards with approximately $3 \times 10^7 \text{ CFU ml}^{-1}$ at 24 h and $8 \times 10^7 \text{ CFU ml}^{-1}$ at 48 h (fig 3-1b). Thus, these results indicated most biofilm cells were killed at early phase upon exposure to colistin, while small populations of bacterial cells were able to survive and proliferate after adapting to colistin-containing environment at 12 h time-point.

From 24 h to 32 h time-point, viable aggregates of cells, but not the single cells, were seen moving toward and attached onto the dead mushroom-shape biofilms (fig 3-1a). This migration was further validated by particle tracking, showing the movement trajectory and track displacement of colistin-tolerant aggregates moving and pointing towards the dead mature biofilms, while the single cells appeared to move at random directions (fig 3-1c). The top-view and side-view videos of development and migration of colistin-tolerant subpopulations in flow chamber biofilms after exposure to colistin treatment for 24 h to 32 h were presented in supplementary video A3 and A4 respectively. Thus, these results suggested a coordinated social behaviour among the viable cells aggregates, but not in the viable single cells.

a

b

c

Cell aggregate movement trajectories

Track displacement

Figure 3-1. The development and migration of colistin-tolerant subpopulations in flow chamber biofilm. (a) 32 h of real-time CLSM monitoring of the development and migration of colistin-tolerant subpopulations in GFP-tagged PAO1 biofilms in flow chamber after exposure to colistin. The green fluorescence represents the live cells, while red or yellow fluorescence represent the dead cells. Scale bar, 50 μm . Representative image from each time-point was shown. (b) The viable cells in biofilms after treatment with colistin for 0 h, 12 h, 24 h and 48 h were expressed in CFU ml^{-1} . The results were presented as mean $\pm$ s.d from 3 individual experiments. (c) The movement trajectories and track displacement of the colistin-tolerant cell aggregates and single cells in colistin-treated biofilms. Scale bar, 10 μm . Reproduced with permission from (Chua et al 2016).

PAO1/*pmr-gfp* biofilms were grown in flow chamber for 72 h and were challenged with colistin to determine the expression of *pmr* operon as *pmr* operon expression was enhanced in the presence of colistin as reported previously (Barken et al 2008). Transient or low level of *pmr-gfp* expression was seen at 0 h, while a stronger intensity of green fluorescence was observed at 4 h after exposure to colistin (fig 3-2a). The biofilms from colistin-treated and control conditions were retrieved, cultured in planktonic state with or without colistin and followed by plating in LB agar with and without colistin. The results showed that no viable colistin-tolerant cells were presence in the colistin-containing LB agar plates after culturing in ABTGC medium without colistin (fig 3-2b). There was significantly higher *pmr-gfp* expression in colistin-tolerant cells that were retrieved from flow chamber biofilms after culturing in ABTGC medium containing colistin compared to without colistin (fig 3-2c). These

results suggested that the colistin-tolerant cells were capable to revert back to wild-type state when the colistin was depleted from the environment and these cells subsequently became susceptible to killing by colistin. Results from DNA sequencing from both control and colistin-treated biofilms showed no convergent non-synonymous mutation (supplementary table A1). Hence, indicating that these colistin-tolerant cells are phenotypic variations of the wild-type, and not due to resistance strategy.

Figure 3-2. Reversion of colistin-tolerant phenotype to wild-type phenotype. (a) Confocal images of the 72 h old PAO1/*pmr-gfp* biofilms after challenged with colistin for 0 h and 4 h. Green and red fluorescence represent the expression of *pmr-gfp* and total biofilm biomass respectively. The results were presented as representative

CLSM image of each condition from 3 individual experiments. Scale bar, 10 μm . (b) CFU count of the harvested colistin-tolerant cells from colistin-treated biofilms were vortexed vigorously, cultured in ABTGC medium containing 10 $\mu\text{g ml}^{-1}$ colistin or 0.9 % as control, followed by plating on LB agar with or without colistin. (c) GFP expression by *pmr-gfp* colistin-tolerant cells after culturing in ABTGC medium containing 10 $\mu\text{g ml}^{-1}$ colistin or 0.9 % as control for 16 h. The results were presented as mean $\pm$ s.d from 3 individual experiments. (* $P < 0.01$, Student's t-test). Reproduced with permission from (Chua et al 2016).

3.3.2 Pulsed-SILAC-approach to study colistin-tolerant subpopulations in biofilms.

Stable isotope labelling by amino acids in cell culture, SILAC, is a simple yet powerful proteomics-approach with high-accuracy to study proteins expression and is often used in *in vivo* mammalian experiments (Mann 2006, Ong et al 2002, Ong and Mann 2006). This is the first study to utilize SILAC to study colistin-tolerant cells in *P. aeruginosa* biofilms. We hypothesized that the actively expressed proteins in the colistin-tolerant subpopulations provided the survival advantages to the bacteria. In this approach, light C12 L-Lysine and heavy C13 L-Lysine were used to selectively tag proteins of the bacterial during growth in flow chamber. In this approach, bacteria that is incapable of synthesizing lysine amino acids was used so that they required to uptake exogenous lysine for survival. Hence, PAO1 Δ *lysA*, a lysine-mutant that is incapable to synthesize lysine, was tested and results showed that PAO1 Δ *lysA* required the addition of exogenous L-lysine in the ABTG medium to

grow (fig 3-3a). Furthermore, the capability of PAO1 Δ /lysA to form biofilms was not diminished when the growth medium was supplemented with either light C12 L-lysine or heavy C13 L-lysine. Development of colistin-tolerant subpopulations in biofilm was still notable in both conditions after challenged with colistin for 24 h (fig 3-3b).

In this pulsed-SILAC approach to study the colistin-tolerant subpopulations, PAO1 Δ /lysA was supplied with ABTG medium supplemented with light C12- L-lysine, and cultivated for 72 h in flow chamber for the formation of biofilms before challenged with 10 μ g ml⁻¹ colistin for 8 h. After killing the colistin-sensitive population, the remaining surviving colistin-tolerant cells were then treated with fresh medium containing colistin and C13 L-lysine for 48 h to incorporate C13 L-lysine into the newly expressed proteins of only live cells. The schematic diagram of the pulsed-SILAC workflow was shown in figure 3-4a. This pulsed-labelling approach allowed for selective tagging of newly expressed proteins in the colistin-tolerant subpopulations, with C13 L-lysine without prior physical isolation. The level of new protein abundance could be normalized by dividing the relative abundances of C13 L-lysine-tagged proteins (proteins expressed after colistin treatment) with the relative abundances of C12 L-lysine-tagged proteins (proteins expressed before colistin treatment).

The C12 or C13 L-lysine-tagged proteins were subjected Q Exactive MS to analyse the pulsed-SILAC labelled samples, followed by the MaxQuant and Andromeda software as described in the material and methods. Proteins that were regulated in all the three biological replicates (variability < 30 %) were studied (supplementary table A7). The proteins were classified into functional groups and were further

grouped into downregulated functional group (fig 3-4b) and upregulated functional group in colistin-tolerant cells (fig 3-4c). The downregulated and upregulated proteins were shown in supplementary table A5 and supplementary table A6 respectively. From the data, bacterial ribosomes subunits [*rpsS* (ratio H:L = 4.16, reference 20), *rpmI* (ratio H:L = 3.89, reference 26), *rpmC* (ratio H:L = 3.02, reference 69)] were highly upregulated in colistin-tolerant cells (supplementary table A6), in agreement with (Chiang et al 2012, Pamp et al 2008) that these colistin-tolerant cells are metabolically active. Through pulsed-SILAC protein analysis, the bifunctional polymyxin-resistance proteins ArnA (ratio H:L = 3.06, reference 2570) and PmrA (ratio H:L = 2.2, reference 3360) proteins, which conferred resistance against colistin, were upregulated in colistin-tolerant cells (supplementary table A7), in agreement with (Chiang et al 2012, Moskowitz et al 2012, Pamp et al 2008). The *arnBCADTEF* lipopolysaccharide-modification operon had been shown to be controlled by the upstream pmr operon and contribute to the tolerance to polymyxin B and colistin in *P. aeruginosa* (Fernández et al 2010). Hence, both proteins served as positive controls and showed that pulsed-SILAC was highly reliable in detecting proteins important in colistin tolerance. These two markers were consistently detected with high abundance level in the heavy C13 L-lysine-labelled proteins in seven out of eight technical replicates of the three biological replicates except in BR1:R2, where ArnA was not detected in the light C12 condition, and PmrA was not detected in both H and L conditions due to technical variation (supplementary table 7, markers intensity sheet). The missing detection of marker proteins in BR1:R2 might be attributed to growth variations during biofilm cultivation as biofilm experiments normally require a long cultivation period (5 days).

a

b

Figure 3-3. The growth of PAO1 and PAO1 Δ/ysA mutant in planktonic culture and in flow chamber. (a) PAO1 Δ/ysA mutant was unable to grow in ABTG medium without

exogenous L-lysine. PAO1 Δ lysA growth rate is same as PAO1 when 1 mM C12 L-lysine was added to ABTG medium. The results were presented as mean $\pm$ s.d from 3 individual experiments. (b) PAO1 Δ lysA was able to form biofilms with addition of either C12 L-lysine or C13-L-lysine into ABTG medium. The development of colistin-tolerant subpopulations was observed in both conditions after 24 h of exposure to colistin. Green and red fluorescence represent live and dead cells respectively. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 10 μ m. Reproduced with permission from (Chua et al 2016).

a

b **c**

Figure 3-4. Pulsed-SILAC workflow for studying of colistin-tolerant subpopulations. (a) Schematic diagram of the workflow of pulsed-SILAC to study colistin-tolerant cells and colistin-sensitive cells. (b) The downregulated proteins in colistin-tolerant cells were categorised into functional groups. (c) The upregulated proteins in colistin-tolerant cells were categorised into functional groups. Reproduced with permission from (Chua et al 2016).

3.3.3 Contribution of quorum sensing and type IV pili motility to colistin-tolerance phenotype

Proteins such as pilH (supplementary table A6, reference 70) and pilY1 (supplementary table A6, reference 201), involved in the synthesis of type IV pili, were found upregulated in the colistin-tolerant cells (supplementary table A6). Therefore, we hypothesized that type IV pili-driven motility might contribute to the tolerance to colistin since the colistin-tolerant aggregates migrated towards the dead microcolonies in colistin-treated biofilms (fig 3-1c, supplementary video A3 and A4). To validate the contribution of type IV pili to colistin-tolerance, PAO1 Δ *pilA*, a type IV pili mutant, was cultivated in flow chamber for 72 h and challenged with colistin for 48 h.

Approximately Live/dead ratio = ~0.35 and Live/dead ratio = ~1 were observed in colistin-treated PAO1 Δ *pilA* and colistin-treated PAO1 biofilms respectively (fig 3-5a, b). In contrast, phenotype of colistin-tolerant cell aggregate was notable and restored in colistin-treated biofilms of PAO1 Δ *pilA*/pDA2 (Live/dead ratio = ~0.9), PAO1 Δ *pilA* that was complemented with plasmid containing *pilA* gene in pUCP18 vector (fig 3-

5a, b). These results suggested the importance of type IV pili in contributing to the tolerance to colistin.

Quorum sensing (QS) proteins or QS-mediated proteins such as LasB (reference 94) and PhzB (reference 208), were found upregulated in colistin-tolerant cells (supplementary table A6), suggesting the involvement of QS in the *P. aeruginosa* tolerance to colistin. PAO1 Δ *las* Δ *rhl*, PAO1 with mutations in *las* and *rhl* QS system, biofilms were grown in flow chamber prior to challenge by colistin. Colistin-tolerant aggregates were significantly reduced in PAO1 Δ *las* Δ *rhl* biofilms (Live/dead ratio = ~0.3) compared to wild-type PAO1 biofilms (Live/dead ratio = ~1) after colistin treatment (fig 3-5a, b). However, the phenotype of colistin-tolerant was restored similar to PAO1 biofilms in colistin-treated PAO1 Δ *Las* Δ *rhl* biofilms (Live/dead ratio = ~0.65) that were supplemented with 1 μ M N-3-oxo-dodecanoyl-homoserine lactone (OdDHL), a *las* QS system signalling molecule (fig 3-5a, b). Interestingly, the development of colistin-tolerant cells was diminished in PAO1 Δ *pilA* Δ *lasR* Δ *rhlR* (Live/dead ratio = < 0.01), triple mutations in type IV pili motility, *las* and *rhl* QS system, biofilms when challenged with colistin (fig 3-5a, b). Hence, these observations provide strong evidence of the contribution of both *P. aeruginosa* type IV pili-mediated motility and QS activity to the tolerance against colistin.

a

Figure 3-5. The contribution of type IV pili and QS to the colistin-tolerant subpopulations development in biofilms. (a) The left panel showed the 72 h old biofilms of *P. aeruginosa* PAO1, PAO1 $\Delta pilA$, PAO1 $\Delta pilA/pDA2$, PAO1 $\Delta lasI/\Delta rhII$, PAO1 $\Delta lasI/\Delta rhII$ + OdDHL and PAO1 $\Delta pilA\Delta lasR\Delta rhIR$ cultivated in flow chambers. The right panel depicted the respective *P. aeruginosa* biofilms after 24 h treatment with 10 μg ml⁻¹ colistin at 72 h old biofilm time-point. Green and red fluorescence represent live and dead cells respectively. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 50 μm . (b) The Live/dead ratios of respective biofilms of *P. aeruginosa* PAO1, PAO1 $\Delta pilA$, PAO1 $\Delta pilA/pDA2$, PAO1 $\Delta lasI/\Delta rhII$, PAO1 $\Delta lasI/\Delta rhII$ + OdDHL and PAO1 $\Delta pilA\Delta lasR\Delta rhIR$ after treated 24 h with 10 μg ml⁻¹ colistin. Live/dead ratios were tabulated from live and dead biovolumes obtained from IMARIS software. The results were presented as mean $\pm$ s.d from 3 individual experiments. (* $P < 0.01$, Student's t-test). Reproduced with permission from (Chua et al 2016).

As type IV pili-mediated motility and QS system are important in the development of colistin-tolerant subpopulations, further investigation was carried out to determine the order of importance of these genes in their role in tolerance against colistin. *P. aeruginosa* wild-type PAO1/*lasB-gfp*, and type IV pili mutant PAO1 Δ *pilA*/*lasB-gfp*, both carrying *lasB-gfp* translational fusion, a quorum sensing reporter (Hentzer et al 2002), were cultivated 72 h for biofilm development, followed by treatment with colistin for 24 h. *LasB-gfp* expression was observed in PAO1/*lasB-gfp* biofilms, but not in PAO1 Δ *pilA*/*lasB-gfp* biofilms, indicating that type IV pili motility precedes QS for the colistin-tolerant subpopulations development in biofilms (fig 3-6a). Apart from using gene-mutation approach to validate the findings, direct chemical measurement approaches were carried to quantify the QS-regulated virulence factors in the effluents collected from the flow chamber. QS-regulated virulence factors; OdDHL, elastase and pyocyanin were highly expressed in effluents from colistin-treated PAO1 biofilms, but not in the effluents from control PAO1 biofilms (fig 3-6b, c, d). However, both colistin-treated and control PAO1 Δ *las*/ Δ *rhII* biofilms have no significant difference in expression of OdDHL, elastase and pyocyanin (fig 3-6b, c, d). This provides further evidence to support the findings from the gene-mutation and pulsed-SILAC approaches that QS systems are highly upregulated in colistin-tolerant subpopulations.

Figure 3-6. The upregulation of QS-regulated virulence factors in colistin-tolerant subpopulations. (a) 72 h old PAO1/*lasB-gfp* and PAO1 $\Delta pilA$ /*lasB-gfp* biofilms were exposed to 10 μ g ml⁻¹ colistin for 24 h. Strong expression of *lasB-gfp* was observed in PAO1/*lasB-gfp* biofilms but not in PAO1 $\Delta pilA$ /*lasB-gfp* biofilms. Green fluorescence represents the expression of *lasB-gfp* translational fusion, while red or yellow fluorescence represent dead cells. The results were presented as

representative CLSM image of each condition from 3 individual experiments. Scale bar, 50 μm . The increased production of OdDHL (b), elastase (c) and pyocyanin (d) in cells from colistin-treated biofilms compared to cells from control biofilms. The results were presented as mean $\pm$ s.d from 3 individual experiments. (* $P < 0.01$, Student's t-test). Reproduced with permission from (Chua et al 2016).

It was hypothesized that the migration of the colistin-tolerant cell aggregates to the top of the dead microcolonies in colistin-treated biofilms was due to the need for acquisition of iron since iron is vital for *P. aeruginosa* to sustain growth (Andrews et al 2003). *P. aeruginosa* utilized several strategies to uptake iron including; producing siderophores (pyoverdine and pyocyanin) to uptake extracellular iron(3+) (Lamont et al 2002); or heme uptake system (Ochsner et al 2000); or feo system (Wandersman and Delepelaire 2004); or uptake of xenosiderophore (Traxler et al 2012). It has been demonstrated that the available iron in the environment enhanced *P. aeruginosa* biofilm resistance against antibiotics (Oglesby-Sherrouse et al 2014). Hence, 2,2-dipyridyl (DIPY), an iron-chelator (Chua et al 2014), was introduced to the colistin monotherapy to treat the biofilms. Depleting the availability of iron in the colistin-containing nutrient medium was found to repress the development of these colistin-tolerant subpopulations (fig 3-7), supporting the hypothesis that the cell aggregates migrate to the top of dead microcolonies in search for iron availability. These observations were in agreement with previous study where the addition of iron chelator to the antibiotic treatment decreased the dispersed cells survivability in the flow chamber system (Chua et al 2014).

Figure 3-7. Presence of iron-chelator affects the migration of colistin-tolerants cell aggregates to the top of dead microcolonies. The 72 h old PAO1 flow chamber biofilms were challenged with; 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ DIPY; or 10 $\mu\text{g ml}^{-1}$ and 100 $\mu\text{g ml}^{-1}$ DIPY for 48 h. Green and red fluorescence represent live and dead cells respectively. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 10 μm . Reproduced with permission from (Chua et al 2016).

Figure 3-8. Proposed model of colistin-tolerant cells development in *P. aeruginosa* biofilms. (a) The treatment of *P. aeruginosa* biofilm with colistin kills majority of the biofilms, leaving a few live cells. (b) The live cell populations grow in number and migrate to the top of the dead microcolonies via type IV pili-mediated motility for iron. (c) The colistin-tolerant cells aggregate at the top of the dead microcolonies and upregulate QS signalling molecules and QS-mediated virulence factors. Reproduced with permission from (Chua et al 2016).

3.4 Discussion

The development of colistin-tolerant subpopulations in *in vitro* flow chamber biofilms have been described by several studies (Chiang et al 2012, Haagensen et al 2007, Pamp et al 2008). In this study, we monitored the dynamic development of colistin-tolerant subpopulations in the biofilms for 32 h and tracked the colistin-tolerant cells throughout the development process (fig 3-1, supplementary video A1, A2, A3 and A4). In agreement with (Haagensen et al 2007), *pmr* operon was only highly active in colistin-tolerant cells in the presence of colistin (fig 3-2a). The development of colistin-tolerant subpopulations is not due to genetic mutations caused by colistin as no convergent non-synonymous mutation was observed (supplementary table A1) and this colistin-tolerant phenotype can be reverted back to wild-type phenotype (fig 3-2b).

We applied a non-destructive pulsed-SILAC to understand the proteome of the colistin-tolerant and colistin-sensitive subpopulations. This proteomic-approach is able to selectively label colistin-tolerant and colistin-sensitive subpopulations using heavy C13 L-lysine and light C12-L-lysine respectively (fig 3-4a). Through the use of pulsed-SILAC, many proteins have been identified to be crucial in the development of colistin-tolerant subpopulations (fig 3-4b, c). It was noted that proteins involved in type IV pili-mediated motility and QS-mediated virulence factors were highly expressed in colistin-tolerant cells (supplementary table A6), which were in agreement with other studies (Cummins et al 2009, Haagensen et al 2007).

Both type IV pili-mediated motility and QS-mediated virulence factors contribute to the development of colistin-tolerant subpopulations as the development of colistin-

tolerant subpopulations were affected in *PAO1ΔpilA* and *PAO1ΔlasIΔrhII* biofilms when challenged with colistin. The presence of colistin-tolerant subpopulations was deemed negligible in the *PAO1ΔpilAΔlasRΔrhIR* biofilms (fig 3-5). The type IV pili preceded the QS since no *lasB-gfp* was observed in type IV pili-null mutant biofilms after challenged with colistin (fig 3-6a). QS-mediated virulence factors; OdDHL, elastase and pyocyanin were strongly induced in these colistin-tolerant cells (fig 3-6b) was in agreement with (Cummins et al 2009).

Iron is required for *P. aeruginosa* biofilm development (Banin et al 2005, Yang et al 2007). Here, we demonstrated that these colistin-tolerant cell aggregates moved to the top of dead microcolonies via type IV pili-mediated motility for available iron (supplementary video A3 and A4, fig 3-1 and 3-7), and addition of DIPY to the colistin-containing medium led to the absence of colistin-tolerant subpopulations at the top of dead microcolonies (fig 3-7).

Here, we proposed a model of the colistin-tolerant subpopulations development in *P. aeruginosa* biofilms (fig 3-8). The colistin kills majority of the biofilms and a few colistin-tolerant cells survive (fig 3-8a). Next, these colistin-tolerant cells expand and migrate to the top of the dead microcolonies via type IV pili-mediated motility for available iron (fig 3-8b). The colistin-tolerant subpopulations at the top of the dead microcolonies highly expressed QS-mediated virulence factors including odDHL, elastase and pyocyanin (fig 3-8c).

CHAPTER 4. THE EFFECT OF *PSEUDOMONAS AERUGINOSA* C-DI-GMP SIGNALLING ON HOST-PATHOGEN INTERACTIONS

4.1 Introduction

Biofilm-associated infections have been associated to chronic infections, including infections in lung of cystic fibrosis patients (Hoiby et al 2010), endocarditis (Hoiby et al 1986) and medical devices such as catheters (Cross et al 1983, Tacconelli et al 2009). The presence of bacterial cell aggregates is often recognised on the surface of these medical devices or in the sputum of CF patients. Nevertheless, the physiology of these cell aggregates in the biofilm-associated infections has remained unclear. There is a lack of physiological characterization of the cell aggregates in the infection to address the impact of these cell aggregates on the infection. For example, ocular infection by microorganism has been the major cause for eye diseases and blindness (Whitcher et al 2001). The risk factors include wearing of contact-lens, trauma to the cornea or eye-related surgery (Green et al 2008). Bacterial keratitis is defined as an infection of the cornea [eye's most outer layer (fig 4a)] characterized by growing of bacteria and recruitment of polymorphonuclear leukocytes (PMNs) in the cornea tissue, which can lead to blindness.

Pseudomonas aeruginosa is the major pathogen that contributes to approximately 40 - 70 % of the microbial keratitis cases in United States (Green et al 2008, Schornack et al 2008, Willcox et al 2004). There are increasing evidence demonstrating the importance of bacterial biofilms in ocular infections (Zegans et al 2002). *In vitro* studies showed *P. aeruginosa* clinical isolates produced more virulence factors than laboratory strains though both form biofilms on contact-lens

and are less susceptible to killing by contact-lens care products than planktonic cells (Sewell et al 2014, Szczotka-Flynn et al 2009). However, these *in vitro* studies showed indirect evidence to demonstrate the presence of *P. aeruginosa* biofilm during *P. aeruginosa* keratitis. Recently, there are *in vivo* studies showing the presence of cell aggregates on the mice corneas that were infected by *P. aeruginosa* (Tam et al 2011). However, these studies are unable to address the physiological state of the *P. aeruginosa* cell aggregates in the ocular infection. The studies of the bacterial physiology during the infections, allow further understanding of the bacterial pathogenesis in the ocular infection.

The development of *P. aeruginosa* biofilm is regulated by intracellular c-di-GMP contents, which in turn, is regulated by diguanlyate cyclases (DGCs) and phosphodiesterases (PDEs) (Hengge 2009). However, it remains unclear whether c-di-GMP is involved in the biofilm formation during the ocular infections and whether any difference in intracellular c-di-GMP content in *P. aeruginosa* has any impact on the ocular infections.

In this chapter, we systematically characterize the role of *P. aeruginosa* intracellular c-di-GMP signalling in mouse *Pseudomonas* keratitis model. Transcriptomics data showed that *P. aeruginosa in vivo* cells have different physiology compared to *in vitro P. aeruginosa* planktonic and biofilm cells. The synthesis of c-di-GMP was induced in *P. aeruginosa* during ocular infection, showing the physiology of the biofilm in the mouse ocular infection model. Using *P. aeruginosa* strains with different intracellular c-di-GMP contents, we showed that c-di-GMP is necessary for the microcolonies establishment in mouse ocular infection model.

Figure 4a. Schematic diagram showing the anatomy of the eye. Diagram taken from National Eye Institute, National Institutes of Health (NIH) (<https://nei.nih.gov/health/cornealdisease>).

4.2 Materials and Methods

4.2.1 Bacterial strains and culture media

The *P. aeruginosa* bacterial strains used in this chapter are listed in table 4-1. Bacterial strains are grown in LB medium with; 30 $\mu\text{g ml}^{-1}$ gentamycin (Gm), 50 $\mu\text{g ml}^{-1}$ tetracycline (Tc), 100 $\mu\text{g ml}^{-1}$ streptomycin (Strep) or 200 $\mu\text{g ml}^{-1}$ carbenicillin (Cb) added appropriately for marker selection. Primers used for generation of *P. aeruginosa* mutants are listed in table 2.

Table 4-1. List of *P. aeruginosa* strains used in chapter 4.

Strains	Relevant characteristic(s)	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototypic wild-type strain	(Holloway and Morgan 1986)
PAO1 Δ <i>wspF</i>	<i>wspF</i> derivative of PAO1 constructed by allelic exchange	(Rybtke et al 2012)
PAO1 Δ <i>siaD</i>	<i>siaD</i> derivative of PAO1 constructed by allelic exchange	(Chua et al 2015b)
PAO1 Δ 5442	PA5442 derivative of PAO1 constructed by allelic exchange	This study
PAO1/p _{lac} - <i>yhjH</i>	Tc ^r ; PAO1 containing the p _{lac} - <i>yhjH</i> vector	(Chua et al 2013)
PAO1/p _{BAD} - <i>yhjH</i>	Gm ^r ; PAO1 containing the p _{BAD} - <i>yhjH</i> vector	(Chua et al 2013)
PAO1/p _{cdrA} - <i>gfp</i>	Gm ^r ; PAO1 carrying the p _{cdrA} - <i>gfp</i> reporter	(Rybtke et al 2012)

PAO1-miniTn7- <i>gfp</i>	Gm ^r ; PAO1 tagged by the miniTn7- <i>gfp</i>	(Chua et al 2013)
PAO1 Δ <i>wspF</i> -miniTn7- <i>gfp</i>	Gm ^r ; Δ <i>wspF</i> tagged by the miniTn7- <i>gfp</i>	(Chua et al 2013)
PAO1/p _{<i>lac</i>} - <i>yhjH</i> -miniTn7- <i>gfp</i>	Gm ^r ; Tc ^r ; PAO1/p _{<i>lac</i>} - <i>yhjH</i> tagged by the miniTn7- <i>gfp</i>	(Chua et al 2013)
PAO1/p _{<i>BAD</i>} - <i>yhjH</i> -miniTn7- <i>gfp</i>	Gm ^r ; Strep ^r ; PAO1/p _{<i>BAD</i>} - <i>yhjH</i> tagged by the miniTn7- <i>gfp</i>	(Chua et al 2013)
PAO1/ <i>lasB</i> - <i>gfp</i>	Gm ^r ; PAO1 carrying the miniTn5- <i>lasB</i> - <i>gfp</i> reporter	(Hentzer et al 2002)
PAO1/ <i>exoT</i> - <i>gfp</i>	Gm ^r ; PAO1 carrying the miniTn7- <i>exoT</i> - <i>gfp</i> reporter	(Almblad et al 2015)

Table 4-2. List of primers used for constructing the deletion mutants in chapter 4.

PA5442-1	AGCTCGGTACCCGGGTGAACTGCTGGATGTTCTCG
PA5442-2	TTCAACGAGCCTGGCGCAAAGTCATAGGCGCTTGTTCTTG
PA5442-3	CAAGAACAAGCGCCTATGACTTTGCGCCAGGCTCGTTGAA
PA5442-4	CGACGGCCAGTGCCAATCTTTTCCGGTAGCGCTTC
PA5442-F	AACGGTAGTTCTGCTCCAG
PA5442-R	CTCGCCCTCGAAGAATTCTG

Table 4-3. List of primers for qRT-PCR analysis used in chapter 4.

pelA-F	GACAACCTGGATCGCTGCACG
pelA-R	CTTGAGTTTCTGCGACAGCC
cupA1-F	TATCACATTTCAGCGGCGAAG
cupA1-R	TCGGTGAGTTGCAGGGTGAAG
dipA-F	AAGAACCACGAGCCTTACTG
dipA-R	CCAGCTTGTTCTGCGTGATG
nbdA-F	CGGCATTCAACGAAGTGTTTC
nbdA-R	TCGTTGATCCGCTTGAAGTG
PA0575-F	CCGAGTACGCCATCCACTAC
PA0575-R	TGATCGGGGTGACCAAATCG
PA1181-F	TCAACAGCGACATCACCG
PA1181-R	CCGATGGAATACAGGGTCAC
PA2072-F	GGCATTTCTGTCTTCACCTATC
PA2072-R	CCTTCCAGCCACTTGACGAT
mucR-F	TCAGCCAGCCGTACCAGATA
mucR-R	CTGGTCCTTGGCGTGATACA
GAPDH-F	GACCGTTCCTCGACAAGTTC
GAPDH-R	TCAGCAGGCCTTCGAGATAG
proC-F	ATCAGTTGCTCCGGCTTCAG
proC-R	ATCGACGTGGTCGAGTCCAA
rpoD-F	GGGGCTGTCTCGAATACGTT
rpoD-R	GGGATACCTGACTTACGCGG

4.2.2 Mouse keratitis experiment

Female C57BL/6 (6-8 weeks old, 20-30 g) mice (The Jackson Laboratory) with healthy corneas were anesthetized subcutaneously with 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylene prior subjected to the scratching of cornea. Corneal scratches (n=3, each 1 mm long) were created using Beaver® miniblade (BD-Beaver, United States) without penetration the superficial stroma on the right eye while left eye remain as control (Cole et al 2005, Karicherla P 2010). Ten µl of the *P. aeruginosa* suspension, containing 1 x 10⁵ cells per µl, was topically added to the scratched cornea for infections to take place.

For imaging of the expression of quorum sensing PAO1/*lasB-gfp* and type III secretion system PAO1/*exoT-gfp* reporter fusion strains in mouse keratitis, the mice were infected with either PAO1/*lasB-gfp* or PAO1/*exoT-gfp* for 2 days before they are euthanized using 100 mg kg⁻¹ pentobarbital (i.p.). The infected corneas were dissected and stained with 20 µl of 5 µM SYTO62® Red Fluorescent Nucleic Acid Stain (ThermoFisher) for 15 min before viewing under CLSM using 63x objective lens and argon laser (excitation, 488nm) for GFP expression and helium laser (excitation, 633 nm) for red fluorescence observation. For controls, PAO1/*lasB-gfp* and PAO1/*exoT-gfp* reporter fusion strains were cultured in ABTGC medium using µ-Slide 8-well imaging chamber (ibidi, Germany) for 18 h in 37°C condition for biofilm development before staining by SYTO62® for 15 min and observation under CLSM for presence of GFP and red fluorescence.

For the observation of biofilm physiological state of PAO1 in mouse corneal, the mice corneas were infected with *P. aeruginosa* PAO1/*p_{cdrA}-gfp* and PAO1/*p_{lac}-gfp* strains. The infected corneas were dissected and stained with SYTO62® for 15 min

at 2 hour post-infection (hpi), 4 hpi, 8 hpi, 2 day post-infection (dpi), 4, dpi, and 7 dpi time-point for GFP and red fluorescence observation under CLSM. For planktonic controls, *P. aeruginosa* PAO1/p_{cdrA-gfp} and PAO1/p_{lac-gfp} strains were cultured in LB medium for 6 h (mid-log phase) at 37°C, 200 rpm shaking condition before staining by SYTO62® for 15 min and observation under CLSM for GFP and red fluorescence.

4.2.3 Construction of DGC mutants

The DGC PA5442 upstream and downstream sequences were obtained from *Pseudomonas* genome database (<http://www.pseudomonas.com/>). The primers used were listed in table 4-2. PA5442 were amplified using up-flanking fragments; PA5442-1 & PA5442-2 and down-flanking fragments; PA5442-3 & PA5442-4. The PCR products underwent purification and assembly with *Bam*HI and *Hind*III-digested pK18 (Gm^r) plasmid using Gibson Assembly Master Mix (New England Biolab®, United States), followed by transformation into *E. coli* DH5 α competent cells. The positive transformants were selected and cultured in LB medium containing 30 μ g ml⁻¹ gentamicin. The mutants were generated by triparental conjugation using RK600. For confirmation, the mutants were amplified with primers PA5442-F and PA5442-R, followed by sequencing. Other mutants were generated in the same procedure.

4.2.4 *P. aeruginosa* colonizing pattern and viable cells in mouse

keratitis

Healthy female C57BL/6 mice corneas were infected by p_{lac} -*gfp*-tagged *P. aeruginosa* PAO1 wild-type, PAO1 Δ *wspF* mutant or the PAO1/ p_{lac} -*yhjH* strain as described above. For the observation of the *P. aeruginosa* strains colonizing pattern in the mouse keratitis, the mice corneas were sacrificed and dissected at 2 dpi and 7dpi as mentioned above. The infected corneas were stained 15 min with 1 μ M of LysoTracker® Red DND-99 (Thermo Fisher Scientific, United States), a red fluorescent stain that stain for lysosome and acidic organelles in eukaryotic cells, and approximately 0.165 μ M Alexa Fluor® 635 phalloidin (Thermo Fisher Scientific, United States), a far-red fluorescent stain that stains for F-actin in eukaryotic cells. The stained corneas were observed under CLSM for presence of LysoTracker® Red DND-99 (excitation 561 nm, emission 590 nm) and Alexa Fluor® 635 phalloidin (excitation 633 nm, emission 647 nm).

For enumeration of the viable bacterial cells in the mouse keratitis, the infected corneas were dissected at 2 dpi and 7 dpi, homogenized using micro pestle (Cat no. SIAL501ZZ0, sigma-aldrich, United States) and underwent bead beating using 3 mm solid-glass beads (Cat no. Z143928, sigma-aldrich, United States) by vortexing vigorously for 5 min to disrupt the bacterial microcolonies. The bacterial cells were then serially diluted, plated on LB agar and incubated overnight in 37°C for CFU count. The results were presented as mean $\pm$ s.d from 3 individual experiments.

4.2.5 Transcriptomic analysis

4.2.5.1 RNA preparation

Healthy female C57BL/6 mice were infected by PAO1 wild-type, PAO1 Δ wspF mutant or the PAO1/p_{lac}-yjhH strain as mentioned above. The infected corneas were dissected on 2 dpi and 7 dpi and total RNA, including mouse and bacterial RNA, was extracted using MiRNeasy mini kit (Qiagen, Netherlands). The RNA were subjected DNase treatment using TURBO DNA-free™ (Thermo Fisher Scientific, United States) prior to quality checking of RNA integrity using Qubit 2.0 Fluorometer (Invitrogen, United States) and Agilent 2200 TapeStation (Agilent Technologies, United States). Three biological replicates were used for the transcriptomic analysis for both 2 dpi and 7 dpi samples.

4.2.5.2 RNA sequencing and data analysis

RNA-sequencing (RNA-Seq) was conducted using Illumina RNA sequence technology. RNA libraries were created using TruSeq Stranded mRNA Sample Prep Kit (Illumina, United States) and were sequenced using Illumina HiSeq 2500 system (Illumina, United States) with a paired-end protocol and read lengths of 100 nucleotides (nt).

Approximately 20 million reads were obtained for each sample and the raw reads were processed by removal of the adaptor sequences and reads below 50 base-pair (bp). After which, the reads were mapped on *Pseudomonas aeruginosa* reference genome (www.pseudomonas.com) using CLC genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark). The following settings were used; minimum length fraction of 0.9,

maximum number of hits for a read of 1, maximum number of two mismatches and minimum similarity fraction of 0.8. To prevent any problem caused by 0, a constant of 1 was added to the raw transcript count value.

The raw count table of transcripts was input into DESeq R for differential gene expression analysis based on the negative binomial distribution (Love et al 2014). The raw counts were normalized against the sample library size, followed by identifying differentially expressed genes through negative binomial test. Transcripts were considered differentially expressed when the fold-change value was larger than 5 and P-value < 0.05 in the pairwise comparison. The transcripts were $\log_2(N+1)$ transformed before Principal Component Analysis (PCA).

4.2.6 qRT-PCR analysis

Total RNA was extracted from the infected mouse corneas, subjected to DNase treatment and RNA integrity quality check as described above. The two-step quantitative reverse transcriptase PCR (qRT-PCR) was performed on the RNA. SuperScript III First-Strand Synthesis SuperMix kit (Cat. No. 18080-400, Invitrogen) was used to synthesize First-Strand cDNA and the First-Strand cDNA was used as template for qRT-PCR together with SYBR Select Master Mix kit (Cat. No. 4472953, Applied Biosystems by Life Technologies) on an Applied Biosystems StepOnePlus Real-Time PCR System. The control endogenous genes; *rpoD*, *proC* and *GAPDH* were used (table 4-3). Primer pairs with efficiency between 90-110% were used for qRT-PCR (table 4-3). The specific single-product amplification was confirmed using

melting curve analysis. The results were presented as mean $\pm$ s.d from 3 individual experiments.

4.2.7 Accession Code

The mouse and bacterial RNA-Seq data generated in this chapter have been deposited in the NCBI Short Read Archive (SRA) database under the accession code SRP078905. The RNA-Seq data of *P. aeruginosa in vitro* planktonic cells, biofilm cells and dispersed cells were previously published (Chua et al 2014) and deposited in the NCBI Short Read Archive (SRA) database with the accession code SRP041868.

4.2.8 Ethic statement

The mouse keratitis model experiments were conducted in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, the guide for the Care and Use of laboratory animals (National Research Council) under SingHealth Institutional Animal Care and Use Committee (IACUC) protocol number #2014/SHS/901, SingHealth Institutional Biosafety Committee (IBC) approval number SHSIBC-2014-015 and under the supervision of SingHealth Experimental Medical Centre (SEMC).

4.3 Results

4.3.1 Transcriptomes of *in vivo* and *in vitro* *P. aeruginosa* biofilms

To understand the physiological state of *P. aeruginosa* in the mouse keratitis model, *P. aeruginosa* strains with different intracellular c-di-GMP contents were used for the mouse cornea infection model. Three *P. aeruginosa* strains, namely; PAO1 Δ *wspF*, cells with “locked-high” intracellular c-di-GMP contents due to the continuous activation of diguanlylate cyclase *wspR* (Hickman et al 2005); PAO1/*p_{lac}-yhjH*, cells with “locked-low” intracellular c-di-GMP contents due to the continuous expression of phosphodiesterase *yhjH* (Gjermansen et al 2005); and wild-type PAO1 were used to infect the mouse corneas for 2 and 7 days since biofilm-like *P. aeruginosa* cell aggregates were reported after 2 day post-infection (dpi) (Saraswathi and Beuerman 2015). The RNA of *in vivo* *P. aeruginosa* PAO1 Δ *wspF*, PAO1/*p_{lac}-yhjH* and PAO1 were extracted, purified and subjected to RNA-Seq via illumina Hiseq 2500 platform. Read number ranging from 404,204 to 1,192,838 were mapped to the *P. aeruginosa* PAO1 reference genome (supplementary table B1). Approximately 0.21 – 0.26 % of total fragments were mapped to *P. aeruginosa* genome for PAO1 from 2 dpi, while approximately only 0.02 – 0.05 % were mapped to *P. aeruginosa* genome for PAO1 from 7 dpi (supplementary table B1). Due to insufficient raw reads mapping to *P. aeruginosa* genome, 7 dpi samples were unable to be further analysed. This is probably because of the high abundant of raw reads from eukaryotic cells where approximately 29.02 – 34.72 % of total fragments were mapped to mouse from 7 dpi samples (supplementary table B1).

The transcriptomes of 2 dpi *in vivo* *P. aeruginosa* cells were then compared to the transcriptomes of the *in vitro* *P. aeruginosa* biofilm cells, dispersed cell, planktonic cell, that were characterized previously (Chua et al 2014). Interestingly, the principal component analysis (PCA) plot derived from transcriptomes of both *in vivo* and *in vitro* *P. aeruginosa* cells depicted a significant difference in physiology between *in vivo* and *in vitro* *P. aeruginosa* along PC1, while *in vivo* PAO1 Δ *wspF*, PAO1/*p_{lac}-yhjH* and PAO1 had similar physiology as *in vitro* biofilm cells along PC2 (fig 4-1a). The results from negative binominal test showed an upregulation of 348 genes (supplementary table B2) and downregulation of 301 genes (supplementary table B3) *in vivo* PAO1 compared to *in vitro* PAO1 biofilm. Of these upregulated genes, genes involved in type III secretion system (T3SS) [*exoT* (Log₂ Fold Change= 4.83), *exoS* (Log₂FC= 5.99), *pcrV* (Log₂FC= 5.58 and *pscF* (Log₂FC= 5.25)] were found highly expressed, in agreement with (Hauser 2009) that *P. aeruginosa* T3SS were involved in the injection of effector proteins into the host cells during infections (supplementary table B2). *P. aeruginosa* PA4834 – PA4837 cluster, involved in iron acquisition and are commonly found upregulated in sputum of CF patients (Beckmann et al 2005, Gi et al 2015, Palmer et al 2005), were also highly expressed in the *in vivo* PAO1 (supplementary table B2). In addition, other iron-uptake genes such as *hasR* (Log₂FC= 4.52), *hasAp* (Log₂FC= 6.51), *hasD* (Log₂FC= 4.62) and *phuR* (Log₂FC= 2.51) were highly upregulated in *in vivo* PAO1 (supplementary table B2). On the other hand, the downregulated genes include genes involved in quorum sensing [*lasB* (Log₂FC= -8.22), *chiC* (Log₂FC= -8.70), *rhIA* (Log₂FC= -4.15) and *hcnB* (Log₂FC= -5.83)] (supplementary table B3). PAO1/*lasB-gfp*, carrying GFP-based reporter fusions of the *lasB* gene; and PAO1/*exoT-gfp*, carrying GFP-based reporter fusions of the *exoT* gene, were used to infect the mouse corneas and

observed under CLSM to validate the findings from RNA-Seq analysis. In agreement with the RNA-Seq analysis, PAO1/*lasB-gfp* showed weak expression of green fluorescence in *in vivo* PAO1, while displayed a strong expression in *in vitro* PAO1 biofilms (fig 4-1b). In contrast, PAO1/*exoT-gfp* depicted an enhanced expression of green fluorescence in *in vivo* PAO1, but a weak expression in *in vitro* PAO1 biofilms (fig 4-1b).

Figure 4-1. Transcriptomic analysis of *P. aeruginosa* strains in *in vivo* and *in vitro* conditions. (a) PCA plot of *in vivo* *P. aeruginosa* (PAO1 Δ *wspF*, PAO1/*p_{lac}-yhjH* and PAO1 from mouse corneas after 2 dpi) and *in vitro* *P. aeruginosa* (biofilm cells, dispersed cells and planktonic cells) from (Chua et al 2014). The dotted line indicates the distinct difference between *in vivo* and *in vitro* *P. aeruginosa* strains. Experiments were performed with three technical replicates. (b) Expression of *lasB-gfp* reporter fusion and *exoT-gfp* reporter fusion in mouse cornea after 2 dpi and *in vitro* biofilms. Green fluorescence indicates the expression of GFP reporter fusion, and red fluorescence indicates the total biomass by SYTO62® stain. The results

were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 10 μ m.

4.3.2 Induction of c-di-GMP in *P. aeruginosa* during corneal infections

We demonstrated that *in vivo* *P. aeruginosa* (PAO1 Δ wspF, PAO1/*p_{lac}-yhjH* and PAO1) have similar physiologies as *in vitro* biofilm cells (fig 4-1a). Based on this transcriptomics analysis, we hypothesized that *P. aeruginosa* PAO1 might form biofilms on the infected mouse corneas as biofilm-like structure was also observed on mouse corneas previously described (Saraswathi and Beuerman 2015).

In vivo PAO1 were compared to *in vitro* planktonic cells to determine whether any features of biofilm can be identified in *in vivo* PAO1. Through negative binomial test for comparison of these two sets of data, there were upregulation of 330 genes (supplementary table B4) and downregulation of 414 genes (supplementary table B5) in *in vivo* PAO1 compared to planktonic cells. Genes involved in biofilm formation such as pel exopolysaccharide production [*pelA* (Log₂FC= 2.14), *pelB* (Log₂FC= 2.82), *pelC* (Log₂FC= 2.58), *pelD* (Log₂FC= 2.38) and *pelF* (Log₂FC= 2.45)] and cup fimbriae [*cupA1* (Log₂FC= 2.75) and *cupA2* (Log₂FC= 3.67)] were significantly upregulated in *in vivo* PAO1, suggesting the presence of *P. aeruginosa* biofilms on the infected mouse corneal after 2 dpi. These transcriptomics analysis were validated using qRT-PCR, showing the enhanced expression of *pelA* and *cupA1* transcripts in *in vivo* PAO1 compared to planktonic PAO1 (fig 4-2).

Figure 4-2. qRT-PCR of *P. aeruginosa* gene expression *in vivo* cells compared to *in vitro* planktonic cells. The results were presented as mean $\pm$ s.d from 3 individual experiments.

It is well-recognised that high intracellular c-di-GMP contents in *P. aeruginosa* resulted in the formation of biofilm, while the low c-di-GMP contents resulted in dispersion of biofilm (Hengge 2009). Therefore, to further verify the biofilm physiological state of PAO1 in the infected mouse corneas, we used PAO1/*p_{cdrA}-gfp*, PAO1 that harbour a *p_{cdrA}-gfp* c-di-GMP reporter fusion (Rybtke et al 2012), to monitor the GFP expression during the mouse cornea infections. In planktonic state, there was a strong expression of GFP in PAO1/*p_{lac}-gfp* (fig 4-3a), but a weak GFP expression in PAO1/*p_{cdrA}-gfp* (fig 4-3b), in agreement with previous studies (Chua et al 2014, Rybtke et al 2012) that planktonic cells has low level of intracellular c-di-GMP compared to biofilm cells. PAO1/*p_{cdrA}-gfp*-infected mouse corneas were

dissected and observed at time-point 2 hpi, 4 hpi, 8 hpi, 2 dpi 4 dpi and 7 dpi (fig 4-3d - i). Undetectable green fluorescence was observed in uninfected scratched mouse cornea (supplementary fig B1). Green-fluorescent PAO1/*p_{lac}-gfp* cells aggregates were observed at 8 hpi (fig 4-3c). Interestingly, PAO1/*p_{cdrA}-gfp* GFP expression can be observed 2 h after infection (fig 4-3d), suggesting the dynamic of PAO1 in enhancing intracellular c-di-GMP contents and transiting its physiology from planktonic mode to biofilm mode in short period of time after the infection takes place. GFP expression of PAO1/*p_{cdrA}-gfp* were observable at 2 and 4 dpi (fig 4-3g – h), but GFP expression were reduced at 7 dpi (fig 4-3i) that could likely be due to the killing of bacterial cells by the mouse immune system.

Figure 4-3. C-di-GMP reporter fusion p_{cdrA} - gfp expression in *P. aeruginosa* during *P. aeruginosa*-induced mouse ocular infections. The confocal images depicted the GFP expression from controls; planktonic PAO1/ p_{lac} - gfp (a); planktonic PAO1/ p_{cdrA} - gfp (b); and PAO1/ p_{lac} - gfp at 8 hpi (c). GFP expression of PAO1/ p_{cdrA} - gfp were observed in the mouse corneas at; 2 hpi (d); 4 hpi (e); 8 hpi (f); 2 dpi (g); 4 dpi (h); and 7 dpi (i). The green fluorescence represents the expression of GFP from PAO1/ p_{lac} - gfp or PAO1/ p_{cdrA} - gfp , while the red fluorescence represents the total biomass including

cornea cells and bacterial after staining by SYTO62® stain. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 10 μ m. (This figure was co-authored with NTU School of Biological Sciences Graduate Student, Dr Thet Tun Aung)

Pseudomonas aeruginosa intracellular c-di-GMP content is regulated by the activities of DGCs and PDEs in response to the environmental condition. Hence, to determine which DGC(s) is/are involved in the synthesis of intracellular c-di-GMP during the mouse cornea infections, the transcriptome of *in vivo* PAO1 cells from 2 dpi were compared with transcriptome of *in vitro* PAO1 planktonic cells. The transcriptomic analysis results showed DGC *siaD* ($\text{Log}_2\text{FC}= 2.8$) and PA5442 ($\text{Log}_2\text{FC}= 1.39$) were upregulated in *in vivo* PAO1 cells compared to *in vitro* planktonic PAO1 (supplementary table B4). To further validate the involvement of *siaD* and PA5442 in the synthesis of c-di-GMP during ocular infection, *p_{cdrA}-gfp* were inserted into both PAO1 Δ *siaD* and PAO1 Δ PA5442 mutants for mouse cornea infections. The results showed the expression of GFP in mouse corneas infected by PAO1 Δ *siaD*/*p_{cdrA}-gfp* and PAO1 Δ PA5442/*p_{cdrA}-gfp* at 2 dpi (fig 4-4), suggesting that the activities of DGC *siaD* and PA5442 are likely to contribute partially to the enhanced intracellular c-di-GMP pool during ocular infections.

Figure 4-4. Confocal images of mouse corneas infected by PAO1 Δ *siaD*/*p_{cdrA}-gfp* or PAO1 Δ 5442/*p_{cdrA}-gfp* for 2 days. Green fluorescence represents the expression of *p_{cdrA}-gfp*, while the red fluorescence represents the total biomass stained by SYTO62® stain. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 10 μ m.

We further investigate the activities of PDEs in the *in vivo* PAO1 by comparing the transcriptomes of *in vivo* PAO1 and *in vitro* planktonic PAO1. Interestingly, several PDE-coding genes including *dipA* (PA5017) ($\text{Log}_2\text{FC} = -1.87$), *mucR* ($\text{Log}_2\text{FC} = -1.25$),

nbdA (PA3311) (Log₂FC= -3.08), PA0575 (Log₂FC= -3.64), PA1181 (Log₂FC= -1.78), and PA2072 (Log₂FC= -2.70) were found downregulated in *in vivo* PAO1 (supplementary table B5). qRT-PCR analysis results validated the findings from the transcriptomics analysis, showing less *dipA*, *mucR*, *nbdA*, PA0575, PA1181 and PA2072 transcripts in *in vivo* PAO1 cells compared to *in vitro* planktonic cells (fig 4-2). These results suggested that the downregulation of PDE-coding genes were likely contributed to the enhanced intracellular c-di-GMP content in *in vivo* PAO1 during the infections, which further supported that the *P. aeruginosa*-induced mouse keratitis is a biofilm-associated infection.

4.3.3 *P. aeruginosa* high intracellular c-di-GMP content led to the formation of microcolonies during corneal infection

P. aeruginosa biofilm cells have high tolerance towards killing by polymorphonuclear leukocytes (PMNs) and respond aggressively to the PMNs as demonstrated previously (Alhede et al 2009, Bjarnsholt et al 2005). The biofilm “features” such as increased *pel* and *cupA1* gene expression in *in vivo* cells from transcriptomics analysis and the enhanced synthesis of c-di-GMP in *in vivo* cells from confocal imaging of *p_{cdrA}-gfp*, strongly suggested *P. aeruginosa* biofilms presence in the *P.aeruginosa*-induced mouse keratitis. We hypothesized that the different level of intracellular c-di-GMP contents are likely to affect the outcome of the mouse keratitis.

The mouse cornea was infected with *P. aeruginosa* GFP-tagged PAO1Δ*wspF*, PAO1/*p_{lac}-yhjH* or PAO1 for 2 and 7 day prior to observation under CLSM for the bacterial colonization pattern. Interestingly, both PAO1 and PAO1Δ*wspF* formed

huge and densely-packed microcolonies at 2 dpi, while PAO1/*p_{lac}-yhjH* microcolonies seem to be less dense and fewer cells were observed compared to PAO1 and PAO1 Δ *wspF*. The PMNs, stained red by LysoTracker[®] Red DND-99, seem able to penetrate into the PAO1/*p_{lac}-yhjH* microcolonies, while they seem only surrounding PAO1 and PAO1 Δ *wspF* microcolonies. However, at 7 dpi, PAO1 Δ *wspF* densely-packed microcolonies were still visible, while fewer PAO1 were observed in the mouse corneas. PAO1/*p_{lac}-yhjH* was not detectable under CLSM at 7 dpi, suggesting that most of the cells might be killed by the host immune system. To verify the observations obtained from CLSM, the bacterial cells were harvested from the infected corneas at 2 and 7 dpi and were plated on LB agar for CFU count. The number of viable PAO1 and PAO1 Δ *wspF* were approximately 1×10^8 cells per cornea, which were approximately 100 folds more than PAO1/*p_{lac}-yhjH*, at approximately 1×10^6 cells per cornea at 2 dpi (fig 4-5g). Interestingly at 7 dpi, harvested PAO1 and PAO1/*p_{lac}-yhjH* were approximately 5×10^6 and 5×10^3 cells per cornea respectively, while there were about 5×10^7 PAO1 Δ *wspF* per cornea (fig 4-5g). These results suggested that *P. aeruginosa* cells with high intracellular c-di-GMP contents have higher tolerance to killing by PMNs than cell with low intracellular c-di-GMP contents and these could likely be due to the presence of biofilm matrix that interfered the killing activities of PMNs toward the biofilm cells.

Figure 4-5. The microcolonies and viable GFP-tagged *P. aeruginosa* strains in mouse corneas at 2 and 7 dpi. The mouse corneas were infected with; PAO1; or PAO1 Δ *wspF*; or PAO1/*p_{lac}-yhjH* and were harvested at 2 dpi (a – c) and 7 dpi (d – f). *P. aeruginosa* cells appeared green due to the tagged by the *p_{lac}-gfp* at their chromosome, while the PMNs appeared red due to the staining by LysoTracker[®] Red DND-99. The yellow fluorescence represents the Alexa Fluor[®] 635 phalloidin, which stains F-actin of the eukaryotic cells. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 20 μ m. (g) The CFU count of PAO1, PAO1 Δ *wspF* and PAO1/*p_{lac}-yhjH* harvested from the infected mouse corneas at 2 and 7 dpi. The results were presented as mean $\pm$ s.d from 3 individual experiments. (**P* < 0.01, One-way ANOVA). (This figure was co-authored with NTU School of Biological Sciences Graduate Student, Dr Thet Tun Aung)

4.4 Discussion

It is well-recognised that biofilm-associated infections often lead to persistent, chronic infections and are difficult to be cleared by host immune system. There are many *in vitro* studies that showed bacterial biofilms are highly tolerance to antibiotics and phagocytic cells of the host immune systems (Costerton et al 1999a, Donlan and Costerton 2002, Høiby et al 2011). However, the strategies to control the biofilm development are limited due to the lack of characterization of the *in vivo* biofilms.

In this chapter, the *in vivo* *P. aeruginosa* cells were characterized in mouse ocular infection model. Here, we have demonstrated the physiological evidence of *P. aeruginosa* forming biofilms at the site of infection. The transcriptomics analysis and PCA plot (fig 4-1a) showed the physiological differences between *in vivo* cells and *in vitro* cells. Quorum sensing-mediated genes were downregulated in *in vivo* PAO1 cell compared to planktonic cells (fig 4-1b, supplementary table B3). Similar observations were noted in the study by (Hammond et al 2016) that approximately 21 % of the clinical isolates from patients' cornea lacked functional *lasR*, which led to deficiency in quorum sensing-mediated genes expression and were associated with enhanced production of cupA fimbriae. Furthermore, infections caused by LasR-deficient isolates were associated with worse vision outcome (Hammond et al 2016). In contrast, genes involved in T3SS were upregulated in *in vivo*, but not *in vitro* biofilm conditions (fig 4-1b, supplementary table B2). Thus, these suggested that characterizing the physiology of the *in vivo* PAO1 cells is vital, as results from *in vitro* studies might not reflect the actual findings from *in vivo* studies.

Here, we have provided the physiological evidence of *P. aeruginosa* biofilms in the ocular infections by using *p_{cdrA}-gfp* reporter, indicating the expression of intracellular

c-di-GMP as high c-di-GMP content leads to formation of *P. aeruginosa* biofilm (Hengge 2009). We also demonstrated the dynamic of *in vivo* PAO1 cells whereby the synthesis of c-di-GMP was enhanced at 2 hpi and reduced intracellular c-di-GMP content at 7 dpi (fig 4-3). It is most likely that the downregulation of several PDEs led to the increased pool of c-di-GMP in the cells during the infections (fig 4-2).

The enhanced synthesis of intracellular c-di-GMP is important for the formation of microcolonies and survival of *P. aeruginosa* in the mouse ocular infections. Our results showed that the PMNs were able to penetrate into PAO1/*p_{lac}-yhjH* microcolonies but not into PAO1 and PAO1 Δ *wspF* microcolonies at 2 dpi (fig 4-5a, b, c). We have also demonstrated the ability of the host immune system in clearing the bacterial of different intracellular c-di-GMP contents. The host immune system was able to eliminate PAO1/*p_{lac}-yhjH* more efficiently than PAO1 and PAO1 Δ *wspF* during the infections (fig 4-5g). *P. aeruginosa* clinical isolates from lungs of CF patients have phenotype variants that mostly have high c-di-GMP contents (Lam et al 1980, Starkey et al 2009). Here, a “locked-high” c-di-GMP-containing PAO1 Δ *wspF* was used for mouse ocular infections. Unlike PAO1/*p_{lac}-yhjH*, PAO1 Δ *wspF* formed density-packed microcolonies at the site of infections and the PMNs seemed unable to penetrate into the microcolonies (fig 4-5b, e). This phenomenon was also observed previously in chronically *P. aeruginosa*-infected cystic fibrosis lungs (Bjarnsholt et al 2009). PAO1 Δ *wspF* were less susceptible to killing by the host immune systems compared to PAO1/*p_{lac}-yhjH* (fig 4-5g). This could be due to the biofilm-associated properties such as the enhanced production of pel exopolysaccharide, cup fimbriae, iron-acquisition genes and T3SS as shown in the transcriptomics analysis from mouse ocular infections (supplementary table B4).

Therefore, these biofilm-associated properties likely contributed to the decreased killing efficacy of host immune system against the biofilm cells. Interestingly, only pel exopolysaccharide, but not psl exopolysaccharide [*pslF* ($\text{Log}_2\text{FC} = -2.84$), *pslG* ($\text{Log}_2\text{FC} = -2.63$), *pslB* ($\text{Log}_2\text{FC} = -1.95$)] (supplementary table B4), was found upregulated in the *in vivo* cells. *P. aeruginosa* psl exopolysaccharide is crucial for biofilm structure and protects the bacterial against phagocytosis by neutrophils (Ma et al 2006, Ma et al 2009, Mishra et al 2012). In contrast, pel exopolysaccharide is required for biofilm structure scaffold and protect bacterial against antibiotic (Colvin et al 2011, Yang et al 2011a), however it remains unclear on how pel exopolysaccharide is involved in the protection against host immune system. One of the future recommendations is to determine the role of pel exopolysaccharide in the *P. aeruginosa*-induced mouse keratitis.

CHAPTER 5. TARGETED APPROACHES IN ERADICATION OF COLISTIN-TOLERANT SUBPOPULATIONS IN PSEUDOMONAS AERUGINOSA BIOFILMS IN *IN VITRO* AND *IN VIVO* MODELS

5.1 Introduction

Bacterial biofilms are able to withstand and survive under high concentrations of antibiotic treatment. The bacterial in biofilm mode is a major public health concern as it can cause chronic infections such as *P. aeruginosa* biofilms in the lungs of cystic fibrosis patients and patients with *Pseudomonas* keratitis. While aggressive antibiotics therapies are often given to the patients to manage the biofilm-associated infections, the recalcitrance of bacterial biofilms likely to occur when antibiotics treatments are halted.

As described in chapter 3, colistin alone is unable to eliminate the *P. aeruginosa* biofilms completely in the flow chamber experiment due to the development of colistin-tolerant subpopulations. These colistin-tolerant subpopulations are metabolically active and have increased efflux pump activities. These colistin-tolerant cell aggregates required type IV pili-mediated motility to move to the top of dead microcolonies to acquire iron. Furthermore, quorum sensing is important in the colistin-tolerant subpopulations development as there is an increased in expression of QS-mediated virulence factors in these colistin-tolerant cells.

In chapter 4, we demonstrated the physiology of the *P. aeruginosa* in the *P. aeruginosa*-induced keratitis in mouse ocular infection experiment, showing evidence of the presence of biofilms in the infection sites via *P. aeruginosa in vivo* cells

transcriptomics analysis and c-di-GMP reporter. The level of intracellular c-di-GMP affects the fitness of the *P. aeruginosa* in the mouse keratitis model. *P. aeruginosa* cells with “locked-low” level of c-di-GMP contents are more susceptible to the mouse immune system than cells with “locked-high” c-di-GMP contents.

In this chapter, we designed targeted-approaches to eliminate *in vitro* and *in vivo* *P. aeruginosa* biofilms. Type IV pili-driven motility and QS-mediated virulence factors are required for the development of colistin-tolerant subpopulations in the biofilms as demonstrated in chapter 3. Therefore, we blocked the activity of type IV pili-mediated motility and QS signalling by addition of erythromycin, which is known to inhibit motility and QS in *P. aeruginosa* (Pérez-Martínez and Haas 2011), together with colistin, is able to eradicate and repress the development of colistin-tolerant subpopulations in *in vitro* flow chamber biofilms as well as *in vivo* mouse infection implant model. In addition, as demonstrated in chapter 4 that PAO1 with low intracellular c-di-GMP contents are susceptible to host immune system in mouse keratitis model. Therefore, we proposed to target *P. aeruginosa* intracellular c-di-GMP contents to manage the biofilms in both *in vitro* and *in vivo* experiments. Biofilm-dispersal agent and colistin combinatorial treatment was used to manage *P. aeruginosa* flow chamber biofilms and mouse *Pseudomonas* keratitis model.

5.2 Materials and Methods

5.2.1 Bacterial strains and culture media

The bacterial strains used in this chapter are listed in table 3. *P. aeruginosa* strains were cultivated in Luria-Bertani (LB) medium containing; 30 µg ml⁻¹ gentamycin (Gm) or 100 µg ml⁻¹ streptomycin (Strep) for marker selection in *P. aeruginosa*.

Table 5-1. List of *P. aeruginosa* strains used in chapter 5.

Strains	Relevant characteristic(s)	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototypic wild-type strain	(Holloway and Morgan 1986)
PAO1/p _{BAD} -yjhH	Gm ^r ; PAO1 containing the p _{BAD} -yjhH vector	(Chua et al 2013)
PAO1-miniTn7-gfp	Gm ^r ; PAO1 tagged by the miniTn7-gfp	(Chua et al 2013)
PAO1/p _{BAD} -yjhH - miniTn7-gfp	Gm ^r ;Strep ^r ; PAO1/p _{BAD} -yjhH tagged by the miniTn7-gfp	(Chua et al 2013)

5.2.2 Treatment of *P. aeruginosa* biofilms with colistin and erythromycin in flow chamber experiment.

PAO1-gfp biofilms were cultivated for 72 h in flow chamber with ABTG medium as described in chapter 3. The 72 h old biofilms were treated with 10 µg ml⁻¹ colistin or 100 µg ml⁻¹ erythromycin, or 10 µg ml⁻¹ colistin and 100 µg ml⁻¹ erythromycin for 48 h before staining with 300 µl of 20 µM PI to observe the dead cells. The biofilms were

viewed and images acquired using CLSM as described in chapter 3. The results were presented as mean $\pm$ s.d from 3 individual experiments.

5.2.3 Time-dependent killing of planktonic PAO1 with colistin and erythromycin.

The minimal inhibitory concentration of colistin and erythromycin for PAO1 is 1 $\mu\text{g ml}^{-1}$ and 100 $\mu\text{g ml}^{-1}$ respectively. 1×10^{10} PAO1 cells were cultivated in ABTGC medium containing 10 $\mu\text{g ml}^{-1}$ colistin or 100 $\mu\text{g ml}^{-1}$ erythromycin, or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ erythromycin at 37°C, 200 rpm shaking condition for 24 h. At 0 h, 1 h, 3 h and 24 h time-point, bacterial cells from each condition were serially diluted, plating on LB agar and incubated overnight in 37°C for CFU count. The results were presented as mean $\pm$ s.d from 3 individual experiments.

5.2.4 Mouse implant infection model.

Healthy 7-week-old female BALB/c mice (Taconic M&B A/S) were used in this study. *P. aeruginosa* PAO1 biofilms were developed on silicon tubings as described previously (Christensen et al 2007, Chua et al 2015a). Briefly, the sterile silicon tubes (3 mm X 5 mm Ø) were submerged into the 50 ml flask containing 20 ml of OD₆₀₀= 0.01 of overnight PAO1 culture in 0.9 % NaCl . The flasks were incubated at 37°C, 110 rpm shaking condition for 20 h for biofilm development onto the implants. The mice were anesthetized with 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylene prior to the insertion of implants into the peritoneum of the mice. The implants were

washed thrice with 1 ml of 0.9 % NaCl just before insertion into the peritoneum of the anesthetized mice to remove the unbound bacterial cells.

Each group of 5 mice were given; no antibiotic; or 1 mg kg⁻¹ colistin; or 10 mg kg⁻¹ erythromycin; or 1 mg kg⁻¹ colistin and 10 mg kg⁻¹ erythromycin at the opposite site of the peritoneum. The mice were euthanized using 100 mg kg⁻¹ pentobarbital (i.p.) after 1 day infections for harvesting of the implants and spleens. The implants were collected into Eppendorf tubes containing 1 ml of 0.9 % NaCl each and underwent water-bath sonication for 10 min using Elmasonic P120H (Elma, Germany; power = 50% and frequency = 37 KHz) and 3 sets of 10 s of vigorous vortexing to disrupt the biofilms. The spleens were collected and were homogenised with Bio-Gen PRO200 Homogenizer (Pro Scientific, US) at maximum power. The bacterial cells from implants and spleens were serially diluted, plated on LB agar and grew overnight in 37°C for CFU count. Five replicates for each group were performed and the results were shown as mean ± s.d.

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5.2.5 Eradication of biofilms with colistin and biofilm-dispersal agents in flow cell biofilm experiment

P. aeruginosa GFP-tagged PAO1/*p_{BAD}-yhjH* biofilms were grown in flow chamber as described in chapter 3. After 72 h for mature biofilm to develop, the biofilms were treated with; no treatment; or 10 µg ml⁻¹ colistin; or 0.01 % (w/v) arabinose; or 10 µg ml⁻¹ colistin and 0.01 % (w/v) arabinose for 1 day. After treatment, 300 µl of 20 µM PI stain were injected into each flow channel to stain the dead cells. GFP-expressing live cells and dead cells were imaged by using CLSM with an argon laser (488 nm,

emission 535 nm) and helium laser (561 nm, emission 617 nm) respectively. The live and dead biovolumes were obtained using IMARIS from each confocal image. The results were presented as mean $\pm$ s.d from 3 individual experiments.

5.2.6 Chemical treatment of corneal infections

Healthy female C57BL6 mice were infected by the *P. aeruginosa* PAO1/p_{BAD}-*yhjH* as described above in chapter 4. 48 mice were used for arabinose treatment study (n= 12, 4 groups) for different time points (1, 2, 3 and 4 dpi). PAO1/p_{BAD}-*yhjH*-infected mouse corneas were treated topically starting from 1 dpi with the following treatments (3 times per day); 10 μ l of 0.9 % (w/v) NaCl; or 10 μ l of 2 mg ml⁻¹ colistin; or 10 μ l of 1 % (w/v) arabinose in 0.9 % NaCl; or 10 μ l of 1 % (w/v) arabinose and 2 mg ml⁻¹ of colistin. The infected corneas of four mice from each group were euthanized, dissected and plated on LB agar daily to determine the numbers of viable bacteria present in the infected corneas as mentioned above in chapter 4. Log₁₀ number of CFU of bacteria were shown as results (Karicherla P 2010). One-way ANOVA with Post-hoc, Bonferroni test statistical analysis method was used to compare the differences in among the different group of animal experiments. P value $\leq$ 0.05 was considered statistically significant difference.

5.2.7 Ethic Statement

The mouse implant infection model experiments were performed in accordance to the NACLAR Guidelines and Animal and Birds (Care and Use of Animals for Scientific Purposes) Rules by Agri-Food & Authority of Singapore (AVA), with

authorization and approval by the Institutional Care and Use Committee (IACUC) and Nanyang Technological University, under the permit number A-0191 AZ.

All animal ocular infection experiments were conducted in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, the guide for the Care and Use of laboratory animals (National Research Council) under SingHealth Institutional Animal Care and Use Committee (IACUC) protocol number #2014/SHS/901, SingHealth Institutional Biosafety Committee (IBC) approval number SHSIBC-2014-015 and under the supervision of SingHealth Experimental Medical Centre (SEMC).

5.3 Results

5.3.1 Elimination of *in vitro* colistin-tolerant subpopulations via erythromycin and colistin combinatorial treatment

Colistin-tolerant subpopulations development in *P. aeruginosa* flow chamber biofilms required the type IV pili-mediated motility and QS-mediated virulence factors as shown in chapter 3. To prevent the development of these colistin-tolerant cells in the biofilm, erythromycin, a macrolide that was demonstrated to inhibit type IV pili assembly and quorum sensing activity against *P. aeruginosa* (Pérez-Martínez and Haas 2011), was used in combination with colistin. *P. aeruginosa* PAO1 biofilms treated with colistin showed the GFP-expressing cells at the top of the dead microcolonies, while treatment with erythromycin showed little killing of the biofilms (fig 5-1a, b). Interestingly, there is a strong synergistic killing effect of the biofilms in the colistin and erythromycin combinatorial treatment and negligible viable cells were detected (fig 5-1a, b). However, the same concentration of colistin and erythromycin combinatorial treatment used in flow chamber biofilms has no synergistic killing effect for planktonic PAO1 (fig 5-1c). These results suggested that using of erythromycin and colistin combinatorial treatment is a targeted-approach against colistin-tolerant subpopulations in PAO1 biofilms, but not planktonic PAO1.

a

Figure 5-1. Inhibiting type IV pili-mediated motility and QS signalling halt colistin-tolerant subpopulations development in *P. aeruginosa* biofilms in flow chamber. (a) The biofilms were cultured for 72 h with ABTG medium in flow chamber prior to treatment by; no treatment; or 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ erythromycin; or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ erythromycin for 48 h. Negligible amount of live cells were observed under CLSM. Green and red fluorescent represent live and PI-stained dead cells. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 50 μm . (b) The live/dead ratio of the biofilms. Biovolumes of live and dead cells from each confocal image were generated using IMARIS software. The results were presented as mean $\pm$ s.d from 3 individual experiments. (* $P < 0.01$, One-way ANOVA). (c) Time-dependent killing of planktonic PAO1. The planktonic PAO1 were treated with; no treatment; or 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ erythromycin; or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ erythromycin for 24 h. Cells were collected and plated on LB agar for CFU count on 0 h, 1 h, 3 h and 24 h time-point. The results were presented as mean $\pm$ s.d from 3 individual experiments. Reproduced with permission from (Chua et al 2016).

5.3.2 Combating *in vivo* PAO1 biofilms with erythromycin and colistin combinatorial treatment

To test the feasibility of using colistin and erythromycin combinatorial treatment in eliminating the PAO1 biofilms in *in vivo* condition; mouse infection implant model (Christensen et al 2007) was used. This animal model mimics the biofilms in clinic settings since biofilms of similar range of size were noted on *ex vivo* (Bjarnsholt et al

2009, Bjarnsholt *et al* 2013) and *in vivo* (Moreau-Marquis *et al* 2008, Moreau-Marquis *et al* 2009) conditions. The infected mice were given; no treatment; or 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ erythromycin; or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ erythromycin intraperitoneally (i.p) after the insertion of the biofilm-coated implant into the peritoneum. The CFU ml^{-1} of the bacterial harvested from the implant of the control group, erythromycin-treated group and colistin-treated group were approximately 1.5×10^7 , 2×10^6 and 2×10^5 respectively. Interestingly, no bacterial colony was detected from the harvested implants of colistin and erythromycin combinatorial treatment group (fig 5-2a).

The CFU ml^{-1} of the bacterial harvested from the spleens of the control group and erythromycin-treated group are approximately 5×10^7 and 1×10^4 respectively. No bacterial colony was observed in colistin-treated group and combinatorial treatment group, suggesting PAO1 is unable to spread to spleen with the presence of colistin (fig 5-2b). Thus, addition of tolerance-interfering compound to the conventional antibiotic treatment deem to be a potential new therapy for eradicating biofilm-associated infections.

a **b**

Figure 5-2. Targeting *P. aeruginosa* PAO1 type IV pili-mediated and QS signalling prevent colistin-tolerant cells development in mouse infection implant model. The mice with PAO1 biofilm-coated implants inserted into the peritoneum, were treated with; no treatment; or 10 $\mu\text{g ml}^{-1}$ colistin; or 100 $\mu\text{g ml}^{-1}$ erythromycin; or 10 $\mu\text{g ml}^{-1}$ colistin and 100 $\mu\text{g ml}^{-1}$ erythromycin (i.p). The implants (a) and spleen (b) were harvested, homogenised and plated on LB agar for CFU count after serial dilution. Experiments were performed with 5 replicates, and the results were shown as mean $\pm$ s.d. The dotted line indicates the limit of detection. (*P < 0.01, Student's T-test). Reproduced with permission from (Chua et al 2016).

5.3.3 Lowering the intracellular c-di-GMP content enhances the susceptibility of *in vitro* *P. aeruginosa* biofilms to colistin treatment

P. aeruginosa PAO1/*p_{lac}-yhjH* with “locked-low” intracellular c-di-GMP contents were more susceptible than PAO1 Δ *wspF* with “locked-high” intracellular c-di-GMP contents to host immune system as demonstrated in chapter 4. Therefore, to manage the biofilms, we proposed a targeted–approach to target *P. aeruginosa* intracellular c-di-GMP by lowering the intracellular c-di-GMP contents in biofilms and treatment with colistin since planktonic bacterial cells are known to be more susceptible to antibiotic than cells in biofilm.

To test the feasibility of lowering the bacterial c-di-GMP content with biofilm-dispersal agent and colistin to manage the biofilm, we used GFP-tagged PAO1/*p_{BAD}-yhjH* for this *in vitro* biofilm experiment. PAO1/*p_{BAD}-yhjH*, carry plasmids that contains an

arabinose-inducible *yhjH* (PDE), which breaks down c-di-GMP upon induction when arabinose binds to P_{BAD} promoter (Chua et al 2013). Thus, addition of arabinose to the medium results in lowering intracellular c-di-GMP content in PAO1/p_{BAD}-*yhjH* biofilm, which leads to biofilm dispersal. PAO1/p_{BAD}-*yhjH* biofilms were developed in flow chamber for 72 h. The 72 h old biofilms were then treated with; no treatment; or colistin; or arabinose or colistin and arabinose combinatorial treatment.

The biovolumes of the GFP-expressing cells in control group, arabinose-treated group and colistin-treated group were approximately $3.7 \times 10^6 \mu\text{m}^3$, $2.8 \times 10^6 \mu\text{m}^3$ and $8 \times 10^5 \mu\text{m}^3$. Negligible amount of GFP-expressing cells was observed in arabinose and colistin combinatorial treatment group (fig 5-3a - e). These results strongly suggested the synergistic biofilm-killing effect of the arabinose and colistin combinatorial treatment (fig 5-3a - e).

Figure 5-3. Lowering intracellular c-di-GMP content repressed colistin-tolerant subpopulation development in *P. aeruginosa* biofilms in flow chamber. The GFP-tagged PAO1/*p_{BAD}-yhjH* was grown with ABTG medium in flow chamber for 72 h. At 72 h time-point, the biofilms were treated with; no treatment (a); or 10 μg ml⁻¹ colistin (b); or 0.01 % arabinose (w/v) arabinose (c); or 10 μg ml⁻¹ colistin and 0.01 % arabinose (w/v) arabinose combinatorial treatment (d) for 24 h. After which, PI stain was injected into each flow channel and biofilms were observed under CLSM. The green and red fluorescent represent live and PI-stained dead cells respectively. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 20 μm. (e) Biovolumes of live and dead cells of each treatment condition were obtained using IMARIS software from each confocal image. The results were presented as mean ± s.d from 3 individual experiments. (**P < 0.01, One-way ANOVA).

5.3.4 Lowering the intracellular c-di-GMP content improves the effectiveness of colistin treatment of mouse cornea *P. aeruginosa* infections

The success in controlling the *in vitro* *P. aeruginosa* flow chamber biofilms with biofilm dispersal agent and colistin combinatorial treatment led to the feasibility testing of the effectiveness of this combinatorial treatment in controlling biofilm infections in *in vivo* experiment. For evaluating the *yhjH*-based c-di-GMP reduction for the treatment of mouse corneal infections, *P. aeruginosa* PAO1/*p_{BAD}-yhjH* was topically administered to the scratched mouse corneas as *P. aeruginosa* biofilm physiology was noted on the infected mouse corneal as demonstrated in chapter 4. The mouse corneas were infected with PAO1/*p_{BAD}-yhjH* for one day for biofilm to develop prior to the treatment (3 times per day) with; 0.9 % NaCl; or colistin; or arabinose; or colistin and arabinose combination treatment. Every day, 4 mice from each treatment group were sacrificed and bacterial cells were plated on LB agar as described in Materials and Methods. Treatment with colistin showed a log₁₀ reduction of 1.29, 1.08, and 2.62 on 2 dpi, 3 dpi and 4 dpi in the corneal bacterial loads, respectively, compared to the controls. The arabinose and colistin combination treatment had the best killing effect on PAO1/*p_{BAD}-yhjH*, with corneal bacterial loads significantly decreased, by log₁₀ reductions of 2.31, 2.41 and 3.07 on 2 dpi, 3 dpi and 4 dpi, respectively, compared to the controls. The results showed a significant reduction ($P < 0.05$, one-way ANOVA, Bonferroni test) in viable PAO1/*p_{BAD}-yhjH* in colistin and arabinose combination treatment group as compared to control, arabinose-alone and colistin-alone group (fig 5-4). Thus, suggested that therapy of lowering the intracellular c-di-GMP contents in *P. aeruginosa* biofilm by

dispersal agent and treatment with colistin is feasible in controlling *P. aeruginosa* in infected mouse cornea.

Figure 5-4. Quantification of viable PAO1/p_{BAD}-yjhH cells in *P. aeruginosa*-induced keratitis in mouse cornea after treatment. The PAO1/p_{BAD}-yjhH-infected mouse corneas were treated (3 times per day) with 10 μ l of respective chemicals; 0.9 % NaCl; or 10 μ g ml⁻¹ colistin; or 0.1 % (w/v) arabinose; or 10 μ g ml⁻¹ colistin and 0.1 % (w/v) arabinose. Four mice from each group were sacrificed daily for the harvesting of the infected corneas. The infected corneas were homogenized and the bacterial cells were plated on LB agar and incubated overnight in 37°C for CFU count. Experiments were performed with four replicates, and the results were shown as mean $\pm$ s.d. ($P < 0.05$, One-way ANOVA, Bonferroni test).

5.4 Discussion

The bacterial biofilms are highly tolerance to antibiotics and host immune systems. In recent years, many studies have recognised and understand the importance of the signalling pathways such as quorum sensing signalling and c-di-GMP signalling pathways involved in the development of biofilms (Fuqua et al 1994, Hengge 2009, Jenal and Malone 2006, Miller and Bassler 2001, Römling et al 2013, Singh et al 2000, Waters and Bassler 2005). Many studies have suggested targeting these signalling pathways for better controlling or eliminating of the biofilms in clinical or industrial settings (Barraud et al 2009a, Barraud et al 2009b, Chua et al 2014, Hentzer et al 2003, Høiby et al 2010a, Høiby et al 2011, Rutherford and Bassler 2012). Here, we provided evidence that the targeted-approaches against quorum sensing and c-di-GMP signalling pathways were deemed effective in controlling the *P. aeruginosa* biofilms in *in vitro* and *in vivo* studies.

In chapter 3, the colistin-tolerant subpopulations developed in the flow chamber experiments though the *P. aeruginosa* biofilms were treated with 10 times colistin MIC. We observed the colistin-tolerant subpopulations development dynamic and noticed that the surviving colistin-tolerant cells, expanded in number at early colistin treatment phase, followed by migration of these cell aggregates toward the top of dead microcolonies. Through pulsed-SILAC-approach, we noted that both QS activity and type IV pili-mediated motility were crucial in development of the colistin-tolerant subpopulations. Therefore, in this chapter, we designed a targeted-approach by targeting both QS system and type IV pili-mediated motility by addition of erythromycin to the colistin treatment process. This combinatorial treatment led to the repression and elimination of colistin-tolerant subpopulation in the flow chamber

PAO1 biofilm (fig 5-1). The feasibility of this combinatorial treatment against bacterial biofilm in *in vivo* model was verified using mouse implant infection model experiments since biofilms were often observed on the catheters or other medical devices in clinical settings. Given non-lethal dosage of both erythromycin and colistin to the infected mice, led to the significant reduction of bacterial load on the biofilm-coated implants (fig 5-2). Therefore, interfering the development of colistin-tolerant subpopulation by supplementing erythromycin or other tolerant-interfering compound to the colistin treatment is found to be a promising therapy to clear the biofilm-associated infections.

In chapter 4, we did comparative transcriptomics analysis of *in vivo* PAO1 and *in vitro* PAO1 and found that *in vivo* PAO1 has features of biofilm, which include upregulation of *pel*, *cupA* and *cdrA* gene expression. Thus, we hypothesized that PAO1 biofilms are likely to be developed on the infected mouse corneas. C-di-GMP, an important bacterial secondary messenger, is crucial in regulating biofilm formation, cell cycle, virulence factors, motility and other cellular functions. We demonstrated the physiology of the biofilms on the infected cornea via c-di-GMP reporter. Moreover, we found that mouse immune systems are able to eliminate PAO1 with low intracellular c-di-GMP contents are more efficiently than wild-type PAO1 or PAO1 with high c-di-GMP concentration during the infections.

Hence, in this chapter, we hypothesized that by lowering the intracellular c-di-GMP content in PAO1, together with conventional antibiotic, we can eradicate the PAO1 biofilm-associated infections. This concept was demonstrated in *in vitro* PAO1/*p_{BAD}-yhjH* biofilm where intracellular c-di-GMP content was lowered via genetic-approach using arabinose-inducible PDE *yhjH*. The biofilm-dispersal agent and colistin

combinatorial treatment significantly reduced the colistin-tolerant subpopulations in the flow chamber PAO1/*p_{BAD}-yhjH* biofilm (fig 5-3). The combinatorial treatment feasibility test was carried out in the mouse *Pseudomonas* keratitis model. The treatment group that was given non-lethal dosage of combinatorial treatment has significant reduced bacterial load in the infected corneas compared to the single-compound treatment group (fig 5-4). Hence, supplementing biofilm-dispersal or c-di-GMP-lowering agent to the conventional colistin treatment was found to be promising in elimination of biofilm-associated keratitis or perhaps other biofilm-associated infections.

CHAPTER 6. CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 *Summary and Conclusions*

The quorum sensing and c-di-GMP signalling are the two major signalling pathways in *P. aeruginosa* that regulate a wide range of cellular functions in order for the bacterial to adapt and survive in different environmental conditions. This study focused on the investigation of the effects of *P. aeruginosa* quorum sensing and c-di-GMP cell signalling in *in vitro* experiments as well as on host-pathogen interactions during infections.

Role of quorum sensing and type IV pili in colistin-tolerant subpopulations.

The development of colistin-tolerant subpopulations in *P. aeruginosa* biofilms were observed in several studies (Chiang et al 2012, Pamp et al 2008). However, none of these studies is able to address the mechanism for the development of these subpopulations during the colistin treatment though these studies have demonstrated that *pmr* gene and efflux pumps-encoding genes are highly upregulated in these colistin-tolerant subpopulations. In agreement with these studies, there was an increased expression of *pmr* when *P. aeruginosa* biofilms were exposed to colistin (fig 3-2). The development of these colistin-tolerant subpopulations was real-time monitored until 32 h upon exposure to colistin (fig 3-1a and supplementary video A1 - A4). There was a huge reduction in viable biofilm cells at the early phase of colistin treatment, followed by an increased in cell number at the late phase of treatment, suggesting the strong adaptability of the remaining

viable cells to the colistin-containing condition (fig 3-1b). Furthermore, viable cell aggregates, but not single cells, migrated towards the dead microcolonies, suggesting a coordinated social behaviour among these aggregate of cells (fig 3-1c). We demonstrated that the migration of these cell aggregates occurred due to the need for iron for survival (fig 3-7). Interestingly, the development of the colistin-tolerant cell aggregates was a tolerance strategy used by the bacterial as shown in DNA sequencing analysis (supplementary table A1) and these cells were able to revert from colistin-tolerance phenotype back to wild-type phenotype when colistin was depleted from the medium (fig 3-2b)

Using pulsed-SILAC (fig 3-4a), a powerful proteomics technique, proteins that were highly expressed in these colistin-tolerant subpopulations could be determined without causing any disturbance to the biofilms (Fig 3-4b, supplementary table A5 and A6). As expected, proteins involved in type IV pili-mediated motility were found highly upregulated in these tolerant cells (supplementary table A6) since functional type IV pili is necessary for development of mature biofilms (Barken et al 2008). QS-mediated virulence factors were also highly expressed in these tolerant cells (supplementary table A6). Gene-mutation approach validated the importance of type IV pili-mediated motility and quorum sensing-mediated virulence factors in the development of colistin subpopulations. The development of colistin-tolerant subpopulations was significantly affected in type IV pili-mediated motility mutant (PAO1 Δ *pilA*) biofilms and *las* and *rhl* quorum sensing systems-deficient mutant (PAO1 Δ *lasI* Δ *rhlI*) biofilms, while the development of the colistin-tolerant cells were repressed in triple gene mutation mutant (PAO1 Δ *pilA* Δ *asR* Δ *rhlR*) biofilms after exposed to colistin (fig 3-5). This demonstrates the importance of both type IV pili-

mediated motility and QS activity in contributing to the colistin-tolerant subpopulations development. Moreover, type IV pili-mediated motility precede QS-mediated gene products during the development of colistin-tolerant subpopulations (fig 3-6a) and the presence of QS-regulated virulence factors were further validated using bioassays (fig 3-6b, c, d).

The role of c-di-GMP signalling in *Pseudomonas aeruginosa*-induced mouse keratitis

Biofilm-like cell aggregates were observed in cornea that was infected by *P. aeruginosa*. The comparison of transcriptomics analysis *in vivo* cells and *in vitro* cells depicting the difference in physiology between the *in vivo* and *in vitro* cells along PC1, while similar physiologies along PC2, which is the biofilm physiology (fig 4-1a). Iron uptake systems and type III secretion systems were upregulated (supplementary table B2 and fig 4-1b), while quorum sensing system were downregulated *in vivo* PAO1 compared to *in vitro* PAO1 (supplementary table B3 and fig 4-1b). The biofilm-features including *pel* polysaccharides and cup fimbriae were highly expressed in *in vivo* PAO1 (supplementary table B4 and fig 4-2), prompting the possibility of the biofilm physiology during the cornea infection. Since high level of intracellular c-di-GMP regulates the formation of *P. aeruginosa* biofilm, c-di-GMP reporter strain was used for the cornea infection. The enhancement of intracellular c-di-GMP content from 2 hpi to 4 dpi and the presence of biofilm-like aggregations at the surface of the infected corneas (fig 4-3), demonstrating both physiological and physical presence of *P. aeruginosa* biofilms during the infections. The enhancement of c-di-GMP content was possibly due to the downregulation of a

range of PDEs instead of upregulation of DGCs (fig 4-2 and 4-4). Apart from forming biofilms on the infected cornea, intracellular c-di-GMP signalling also modulate resistance against host immune system. *P. aeruginosa* with high intracellular c-di-GMP contents were more resistance to killing by host immune system likely due to the biofilm properties such as enhanced production of exopolysaccharide (fig 4-5).

Combinatorial treatments against *P. aeruginosa* biofilms

Quorum sensing system and c-di-GMP signalling pathways have been demonstrated for their role in developing tolerance against antibiotic and host immune systems respectively in this study. Inference of these cell-signalling pathways led to less tolerance and resistance against antimicrobial agents.

The combination treatment of using erythromycin, which acts as an inhibitor of quorum sensing activity and type IV pili motility, together with colistin, specifically targets colistin-tolerant cells since the combined treatment repressed the development of colistin-tolerant cells in flow chamber experiment (fig 5-1a, b). The erythromycin single treatment, at the same concentration used in flow cell chamber, has no killing effect on planktonic PAO1 (fig 5-1c). Using the same combination treatment in *in vivo* experiment, viable *P. aeruginosa* on the implants were drastically reduced while erythromycin-alone and colistin-alone treatment reduced the bacterial load to 10-fold and 100-fold respectively (fig 5-2a). Thus, this highlights the importance of understanding the mechanism of the development of colistin-tolerant cells, which allows designing of new therapeutic to repress the development. The erythromycin and colistin combinatorial treatment can be potential anti-biofilm

therapy to treat other chronic infections such as skin infection and lung infection that are often caused by *P. aeruginosa* infection (fig 6a).

P. aeruginosa intracellular c-di-GMP contents were enhanced during the cornea infections, and contributed to the resistance against killing by host immune systems (fig 4-5). This prompted the use of biofilm-dispersal agent that lowers the c-di-GMP content to manage the infections. Arabinose, the biofilm-dispersal agent that lowers the c-di-GMP level in PAO1/*p_{BAD}-yhjH*, caused the dispersion of biofilm, but not capable to kill biofilm cells in flow chamber experiments (fig 5-3b, e), while colistin-tolerant subpopulations were presence in colistin treatment (fig 5-3c, e). In contrast, arabinose and colistin combinatorial treatment drastically reduced the colistin-tolerant subpopulations (fig 5-3d, e). This combinatorial approach was effective in reducing bacterial load in the infected corneas compared to single treatment (fig 5-4). This highlight the importance of c-di-GMP signalling pathway in contributing to resistance against clearance by host immune systems in *P. aeruginosa*-induced keratitis. This biofilm-dispersal agent and colistin combinatorial treatment can potentially be new therapy in treating cornea infections by *P. aeruginosa* and possibly other Gram-negative pathogens (fig 6b).

Figure 6. Summary diagram of the quorum sensing and c-di-GMP signalling in *P.aeruginosa* affecting biofilm viability. (a) Colistin treatment on biofilms leads to the development of colistin-tolerant subpopulations, where these subpopulations have increased quorum sensing activity and type IV pili-mediated motility. Erythromycin,

which inhibits QS activity and motility, when used in combination with colistin, is able to eradicate and repress the colistin-tolerant subpopulations development in both *in vitro* and *in vivo* models. (b) *P. aeruginosa* with high intracellular c-di-GMP content are able to form densely-packed microcolonies and establish infection on mouse ocular infection model, while cells with low c-di-GMP content form loosely-packed microcolonies and do not establish infection well on mouse ocular infection model. Lowering intracellular c-di-GMP enhances the effectiveness of colistin treatment on mouse *Pseudomonas* keratitis.

6.2 Future recommendations

Investigation the role of *pel* exopolysaccharides in ocular infection

Pel exopolysaccharide, not psl exopolysaccharide, was upregulated during the ocular infections. Thus, the hypothesis is that pel, but not psl exopolysaccharide, is crucial for the development of *P. aeruginosa* biofilms in the scratched cornea and also contribute to the protection of the biofilm cells against host immunity. The future work will include infecting the mouse cornea with *P. aeruginosa* mutants that are deficient in synthesizing pel or psl exopolysaccharides to determine whether the absence of pel exopolysaccharides in the EPS would lead to effective clearance by the host immunity comparing to wild-type PAO1 and psl-defective mutant during the ocular infections. If the hypothesis is true, pel exopolysaccharide can be a potential therapeutic target.

Investigation of the role of type III secretion system (T3SS) and iron-uptake system during ocular infection

T3SS and iron-uptake systems were upregulated in *in vivo* PAO1 as compared to *in vitro* biofilm cells. These results suggested the importance of T3SS and iron-uptake systems during mouse ocular infection. Thus, the future work includes using T3SS and iron-uptake mutants for mouse ocular infection and compare the viability of these mutants against *P. aeruginosa* wild type during mouse keratitis.

New combinatorial treatments against *P. aeruginosa*-induced keratitis

In this study, biofilm-dispersal agent and colistin combinatorial treatment work in synergistic manner in killing the biofilm cells during ocular infections. Although there was significant reduction of bacterial load in the combined treatment than single treatment, there was still viable *P. aeruginosa* in the combined treatment group during the infections. We hypothesize that it could be due to both the physical barrier by the cornea and amount of dosage used during the treatment. Unlike flow chamber experiment where most of the *P. aeruginosa* biofilm surface was exposed to the consistent continuous flow of antimicrobial agents-containing medium, biofilm cells deep in the cornea epithelial cells may not be exposed to the same amount of dosage as the biofilm cells at the surface do. Thus, this likely leads to the less killing efficiency of the combinatorial treatment against *P. aeruginosa* biofilms in ocular infections than flow chamber experiment. DIPY, an iron-chelator, is proposed to be included to the biofilm-dispersal and colistin combinatorial treatment since iron-uptake genes were also upregulated in *in vivo* PAO1 cells (supplementary table B2). It has been demonstrated the effective use of iron-chelator in managing the dispersal biofilm cells previously (Chua et al 2014). Hence, this multi-pronged approach is likely to be more effective in killing *P. aeruginosa* biofilm cells than biofilm-dispersal and colistin combinatorial treatment.

Colistin and biofilm-dispersal agent combination treatment against *P. aeruginosa* biofilm-associated infections

In this thesis, we demonstrated the proof-of-concept of lowering intracellular c-di-GMP in *P. aeruginosa* biofilm by using biofilm dispersal agent, which increased the susceptibility of *P. aeruginosa* biofilm towards colistin and host immune system in mouse *Pseudomonas* keratitis model. This concept can be potentially be applied on other *P. aeruginosa* biofilm-associated infections such as in CF lung infection, medical devices, contact lenses, or patients with chronic obstructive pulmonary diseases, native valve endocarditis, chronic otitis media, chronic sinusitis and chronic (diabetes) wound infections.

Challenges of using colistin and biofilm-dispersal agent combination treatment against *P. aeruginosa* biofilm-associated infections

In this study, we demonstrated the proof-of-concept where we utilized genetic-based biofilm dispersal approach to lower intracellular c-di-GMP content in the cell by inducing p_{BAD}-*yhjH* (PDE) via the presence of arabinose, to breakdown c-di-GMP, which increased susceptibility of bacteria to clearance by colistin and host immune system. However, in clinical setting, there is a need to design chemical-based biofilm dispersal approach as *P. aeruginosa* clinical isolates do not carry arabinose-inducible *yhjH* (PDE). Studies have demonstrated the use of nitric oxide (NO) donor compound to disperse biofilm in industrial setting. Low concentration of NO activates the activity of PDEs, which leads to lowering of intracellular c-di-GMP, resulting in biofilm dispersion (Barraud et al 2009a). An unpublished data has shown the

combination use of colistin and sodium nitroprusside (SNP), an NO donor, is effective in reducing viable *P. aeruginosa* in *in vitro* biofilm and in *P. aeruginosa* keratitis model.

The challenge of introducing this proof-of-concept into the clinical practise and care would be the introducing NO-donor compounds that do not have or have low toxicity to human health. The NO-donor compound should have a targeted delivery of NO to the site of infection as well as a controlled-release of low concentration of NO to the infection. Another challenge is determine the optimal concentration of NO for clinical use as NO is also an important cellular signaling molecule in human as it involved in many physiological and pathological processes (Hou et al 1999).

Another potential approach for biofilm dispersal is the targeting of diguanylate cyclases (DGCs), the enzymes involved in the synthesis of intracellular c-di-GMP. Blocking the active site(s) of DGC can prevent the enzyme from synthesizing c-di-GMP. However, the main problem is that the structural information of the DGCs is still currently unsolved, which is difficult for drugs to be designed to target the active site(s) of the DGCs.

CHAPTER 7. APPENDIX

7.1 *Appendix A*

Supplementary method A1. Parameters used in the CLC genomics analysis.

Reproduced with permission from (Chua et al 2016).

1. Import reads using Illumina paired importer:

Discard read names = Yes

Discard quality scores = No

Paired orientation = Paired reads (forward-reverse)

Minimum distance = 1

Maximum distance = 500

Quality score = NCBI/Sanger or Illumina Pipeline 1.8 and later

Remove failed reads = Yes

MiSeq de-multiplexing = No

2. Trim sequences

Trim adapter list = Illumina TruSeq Trim Adapter List

Ambiguous trim = Yes

Ambiguous limit = 2

Quality trim = Yes

Quality limit = 0.05

Use colorspace = No

Create report = Yes

Also search on reversed sequence = Yes

Save discarded sequences = Yes

Remove 5' terminal nucleotides = No

Minimum number of nucleotides in reads = 30

Discard short reads = Yes

Remove 3' terminal nucleotides = No

Discard long reads = No

Save broken pairs = No

3. Map reads to reference:

References = NC_002516(PA01 genome)

Masking mode = No masking

Mismatch cost = 2

Cost of insertions and deletions = Linear gap cost

Insertion cost = 3

Deletion cost = 3

Insertion open cost = 6

Insertion extend cost = 1

Deletion open cost = 6

Deletion extend cost = 1

Length fraction = 0.5

Similarity fraction = 0.8

Global alignment = No

Auto-detect paired distances = Yes

Non-specific match handling = Map randomly

Output mode = Create reads track

Create report = Yes

Collect un-mapped reads = No

4. Low frequency variant detection:

Required significance (%) = 1.0

Ignore positions with coverage above = 100,000

Restrict calling to target regions = Not set

Ignore broken pairs = Yes

Ignore non-specific matches = Reads

Minimum coverage = 10

Minimum count = 2

Minimum frequency (%) = 1.0

Base quality filter = Yes

Neighborhood radius = 5

Minimum central quality = 20

Minimum neighborhood quality = 15

Read direction filter = Yes

Direction frequency (%) = 5.0

Relative read direction filter = Yes

Significance (%) = 1.0

Read position filter = Yes

Significance (%) = 1.0

Remove pyro-error variants = Yes

In homopolymer regions with minimum length = 3

With frequency below = 0.8

Create track = Yes

Create annotated table = Yes

Create report = Yes

Supplementary videos

Supplementary video A1. Treatment of *P. aeruginosa* biofilms by colistin. Top view of 72 h old GFP-tagged *P. aeruginosa* PAO1 biofilms were treated with colistin and observed from 2 h to 20 h. Scale bars, 10 μ m. Reproduced with permission from (Chua et al 2016). Weblink:

<http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s6.mov>

Supplementary video A2. Treatment of *P. aeruginosa* biofilms by colistin. Side view of 72 h old GFP-tagged *P. aeruginosa* PAO1 biofilms were treated with colistin and observed from 2 h to 20 h. Scale bars, 10 μ m. Reproduced with permission from (Chua et al 2016). Weblink:

<http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s7.mov>

Supplementary video A3. The development of colistin-tolerant subpopulations in biofilm. Top view of 72 h old GFP-tagged *P. aeruginosa* PAO1 biofilms were treated with colistin and observed from 24 h to 34 h of treatment. Scale bars, 10 μ m. Reproduced with permission from (Chua et al 2016). Weblink:

<http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s8.mov>

Supplementary video A4. The development of colistin-tolerant subpopulations in biofilm. Side view of 72 h old GFP-tagged *P. aeruginosa* PAO1 biofilms were treated with colistin and observed from 24 to 34 h of treatment. Scale bars, 10 μ m. Reproduced with permission from (Chua et al 2016). Weblink:

<http://www.nature.com/article->

[assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s9.mov](http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s9.mov)

Supplementary table A1. Comparing the variants of colistin-treated biofilms samples with untreated biofilm samples. Reproduced with permission from (Chua et al 2016). The complete list of data can be found in website: <http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s2.xlsx>

*: Parallel variants in the three colistin-treated biofilm samples: same variants found in all of three samples or different variants found in the same genes of the three samples.

**: Consolidated variants in the three untreated control biofilm samples: variants found in at least one of the three samples.

Marked in grey: the parallel variants found in colistin-treated biofilm samples were also found in at least one of the control biofilm samples. Therefore, these variants can be regarded as 1) false positive due to sequencing error; or 2) variants not caused by colistin treatment.

Parallel variants in the three colistin-treated biofilm samples*		Consolidated variants in the three untreated biofilm samples**	
Protein Number	Coding region change	Protein Number	Coding region change
NP_248849.1	NP_248849.1:c.930T>G	NP_248849.1	NP_248849.1:c.930T>G
NP_249374.1	NP_249374.1:c.217_218insC	NP_249374.1	NP_249374.1:c.217_218insC
NP_249408.1	NP_249408.1:c.76_77delGGinsAT	NP_249408.1	NP_249408.1:c.77G>T
NP_249408.1	NP_249408.1:c.145C>T	NP_249408.1	NP_249408.1:c.145C>T
NP_249409.1	NP_249409.1:c.50C>G	NP_249409.1	NP_249409.1:c.50C>G
NP_249409.1	NP_249409.1:c.80T>C	NP_249409.1	NP_249409.1:c.80T>C
NP_249409.1	NP_249409.1:c.146G>A	NP_249409.1	NP_249409.1:c.146G>A
NP_249410.1	NP_249410.1:c.107C>T	NP_249410.1	NP_249410.1:c.107C>T
		NP_249410.1	NP_249410.1:c.290delT
		NP_249410.1	NP_249410.1:c.292delC
NP_249410.1	NP_249410.1:c.332C>A	NP_249410.1	NP_249410.1:c.332C>A
NP_249410.1	NP_249410.1:c.334C>T	NP_249410.1	NP_249410.1:c.334C>T

NP_2494 10.1	NP_249410.1:c.344C>T	NP_2494 10.1	NP_249410.1:c.344C>T
NP_2494 11.1	NP_249411.1:c.320_321delTGin sCC	NP_2494 11.1	NP_249411.1:c.320_321delTGin sCC
NP_2494 11.1	NP_249411.1:c.356_357delTGin sCA	NP_2494 11.1	NP_249411.1:c.356_357delTGin sCA
NP_2494 11.1	NP_249411.1:c.415A>T	NP_2494 11.1	NP_249411.1:c.415A>T
NP_2494 12.1	NP_249412.1:c.85G>A	NP_2494 12.1	NP_249412.1:c.85G>A
NP_2494 15.1	NP_249415.1:c.560_561delATin sGC	NP_2494 15.1	NP_249415.1:c.560_561delATin sGC
NP_2494 15.1	NP_249415.1:c.745A>G	NP_2494 15.1	NP_249415.1:c.745A>G
NP_2494 15.1	NP_249415.1:c.755G>A	NP_2494 15.1	NP_249415.1:c.755G>A
NP_2494 15.1	NP_249415.1:c.817_819delGGC	NP_2494 15.1	NP_249415.1:c.817_819delGGC
		NP_2494 15.1	NP_249415.1:c.833G>A
NP_2494 15.1	NP_249415.1:c.1032C>A	NP_2494 15.1	NP_249415.1:c.1032C>A
		NP_2494 17.1	NP_249417.1:c.107A>C
		NP_2494 17.1	NP_249417.1:c.500T>G
		NP_2494 17.1	NP_249417.1:c.724T>G
NP_2494 17.1	NP_249417.1:c.761C>G	NP_2494 17.1	NP_249417.1:c.761C>G
NP_2494 17.1	NP_249417.1:c.841A>G	NP_2494 17.1	NP_249417.1:c.841A>G
NP_2494 17.1	NP_249417.1:c.1005C>G	NP_2494 17.1	NP_249417.1:c.1005C>G
NP_2494 17.1	NP_249417.1:c.1110G>C	NP_2494 17.1	NP_249417.1:c.1110G>C
NP_2494 17.1	NP_249417.1:c.1132A>G	NP_2494 17.1	NP_249417.1:c.1132A>G
NP_2494 17.1	NP_249417.1:c.1144C>G	NP_2494 17.1	NP_249417.1:c.1144C>G
NP_2494 17.1	NP_249417.1:c.1166T>C	NP_2494 17.1	NP_249417.1:c.1166T>C
NP_2494 17.1	NP_249417.1:c.1199C>T	NP_2494 17.1	NP_249417.1:c.1199C>T
		NP_2494 17.1	NP_249417.1:c.1232delT
NP_2494 17.1	NP_249417.1:c.1269G>C	NP_2494 17.1	NP_249417.1:c.1269G>C

		NP_249418.1	NP_249418.1:c.10A>C
NP_249418.1	NP_249418.1:c.174_175delACinsGT	NP_249418.1	NP_249418.1:c.174_175delACinsGT
NP_249418.1	NP_249418.1:c.388C>A	NP_249418.1	NP_249418.1:c.388C>A
NP_249418.1	NP_249418.1:c.509_510delAAinsCG	NP_249418.1	NP_249418.1:c.509_510delAAinsCG
		NP_249418.1	NP_249418.1:c.509_511delAAC
NP_249418.1	NP_249418.1:c.516_517delTCinsG	NP_249418.1	NP_249418.1:c.516_517delTCinsG
NP_249418.1	NP_249418.1:c.519_520insT	NP_249418.1	NP_249418.1:c.519_520insT
NP_249418.1	NP_249418.1:c.796C>A	NP_249418.1	NP_249418.1:c.796C>A
NP_249418.1	NP_249418.1:c.809_810delTCinsAT	NP_249418.1	NP_249418.1:c.809_810delTCinsAT
NP_249418.1	NP_249418.1:c.932delA	NP_249418.1	NP_249418.1:c.932delA
NP_249418.1	NP_249418.1:c.934A>G	NP_249418.1	NP_249418.1:c.934A>G
NP_249418.1	NP_249418.1:c.936_937insA	NP_249418.1	NP_249418.1:c.936_937insA
NP_249418.1	NP_249418.1:c.940_943delAAGCinsCGTG	NP_249418.1	NP_249418.1:c.940_943delAAGCinsCGTG
NP_249418.1	NP_249418.1:c.952_953insG	NP_249418.1	NP_249418.1:c.952_953insG
NP_249418.1	NP_249418.1:c.956C>G	NP_249418.1	NP_249418.1:c.956C>G
NP_249418.1	NP_249418.1:c.960_961delGGinsA	NP_249418.1	NP_249418.1:c.960_961delGGinsA
		NP_249418.1	NP_249418.1:c.968_969delAT
NP_249418.1	NP_249418.1:c.969_970delTCinsCG	NP_249418.1	NP_249418.1:c.969_970delTCinsCG
		NP_249418.1	NP_249418.1:c.1277G>A
NP_249720.1	NP_249720.1:c.1_2insC	NP_249720.1	NP_249720.1:c.1_2insC
NP_249841.1	NP_249841.1:c.167A>G		
NP_249841.1	NP_249841.1:c.193C>A		
NP_249841.1	NP_249841.1:c.415T>G		
NP_249841.1	NP_249841.1:c.428G>A		

		NP_249841.1	NP_249841.1:c.643G>A
NP_249841.1	NP_249841.1:c.1173A>C		
NP_249841.1	NP_249841.1:c.1603C>A		
NP_249841.1	NP_249841.1:c.144_145delTAinsCC		
NP_249841.1	NP_249841.1:c.175_176delAGinsGA		
		NP_249865.1	NP_249865.1:c.32_33delTCinsA
NP_249865.1	NP_249865.1:c.32delT	NP_249865.1	NP_249865.1:c.32delT
NP_250150.1	NP_250150.1:c.101G>C	NP_250150.1	NP_250150.1:c.101G>C
NP_250831.1	NP_250831.1:c.515delC	NP_250831.1	NP_250831.1:c.515delC
NP_251181.1	NP_251181.1:c.745G>A	NP_251181.1	NP_251181.1:c.745G>A
NP_251182.1	NP_251182.1:c.226_233delCGGCCAGC	NP_251182.1	NP_251182.1:c.226_233delCGGCCAGC
NP_251182.1	NP_251182.1:c.514T>A	NP_251182.1	NP_251182.1:c.514T>A
		NP_251182.1	NP_251182.1:c.514T>C
NP_251358.1	NP_251358.1:c.40_41insG	NP_251358.1	NP_251358.1:c.40_41insG
NP_252449.1	NP_252449.1:c.1907A>G	NP_252449.1	NP_252449.1:c.1907A>G
NP_253031.1	NP_253031.1:c.474A>C	NP_253031.1	NP_253031.1:c.474A>C
NP_253050.1	NP_253050.1:c.3_4insG		
NP_253050.1	NP_253050.1:c.1_2insC	NP_253050.1	NP_253050.1:c.1_2insC
NP_253084.1	NP_253084.1:c.531_532delCGinsGC	NP_253084.1	NP_253084.1:c.531_532delCGinsGC
NP_253711.1	NP_253711.1:c.410_411insCGG	NP_253711.1	NP_253711.1:c.410_411insCGG
NP_253787.1	NP_253787.1:c.1292_1293delCGinsGC	NP_253787.1	NP_253787.1:c.1292_1293delCGinsGC
NP_254121.1	NP_254121.1:c.858A>C	NP_254121.1	NP_254121.1:c.858A>C
		NP_064725.1	NP_064725.1:c.91G>T
		NP_064728.1	NP_064728.1:c.268G>T

		NP_248701.1	NP_248701.1:c.359A>C
		NP_248706.1	NP_248706.1:c.953A>C
		NP_248706.1	NP_248706.1:c.782G>A
		NP_248716.1	NP_248716.1:c.886C>A
		NP_248719.1	NP_248719.1:c.447A>C
		NP_248721.1	NP_248721.1:c.794A>C
		NP_248723.1	NP_248723.1:c.16C>A
		NP_248724.1	NP_248724.1:c.369C>A
		NP_248730.1	NP_248730.1:c.820A>C
		NP_248731.1	NP_248731.1:c.1802A>C
		NP_248731.1	NP_248731.1:c.3736A>C
		NP_248731.1	NP_248731.1:c.4925T>G
		NP_248737.1	NP_248737.1:c.244A>G
		NP_248748.1	NP_248748.1:c.624A>C
		NP_248761.1	NP_248761.1:c.1310C>A
		NP_248768.1	NP_248768.1:c.256A>C
		NP_248771.1	NP_248771.1:c.874A>C
		NP_248771.1	NP_248771.1:c.855_856insCAGCCA
		NP_248780.1	NP_248780.1:c.398G>T
		NP_248780.1	NP_248780.1:c.667G>T
		NP_248780.1	NP_248780.1:c.1916C>A
		NP_248795.1	NP_248795.1:c.259A>C
		NP_248801.1	NP_248801.1:c.469T>G
		NP_248801.1	NP_248801.1:c.482C>A

		NP_248817.1	NP_248817.1:c.10C>A
		NP_248848.1	NP_248848.1:c.2925A>C
		NP_248854.1	NP_248854.1:c.32A>C
		NP_248858.1	NP_248858.1:c.520T>G
		NP_248881.1	NP_248881.1:c.918A>C
		NP_248882.1	NP_248882.1:c.1085T>G
		NP_248885.1	NP_248885.1:c.1034A>C
		NP_248886.2	NP_248886.2:c.74T>G
		NP_248887.1	NP_248887.1:c.82A>C
		NP_248887.1	NP_248887.1:c.443T>G
		NP_248903.1	NP_248903.1:c.710A>C
		NP_248910.1	NP_248910.1:c.407T>G
		NP_248916.1	NP_248916.1:c.189A>C
		NP_248922.1	NP_248922.1:c.112G>T
		NP_248927.1	NP_248927.1:c.46C>A
		NP_248937.1	NP_248937.1:c.20A>C
		NP_248945.1	NP_248945.1:c.581A>C
		NP_248951.1	NP_248951.1:c.1978T>G
		NP_248951.1	NP_248951.1:c.697G>A
		NP_248963.1	NP_248963.1:c.527A>C
		NP_248971.1	NP_248971.1:c.344C>A
		NP_248972.1	NP_248972.1:c.388G>T
		NP_248977.1	NP_248977.1:c.708C>A
		NP_248977.1	NP_248977.1:c.1068C>A

		NP_2489 82.1	NP_248982.1:c.1367C>A
		NP_2489 84.1	NP_248984.1:c.88C>A
		NP_2489 86.1	NP_248986.1:c.365C>T
		NP_2489 92.1	NP_248992.1:c.811T>G
		NP_2489 96.1	NP_248996.1:c.1980T>G
		NP_2489 99.1	NP_248999.1:c.578A>C
		NP_2490 02.1	NP_249002.1:c.526C>A
		NP_2490 07.1	NP_249007.1:c.334G>A
		NP_2490 08.1	NP_249008.1:c.703C>T
		NP_2490 23.1	NP_249023.1:c.173T>C
		NP_2490 33.1	NP_249033.1:c.603G>T
		NP_2490 43.1	NP_249043.1:c.360A>C
		NP_2490 45.1	NP_249045.1:c.754T>C
		NP_2490 51.1	NP_249051.1:c.905T>G
		NP_2490 67.1	NP_249067.1:c.274A>C
		NP_2490 69.1	NP_249069.1:c.344T>G
		NP_2490 77.1	NP_249077.1:c.539A>C
		NP_2490 82.1	NP_249082.1:c.750A>C
		NP_2490 86.1	NP_249086.1:c.356C>A
		NP_2490 97.1	NP_249097.1:c.185G>T
		NP_2491 01.1	NP_249101.1:c.82C>T
		NP_2491 02.1	NP_249102.1:c.569G>T
		NP_2491 04.1	NP_249104.1:c.4733A>C
		NP_2491 16.1	NP_249116.1:c.256A>T

		NP_2491 20.1	NP_249120.1:c.598delG
		NP_2491 26.1	NP_249126.1:c.860T>G
		NP_2491 27.1	NP_249127.1:c.296G>T
		NP_2491 27.1	NP_249127.1:c.445T>G
		NP_2491 30.1	NP_249130.1:c.1079C>A
		NP_2491 49.1	NP_249149.1:c.305T>G
		NP_2491 49.1	NP_249149.1:c.301A>C
		NP_2491 56.1	NP_249156.1:c.923A>G
		NP_2491 58.1	NP_249158.1:c.382T>G
		NP_2491 60.1	NP_249160.1:c.839G>T
		NP_2491 64.1	NP_249164.1:c.697A>C
		NP_2491 83.1	NP_249183.1:c.526A>C
		NP_2491 99.1	NP_249199.1:c.403G>A
		NP_2492 27.1	NP_249227.1:c.776G>T
		NP_2492 30.1	NP_249230.1:c.683G>T
		NP_2492 49.1	NP_249249.1:c.154G>T
		NP_2492 55.1	NP_249255.1:c.124delC
		NP_2492 64.1	NP_249264.1:c.236A>C
		NP_2492 65.1	NP_249265.1:c.857G>T
		NP_2492 75.1	NP_249275.1:c.665A>C
		NP_2492 79.1	NP_249279.1:c.956G>T
		NP_2492 94.1	NP_249294.1:c.940G>T
		NP_2493 07.1	NP_249307.1:c.503T>G
		NP_2493 11.1	NP_249311.1:c.1262G>T

		NP_2493 21.1	NP_249321.1:c.321C>A
		NP_2493 41.1	NP_249341.1:c.600A>C
		NP_2493 79.1	NP_249379.1:c.275C>A
		NP_2493 84.1	NP_249384.1:c.1003C>A
		NP_2493 98.1	NP_249398.1:c.322G>A
		NP_2494 07.1	NP_249407.1:c.481G>T
		NP_2494 23.1	NP_249423.1:c.872G>A
		NP_2494 27.1	NP_249427.1:c.346T>A
		NP_2494 31.1	NP_249431.1:c.462C>A
		NP_2494 34.1	NP_249434.1:c.534delC
		NP_2494 34.1	NP_249434.1:c.525C>A
		NP_2494 35.1	NP_249435.1:c.791A>C
		NP_2494 60.1	NP_249460.1:c.41G>T
		NP_2494 60.1	NP_249460.1:c.251C>A
		NP_2494 68.1	NP_249468.1:c.134A>C
		NP_2494 71.1	NP_249471.1:c.557T>G
		NP_2494 72.1	NP_249472.1:c.82G>T
		NP_2494 80.1	NP_249480.1:c.1117T>G
		NP_2495 06.1	NP_249506.1:c.800T>G
		NP_2495 09.1	NP_249509.1:c.31G>C
		NP_2495 17.1	NP_249517.1:c.575C>A
		NP_2495 24.1	NP_249524.1:c.599A>C
		NP_2495 26.1	NP_249526.1:c.958T>G

Supplementary data A2. The scatter plot between biological replicate 1 and replicate 2. Reproduced with permission from (Chua et al 2016).

Supplementary data A3. The scatter plot of technical replicates within biological replicate 1. Reproduced with permission from (Chua et al 2016).

Supplementary data A4. The scatter plot of technical replicate within biological replicate 2. Reproduced with permission from (Chua et al 2016).

Supplementary table A5. 139 Proteins that were of lower abundance in *P. aeruginosa* PAO1 colistin-tolerant cells than in colistin-sensitive biofilm cells. The protein data were sorted accordingly from least expressed to similar expression level of colistin-tolerant cells compared to colistin-sensitive cells. Reproduced with permission from (Chua et al 2016).

	GI No.	Gene	Description	Peptides	Ratio H/L (medium)	Ratio H/L variability [%]
1	gi 375048424	PA2072	hypothetical protein	13	0.64	24.109
2	gi 9947518	ccoN2	Cytochrome c oxidase, cbb3-type, CcoN subunit	5	0.64	18.633
3	gi 9948883	vacJ	vacJ like lipofamily protein	19	0.64	23.327
4	gi 9951059	PA4793	hypothetical protein	17	0.64	14.613
5	gi 9951332	pilO	type 4 fimbrial biogenesis protein PilO	13	0.64	25.455
6	gi 9946775	PA0876	probable transcriptional regulator	12	0.64	24.288
7	gi 9949332	PA3212	probable ATP-binding component of ABC transporter	17	0.64	24.439
8	gi 9948840	oprQ	probable outer membrane protein precursor	51	0.64	28.872
9	gi 660508913	PA4326	hypothetical protein	5	0.64	15.305
10	gi 9947223	PA1288	probable outer membrane protein precursor	32	0.64	26.179
11	gi 660507371	PA2756	hypothetical protein	11	0.64	24.851
12	gi 9947369	gbuA	guanidinobutyrase	6	0.63	18.756
13	gi 651875866	PA5310	hypothetical protein	20	0.63	16.59
14	gi 651877751	PA1969	hypothetical protein	9	0.63	22.178
15	gi 9949996	PA3822	hypothetical protein	11	0.63	22.821
16	gi 660506157	ccoQ2	cytochrome c oxidase subunit	6	0.63	19.839
17	gi 9951352	phaF	polyhydroxyalkanoate synthesis protein PhaF	32	0.63	27.853
18	gi 651879916	PA2122	hypothetical protein	8	0.63	8.5923
19	gi 9951238	psd	phosphatidylserine decarboxylase	19	0.63	16.315
20	gi 9947412	motC	flagellar motor protein	8	0.63	15.444
21	gi 9947691	pscC	Type III secretion outer	11	0.63	15.755

			membrane protein PscC precursor			
22	gi 9949808	opr86	outer membrane protein Opr86	60	0.63	22.349
23	gi 9951178	PA4902	probable transcriptional regulator	11	0.63	4.5308
24	gi 9950946	PA4689	hypothetical protein	40	0.63	24.192
25	gi 9949934	PA3764	conserved hypothetical protein	19	0.62	21.317
26	gi 660506474	PA1866	hypothetical protein	4	0.62	1.4919
27	gi 375047586	mexA	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein MexA precursor	37	0.62	28.916
28	gi 9946765	mliC	membrane-bound lysozyme inhibitor of c-type lysozyme	12	0.62	27.139
29	gi 9947512	ccoP1	cytochrome c oxidase	26	0.62	22.96
30	gi 9950690	PA4455	probable permease of ABC transporter	9	0.62	27.327
31	gi 9949644	PA3498	probable oxidoreductase	5	0.62	0.46839
32	gi 9948302	soxR	redox-sensitive transcriptional activator	4	0.61	0.071855
33	gi 9948336	ambC	putative regulatory protein	23	0.61	22.493
34	gi 660508978	PA4390	hypothetical protein	16	0.61	24.707
35	gi 9947229	PA1293	hypothetical protein	31	0.61	24.634
36	gi 9951452	PA5151	hypothetical protein	5	0.61	10.804
37	gi 9951235	motA	chemotaxis protein MotA	7	0.61	13.64
38	gi 9946973	shaF	monovalent cation/proton antiporter, MnhG/PhaG subunit	2	0.61	15.224
39	gi 9949205	xcpZ	general secretion pathway protein M	6	0.61	9.1731
40	gi 9951539	PA5231	probable ATP-binding/permease fusion ABC transporter	35	0.61	20.144
41	gi 9950115	cioA	cyanide insensitive terminal oxidase	5	0.61	12.908
42	gi 9951221	hflC	protease subunit HflC	40	0.61	18.426
43	gi 9948619	PA2559	hypothetical protein	6	0.61	17.695
44	gi 9949046	PA2949	probable lipase	21	0.6	16.96
45	gi 9946283	oprM	Major intrinsic multiple antibiotic resistance efflux outer membrane protein OprM precursor	47	0.6	20.649

46	gi 9950615	groEL	chaperone protein	94	0.6	23.653
47	gi 9950910	PA4656	conserved hypothetical protein	14	0.6	9.3294
48	gi 9950264	oprG	Outer membrane protein OprG precursor	19	0.6	29.247
49	gi 9951051	PA4785	probable acyl-CoA thiolase	31	0.6	20.4
50	gi 9950826	PA4579	hypothetical protein	43	0.6	25.109
51	gi 9950025	PA3848	hypothetical protein	30	0.6	28.189
52	gi 237640402	fpvA	ferripyoverdine receptor	63	0.6	23.838
53	gi 9946467	lptD	LPS-assembly protein	98	0.6	23.824
54	gi 9950590	PA4362	hypothetical protein	21	0.6	20.844
55	gi 9948476	PA1231	conserved hypothetical protein	12	0.6	28.235
56	gi 9950963	PA4705	hypothetical protein	18	0.6	18.54
57	gi 9949628	PA3483	hypothetical protein	24	0.59	23.482
58	gi 9946423	PA0554	hypothetical protein	3	0.59	21.475
59	gi 9949386	PA3262	putative peptidyl-prolyl cis-trans isomerase, FkbP-type	27	0.59	23.938
60	gi 9948004	PA2002	conserved hypothetical protein	4	0.59	15.187
61	gi 9948259	pslD	exopolysaccharide transporter	17	0.59	21.871
62	gi 9949555	PA3417	probable pyruvate dehydrogenase E1 component, alpha subunit	16	0.59	15.032
63	gi 9947513	ccoO1	Cytochrome c oxidase, cbb3-type, CcoO subunit	23	0.59	21.938
64	gi 9951058	PA4792	conserved hypothetical protein	26	0.58	19.993
65	gi 9947757	oprF	Major porin and structural outer membrane porin OprF precursor	42	0.58	21.367
66	gi 9948899	PA2815	probable acyl-CoA dehydrogenase	46	0.58	21.479
67	gi 9949995	secD	secretion protein SecD	39	0.58	21.818
68	gi 9948125	PA2111	hypothetical protein	22	0.58	21.166
69	gi 9946957	PA1044	hypothetical protein	1	0.58	11.311
70	gi 9947396	fliO	flagellar protein FliO	4	0.57	18.693
71	gi 9948412	VgrG3	type VI secretion system, Vgr family	8	0.57	16.961
72	gi 9950436	fptA	Fe(III)-pyochelin outer membrane receptor precursor	75	0.57	26.514

73	gi 9951257	PA4974	probable outer membrane protein precursor	66	0.57	24.004
74	gi 9948367	PA2332	probable transcriptional regulator	7	0.57	9.18
75	gi 9950437	PA4222	probable ATP-binding component of ABC transporter	37	0.57	21.893
76	gi 9951673	glcF	glycolate oxidase subunit GlcF	6	0.57	13.638
77	gi 9949334	PA3214	hypothetical protein	18	0.57	5.5438
78	gi 9950418	mexH	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor	38	0.57	22.953
79	gi 9948165	katE	catalase HPII	20	0.57	30.173
80	gi 9950861	PA4610	hypothetical protein	1	0.57	4.9807
81	gi 9949142	PA3038	probable porin	34	0.56	24.216
82	gi 375050024	aaaA	arginine-specific autotransporter of <i>Pseudomonas aeruginosa</i> , AaaA	46	0.56	23.212
83	gi 9948433	PvdP	Tat (twin-arginine translocation) pathway signal sequence domain protein	53	0.56	23.246
84	gi 9947344	PA1398	hypothetical protein	6	0.56	24.242
85	gi 9946331	fiuA	Ferrichrome receptor FiuA	7	0.55	13.021
86	gi 9951459	PA5158	probable outer membrane protein precursor	28	0.55	28.545
87	gi 9949837	PA3674	hypothetical protein	10	0.55	15.867
88	gi 9949622	rhlB	rhamnosyltransferase chain B	17	0.55	29.86
89	gi 9946282	mexB	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexB	32	0.55	26.201
90	gi 9949594	mqaA	malate:quinone oxidoreductase	26	0.54	21.764
91	gi 9948015	liuD	methylcrotonyl-CoA carboxylase, alpha-subunit (biotin-containing)	59	0.54	20.225
92	gi 9950996	PA4735	hypothetical protein	76	0.54	27.658
93	gi 9946859	PA0953	probable thioredoxin	13	0.54	16.775
94	gi 9946933	PA1022	probable acyl-CoA dehydrogenase	3	0.53	9.8495

95	gi 375040205	PA2291	probable glucose-sensitive porin	45	0.53	21.849
96	gi 9947103	oprH	PhoP/Q and low Mg ²⁺ inducible outer membrane protein H1	30	0.53	18.809
97	gi 9951052	PA4786	probable short-chain dehydrogenase	38	0.53	22.106
98	gi 9949333	PA3213	hypothetical protein	23	0.53	20.005
99	gi 528082511	ampdh2	Ampdh2	29	0.53	29.261
100	gi 9950905	PA4651	probable pili assembly chaperone	4	0.53	16.492
101	gi 635575323	azu	azurin precursor	4	0.53	7.6475
102	gi 660505582	oprD	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	7	0.53	8.935
103	gi 9948953	PA2864	conserved hypothetical protein	2	0.52	7.7883
104	gi 9947514	ccoN1	Cytochrome c oxidase, cbb3-type, CcoN subunit	4	0.52	14.034
105	gi 9945985	pcaG	protocatechuate 3,4-dioxygenase, alpha subunit	8	0.52	3.3881
106	gi 9947722	PA1745	hypothetical protein	15	0.5	23.228
107	gi 9951570	hemD	uroporphyrinogen-III synthetase	13	0.5	10.795
108	gi 9948275	bkdB	branched-chain alpha-keto acid dehydrogenase (lipoamide component)	38	0.5	22.076
109	gi 9948191	PA2171	hypothetical protein	3	0.49	14.467
110	gi 9947982	exaA	quinoprotein ethanol dehydrogenase	7	0.49	11.904
111	gi 660508993	PA4405	hypothetical protein	4	0.49	20.802
112	gi 660505087	PA0452	putative stomatin-like protein	2	0.48	10.865
113	gi 375049057	cdrB	cyclic diguanylate-regulated TPS partner B, CdrB	36	0.48	28.953
114	gi 660508934	PA4347	putative beta-lactamase	13	0.48	17.344
115	gi 9946864	oprD	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	46	0.48	29.951
116	gi 9947451	PA1496	probable potassium channel	3	0.48	13.323
117	gi 9947256	cyoB	cytochrome o ubiquinol oxidase subunit I	7	0.47	25.521

118	gi 529481853	oprE	Anaerobically-induced outer membrane porin OprE precursor	52	0.47	26.671
119	gi 9947755	cmpX	conserved cytoplasmic membrane protein, CmpX protein	10	0.46	21.649
120	gi 9949801	PA3641	probable amino acid permease	8	0.46	24.364
121	gi 651877066	opmQ	probable outer membrane protein precursor	29	0.46	13.627
122	gi 9951403	blc	outer membrane lipoprotein Blc	16	0.45	12.133
123	gi 9946417	PA0549	hypothetical protein	15	0.44	0.7934
124	gi 9946410	PA0543	hypothetical protein	7	0.44	11.683
125	gi 9948143	cgrA	cupA gene regulator A	4	0.43	6.5127
126	gi 9950281	cupB5	adhesive protein CupB5	4	0.43	14.987
127	gi 9946422	PA0553	hypothetical protein	4	0.41	27.716
128	gi 660508920	PA4333	putative fumarase	35	0.41	5.3271
129	gi 9948319	PA2289	conserved hypothetical protein	11	0.41	18.127
130	gi 9946125	cysT	sulfate transport protein CysT	6	0.4	9.7107
131	gi 9950350	PA4144	probable outer membrane protein precursor	16	0.38	21.482
132	gi 9951034	lldP	L-lactate permease	2	0.36	10.992
133	gi 9948187	PA2167	hypothetical protein	4	0.32	10.857
134	gi 9947179	aprF	Alkaline protease secretion outer membrane protein AprF	31	0.31	19.287
135	gi 9947985	exaC	NAD+ dependent aldehyde dehydrogenase ExaC	37	0.29	27.581
136	gi 4838479	hasR	heme receptor HasR	21	0.27	29.843
137	gi 9950345	PA4140	hypothetical protein	6	0.19	18.447
138	gi 375051307	PA3866	Pyocin S4	24	0.14	9.356
139	gi 9950344	PA4139	hypothetical protein	5	0.01	8.2145

Supplementary table A6. 250 proteins that were of higher abundance in *P. aeruginosa* PAO1 colistin-tolerant cells than in colistin-sensitive biofilm cells. The protein data were sorted accordingly from highly expressed to lowly expressed proteins in colistin-tolerant cells compared to colistin-sensitive cells. Reproduced with permission from (Chua et al 2016). The complete list of data can be found in website:

[http://www.nature.com/article-](http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s5.xlsx)

[assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s5.xlsx](http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s5.xlsx)

	GI No.	Gene	Description	Peptides	Ratio H/L (medium)	Ratio H/L variability [%]
1	gi 9949838	PA3675	hypothetical protein	10	12.48	12.407
2	gi 9946956	PA1043	hypothetical protein	6	10.77	9.4975
3	gi 375049572	PA1797	hypothetical protein	13	7.15	25.366
4	gi 9948430	pvdR	efflux transporter, RND family, MFP subunit	13	6.42	19.348
5	gi 478247350	ampC	Chain A, Amp-c Beta-lactamase	13	6.06	27.042
6	gi 9948942	PA2854	conserved hypothetical protein	15	5.74	20.107
7	gi 9948829	PA2750	hypothetical protein	2	5.44	12.81
8	gi 9948874	PA2792	hypothetical protein	3	5.29	7.5153
9	gi 9949757	PA3601	conserved hypothetical protein	3	5.17	16.558
10	gi 9950867	PA4616	probable c4-dicarboxylate-binding protein	12	5.14	27.297
11	gi 9949951	PA3779	hypothetical protein	8	5.06	6.9082
12	gi 9950715	PA4778	conserved hypothetical protein	7	4.88	1.4033
13	gi 3237308	asd	aspartate semialdehyde dehydrogenase	32	4.86	4.2686
14	gi 9948498	PA2450	hypothetical protein	12	4.7	12.047
15	gi 9946314	PA0454	conserved hypothetical protein	5	4.66	12.891
16	gi 9949957	PA3785	hypothetical protein	7	4.65	20.407

17	gi 375050918	pra	protein activator	2	4.43	27.792
18	gi 9946374	nirN	probable c-type cytochrome	14	4.3	10.798
19	gi 9948494	gcvH2	glycine cleavage system protein H2	1	4.24	24.155
20	gi 9950476	rpsS	30S ribosomal protein S19	15	4.16	30.967
21	gi 9946409	PA0542	conserved hypothetical protein	5	4.14	19.07
22	gi 9948656	PA2592	probable periplasmic spermidine/putrescine-binding protein	26	4.11	30.543
23	gi 9951833	znuA	probable adhesin	13	4.11	6.7512
24	gi 660509356	recN	DNA repair protein RecN	30	3.95	17.147
25	gi 9948436	PvdO	sulfatase-modifying factor enzyme 1 family protein	14	3.92	30.697
26	gi 9948820	rpmI	50S ribosomal protein L35	3	3.89	17.072
27	gi 9949214	xcpP	secretion protein XcpP	8	3.89	12.343
28	gi 9949880	PA3715	hypothetical protein	6	3.84	30.294
29	gi 9946112	PA0270	hypothetical protein	1	3.81	18.748
30	gi 9951853	pdxY	pyridoxamine kinase	8	3.78	27.322
31	gi 9946843	PA0938	hypothetical protein	32	3.78	24.021
32	gi 375049887	mltD	membrane-bound lytic murein transglycosylase D	3	3.68	3.3808
33	gi 375050769	PA1048	putative outer membrane protein	7	3.67	2.6116
34	gi 9951201	PA4923	conserved hypothetical protein	4	3.64	27.598
35	gi 9947083	PA1160	hypothetical protein	3	3.64	16.245
36	gi 9951600	PA5286	conserved hypothetical protein	8	3.63	25.281
37	gi 9951777	PA5446	hypothetical protein	4	3.61	9.2327
38	gi 9948454	PA2410	hypothetical protein	19	3.58	21.231
39	gi 375049982	PA5330	hypothetical protein	14	3.56	28.652
40	gi 9948366	PA2331	hypothetical protein	16	3.54	10.206
41	gi 9949993	PA3819	conserved hypothetical protein	7	3.52	25.516
42	gi 9947533	PA1571	hypothetical protein	1	3.47	8.9632
43	gi 9951202	PA4924	conserved hypothetical protein	1	3.46	18.593
44	gi 9947237	PA1301	fecR family protein	10	3.39	17.883

45	gi 375048520	PA4035	hypothetical protein	21	3.39	15.253
46	gi 9950944	hitA	ferric iron-binding periplasmic protein HitA	32	3.38	27.362
47	gi 9950260	PA4063	hypothetical protein	9	3.36	17.384
48	gi 9948716	nuoL	NADH-quinone oxidoreductase subunit L	4	3.34	1.6003
49	gi 660505977	pdxB	erythronate-4-phosphate dehydrogenase	22	3.33	8.3805
50	gi 9949779	fdxA	ferredoxin I	4	3.32	18.093
51	gi 375047949	PA1383	hypothetical protein	22	3.27	27.462
52	gi 9949567	PA3427	probable short-chain dehydrogenase	10	3.26	25.587
53	gi 9949078	kdsB	3-deoxy-manno-octulosonate cytidyltransferase	16	3.24	19.345
54	gi 9948451	PA2407	probable adhesion protein	17	3.22	28.118
55	gi 9949708	arnT	inner membrane L-Ara4N transferase ArnT	5	3.21	19.882
56	gi 660507441	PA2822	putative GAF domain-containing protein	7	3.2	29.582
57	gi 9949970	PA3797	conserved hypothetical protein	18	3.19	26.981
58	gi 660509367	PA4774	spermidine synthase	21	3.18	26.696
59	gi 9947248	phnX	2-phosphonoacetaldehyde hydrolase	7	3.14	18.147
60	gi 651877936		hypothetical protein	2	3.14	7.2784
61	gi 9949666	PA3518	hypothetical protein	6	3.08	28.949
62	gi 9949123	PA3020	probable soluble lytic transglycosylase	36	3.08	27.835
63	gi 9949124	PA3021	hypothetical protein	9	3.08	21.255
64	gi 9949768	PA3611	hypothetical protein	11	3.07	21.235
65	gi 9950602	PA4373	hypothetical protein	12	3.07	10.856
66	gi 9950977	PA4718	hypothetical protein	8	3.05	6.9494
67	gi 9948871	PA2789	hypothetical protein	10	3.05	0.24908
68	gi 9951248	PA4966	hypothetical protein	17	3.04	26.162
69	gi 9950472	rpmC	50S ribosomal protein L29	12	3.02	29.996
70	gi 9946264	pilH	probable periplasmic spermidine/putrescine-	6	3.01	21.965

			binding protein			
71	gi 9950694	PA4458	conserved hypothetical protein	14	3.01	12.558
72	gi 9949625	PA3480	probable deoxycytidine triphosphate deaminase	15	3	27.296
73	gi 9951631	PA5314	hypothetical protein	3	3	24.162
74	gi 9947823	PA1837	hypothetical protein	10	2.98	30.338
75	gi 9949932	PA3762	hypothetical protein	5	2.98	27.034
76	gi 9948247	PA2222	hypothetical protein	11	2.97	19.956
77	gi 375041674	PvdQ	3-oxo-C12-homoserine lactone acylase	51	2.94	26.448
78	gi 9950720	gatC	Glu-tRNA(Gln) amidotransferase subunit C	3	2.92	12.795
79	gi 9950555	PA4330	probable enoyl-CoA hydratase/isomerase	10	2.91	30.232
80	gi 9946995	flgD	flagellar basal-body rod modification protein FlgD	2	2.91	10.657
81	gi 9950966	PhuT	Heme-transport protein	23	2.9	25.935
82	gi 9950208	PA4015	conserved hypothetical protein	13	2.88	27.993
83	gi 9951545	PA5236	probable aromatic hydrocarbon reductase	12	2.86	10.265
84	gi 9948700	PA2633	hypothetical protein	8	2.85	19.692
85	gi 375051021	magE	hypothetical protein	29	2.85	19.033
86	gi 9946684	PA0793	hypothetical protein	14	2.84	26.807
87	gi 9951649	pyrE	orotate phosphoribosyltransferase	24	2.83	18.582
88	gi 9951062	PA4796	hypothetical protein	4	2.83	15.085
89	gi 660505098	PA0462	hypothetical protein	11	2.8	27.619
90	gi 9950100	moaE	molybdopterin converting factor, large subunit	5	2.8	10.291
91	gi 9946126	sbp	sulfate-binding protein precursor	13	2.79	23.079
92	gi 9945846	plcB	phospholipase C, PlcB	7	2.78	23.273
93	gi 9946137	aguB	N-carbamoylputrescine amidohydrolase	6	2.78	13.45
94	gi 9949890	lasB	elastase LasB	25	2.75	20.994
95	gi 9946257	pyrR	bifunctional pyrimidine regulatory protein PyrR	11	2.75	11.259
96	gi 9946205	pfpI	protease PfpI	3	2.74	1.252

97	gi 9950825	PA4578	hypothetical protein	1	2.74	0.20746
98	gi 9951209	PA4930	biosynthetic alanine racemase	14	2.73	26.877
99	gi 9949463	PA3332	Uncharacterized PhzA/B-like protein	21	2.73	26.38
100	gi 9945996	PA0164	probable gamma-glutamyltranspeptidase	21	2.73	20.224
101	gi 9950461	rplO	50S ribosomal protein L15	21	2.72	24.9
102	gi 375048364	Fis	global DNA-binding transcriptional dual regulator	5	2.71	28.666
103	gi 9949066	fabD	malonyl-CoA-[acyl-carrier-protein] transacylase	17	2.7	27.681
104	gi 9946934	PA1023	probable short-chain dehydrogenase	24	2.7	15.895
105	gi 9946608	PA0724	phage coat protein A	8	2.7	12.323
106	gi 9947557	PA1593	hypothetical protein	3	2.69	20.543
107	gi 9950020	PA3844	hypothetical protein	10	2.69	7.1171
108	gi 9947790	fabI	NADH-dependent enoyl-ACP reductase	14	2.68	30.503
109	gi 9950898	PA4645	probable purine/pyrimidine phosphoribosyl transferase	11	2.68	27.671
110	gi 9949379	PA3255	hypothetical protein	11	2.68	26.925
111	gi 9949620	rhlI	autoinducer synthesis protein RhlI	10	2.68	25.5
112	gi 9951289	PA5003	hypothetical protein	12	2.68	2.393
113	gi 9946140	spuI	probable glutamine synthetase	26	2.67	23.007
114	gi 9949911	trmD	tRNA (guanine-N1)-methyltransferase	12	2.67	17.766
115	gi 9946617	PA0732	hypothetical protein	7	2.67	9.6614
116	gi 9947591	PA1624	hypothetical protein	20	2.66	27.686
117	gi 9947589	PA1622	probable hydrolase	10	2.66	18.97
118	gi 9949706	arnA	Bifunctional polymyxin resistance protein ArnA	59	2.65	24.402
119	gi 9951224	hfq	RNA chaperone Hfq	10	2.64	30.97
120	gi 9948981	atuE	putative isohexenylglutaconyl-CoA hydratase	7	2.64	17.926
121	gi 9948046	PA2040	Glutamylpolyamine synthetase	21	2.64	10.673
122	gi 9951405	PA5109	hypothetical protein	8	2.63	25.609
123	gi 9949955	PA3783	hypothetical protein	5	2.63	11.961

124	gi 9950059	narL	two-component response regulator NarL	13	2.62	28.997
125	gi 9951374	PA5080	prolyl aminopeptidase	27	2.62	26.558
126	gi 9947030	PA1112	hypothetical protein	12	2.62	13.02
127	gi 9948685	infA	translation initiation factor IF-1	6	2.61	30.892
128	gi 9947770	PA1789	hypothetical protein	22	2.6	28.711
129	gi 9950174	PA3983	conserved hypothetical protein	16	2.59	19.499
130	gi 375050610	KynA	tryptophan 2,3-dioxygenase	10	2.58	25.771
131	gi 9949196	PA3087	hypothetical protein	17	2.58	20.94
132	gi 375046279	PA4533	hypothetical protein	12	2.58	16.615
133	gi 9947132	PA1205	conserved hypothetical protein	9	2.58	3.169
134	gi 9950117	PA3931	conserved hypothetical protein	11	2.56	19.62
135	gi 9947265	ilvA2	threonine dehydratase	14	2.56	12.725
136	gi 9948966	pyrF	orotidine 5-phosphate decarboxylase	17	2.54	24.692
137	gi 9949244	PA3131	probable aldolase	13	2.54	3.9881
138	gi 9949373	PA3250	hypothetical protein	18	2.53	23.434
139	gi 9950197	PA4005	hypothetical protein	6	2.52	4.7585
140	gi 9946875	PA0968	hypothetical protein	5	2.51	1.8625
141	gi 9946279	pasP	PasP	23	2.5	28.092
142	gi 660505194	PA0557	calcium/calmodulin dependent kinase II Association family protein	4	2.5	10.722
143	gi 9946992	PA1076	hypothetical protein	5	2.49	16.078
144	gi 9951427	SecB	Bacterial protein export chaperone SecB	16	2.48	29.311
145	gi 9947580	gpsA	glycerol-3-phosphate dehydrogenase, biosynthetic	21	2.48	18.898
146	gi 660504671	PA0039	hypothetical protein	2	2.48	12.115
147	gi 9951856	PA5519	conserved hypothetical protein	6	2.48	6.2957
148	gi 301598582	mexR	multidrug resistance operon repressor MexR	8	2.47	10.05
149	gi 9947241	PA1304	probable oligopeptidase	38	2.45	29.726
150	gi 9947831	PA1844	hypothetical protein	5	2.45	18.703
151	gi 9951359	hisI	phosphoribosyl-AMP cyclohydrolase	7	2.45	18.017

152	gi 9950736	PA4497	probable binding protein component of ABC transporter	15	2.45	7.9385
153	gi 9947824	cysI	sulfite reductase	49	2.44	29.791
154	gi 9949287	PA3170	hypothetical protein	16	2.44	28.343
155	gi 9947300	PA1358	hypothetical protein	5	2.44	6.3598
156	gi 9946247	PA0394	putative PLP dependent enzyme	16	2.41	30.246
157	gi 9948589	tpx	thiol peroxidase	22	2.41	28.115
158	gi 9947036	PA1117	hypothetical protein	4	2.41	24.476
159	gi 9945838	def	polypeptide deformylase	13	2.41	22.793
160	gi 9946770	phhB	pterin-4-alpha-carbinolamine dehydratase	11	2.41	17.549
161	gi 9950623	PA4392	hypothetical protein	6	2.41	8.5523
162	gi 9949927	PA3758	probable N-acetylglucosamine-6-phosphate deacetylase	17	2.4	23.264
163	gi 9948588	PA2531	probable aminotransferase	22	2.38	29.37
164	gi 9948292	PA2264	hypothetical protein	3	2.38	13.456
165	gi 9951252	cpdA	Cyclic AMP (cAMP) Phosphodiesterase, CpdA	17	2.37	30.222
166	gi 9950911	PA4657	hypothetical protein	17	2.36	19.193
167	gi 9947596	PA1629	probable enoyl-CoA hydratase/isomerase	4	2.36	5.5427
168	gi 9947113	PA1187	probable acyl-CoA dehydrogenase	7	2.35	14.868
169	gi 9948756	PA2684	conserved hypothetical protein	55	2.34	27.939
170	gi 574451362	PA0372	Chain B, Subunit Pa0372 Of Heterodimeric Zinc Protease	38	2.33	27.709
171	gi 9946141	spuA	probable glutamine amidotransferase	7	2.33	8.3791
172	gi 9951196	PA4918	hypothetical protein	8	2.32	28.379
173	gi 660506262	impG	protein ImpG	28	2.32	26.981
174	gi 9951579	hcpB	secreted protein Hcp	16	2.32	23.19
175	gi 9949071	PA2972	conserved hypothetical protein	14	2.32	11.547
176	gi 9946927	PA1016	hypothetical protein	6	2.32	9.4637
177	gi 9949570	PA3430	probable aldolase	5	2.31	28.981

178	gi 660507174	PA2562	conserved hypothetical protein	3	2.31	27.901
179	gi 9946760	PA0862	hypothetical protein	10	2.31	26.395
180	gi 9950023	PA3846	hypothetical protein	11	2.31	20.669
181	gi 9946246	proC	pyrroline-5-carboxylate reductase	9	2.31	17.954
182	gi 9945970	ahpF	alkyl hydroperoxide reductase subunit F	36	2.3	30.202
183	gi 375040428	PA0719	hypothetical protein of bacteriophage Pf1	2	2.3	11.684
184	gi 9947474	PA1517	conserved hypothetical protein	15	2.29	26.976
185	gi 9946129	DesA	delta-9 fatty acid desaturase	2	2.29	14.351
186	gi 9951402	PA5106	conserved hypothetical protein	24	2.28	11.791
187	gi 9947437	cycH	cytochrome c-type biogenesis protein	28	2.27	28.391
188	gi 9951154	PA4880	probable bacterioferritin	19	2.27	19.432
189	gi 9949632	tle5	phospholipase D	25	2.27	15.495
190	gi 9946700	ampDh3	AmpDh3	10	2.27	7.7276
191	gi 660507770	PA3172	phosphoglycolate phosphatase	14	2.26	25.854
192	gi 9949613	PA3469	conserved hypothetical protein	9	2.26	20.195
193	gi 9947575	fabB	beta-ketoacyl-ACP synthase I	31	2.25	29.14
194	gi 9949232	leuD	3-isopropylmalate dehydratase small subunit	17	2.25	26.043
195	gi 9947626	PA1656	hypothetical protein	28	2.24	22.499
196	gi 9950879	hprA	glycerate dehydrogenase	18	2.24	9.0355
197	gi 9947478	PA1521	probable guanine deaminase	23	2.22	23.606
198	gi 9950150	PA3961	probable ATP-dependent helicase	16	2.22	10.68
199	gi 9951640	algC	phosphomannomutase AlgC	50	2.22	7.5224
200	gi 660507816	cyaB	adenylate and Guanylate cyclase catalytic domain protein	4	2.2	13.134
201	gi 1246302	pilY1	type 4 fimbrial biogenesis protein PilY1	52	2.2	6.8205
202	gi 9951245	PA4963	hypothetical protein	17	2.19	30.668
203	gi 9951008	PA4746	conserved hypothetical	3	2.19	11.321

			protein			
204	gi 9948846	PA2766	probable transcriptional regulator	3	2.19	3.1777
205	gi 9949984	hscA	heat shock protein HscA	41	2.18	21.597
206	gi 9950660	sspB	stringent starvation protein B	5	2.18	13.36
207	gi 9949197	PA3088	conserved hypothetical protein	14	2.17	22.697
208	gi 9950424	PhzB	Phenazine biosynthesis protein	21	2.17	22.593
209	gi 9946258	PA0404	conserved hypothetical protein	7	2.16	29.315
210	gi 9949783	surE	survival protein SurE	10	2.15	29.239
211	gi 9950666	rplM	50S ribosomal protein L13	21	2.15	25.362
212	gi 9948091	kynU	kynureninase KynU	21	2.15	15.717
213	gi 9946161	PA0315	hypothetical protein	1	2.14	29.746
214	gi 9949773	PA3615	hypothetical protein	19	2.14	28.473
215	gi 9946807	RsmA	regulation of secondary metabolic process	6	2.14	20.46
216	gi 9950870	PA4618	hypothetical protein	13	2.14	13.583
217	gi 9948955	mttC	secretion protein	13	2.14	9.036
218	gi 9948805	PA2729	hypothetical protein	31	2.13	24.909
219	gi 9951232	orn	oligoribonuclease	10	2.13	21.299
220	gi 9950494	nusG	transcription antitermination protein NusG	20	2.12	29.837
221	gi 9949637	PA3491	probable ferredoxin	20	2.11	29.659
222	gi 9948637	PA2575	hypothetical protein	27	2.11	29.343
223	gi 9950198	PA4006	nicotinic acid mononucleotide adenylyltransferase	7	2.11	29.155
224	gi 9948689	cspD	cold-shock protein CspD	6	2.11	22.721
225	gi 9949704	arnB	UDP-4-amino-4-deoxy-L-arabinose--oxoglutarate aminotransferase, ArnB	35	2.11	18.22
226	gi 660505712	fliL	flagellum-specific ATP synthase	17	2.11	13.32
227	gi 9951408	gloA3	lactoylglutathione lyase	9	2.11	5.2086
228	gi 9949565	PA3426	probable enoyl CoA-hydratase/isomerase	15	2.1	17.88
229	gi 375050788	PA1029	hypothetical protein	4	2.1	7.4422

230	gi 9946100	PA0259	hypothetical protein	36	2.09	30.174
231	gi 9948072	PA2063	hypothetical protein	8	2.09	25.8
232	gi 9950864	katB	catalase	21	2.08	26.793
233	gi 9946010	PA0177	probable purine-binding chemotaxis protein	2	2.08	25.615
234	gi 9949916	PA3748	conserved hypothetical protein	11	2.08	17.002
235	gi 9949228	PA3116	probable aspartate- semialdehyde dehydrogenase	14	2.07	6.7757
236	gi 9950107	PA3922	hypothetical protein	34	2.06	18.529
237	gi 9951555	PA5245	hypothetical protein	22	2.05	28.703
238	gi 9950070	PA3889	probable binding protein component of ABC transporter	2	2.05	0.86756
239	gi 9949760	ErdR	bacterial regulatory s, luxR family protein	15	2.04	28.818
240	gi 37505084 9	Nuh	purine nucleosidase	19	2.04	24.963
241	gi 9947936	PA1940	hypothetical protein	16	2.04	20.311
242	gi 9950473	rplP	50S ribosomal protein L16	19	2.03	27.695
243	gi 9951512	argE	acetylornithine deacetylase	20	2.03	26.029
244	gi 9951651	crc	catabolite repression control protein	22	2.03	23.326
245	gi 9947639	PA1668	hypothetical protein	14	2.03	22.566
246	gi 65187771 9	PA0431	hypothetical protein	9	2.03	21.677
247	gi 9948880	PA2797	hypothetical protein	7	2.02	24.75
248	gi 9947471	PA1514	ureidoglycolate hydrolaseYbbT	6	2.02	10.879
249	gi 9945824	PA0006	hypothetical protein	5	2.01	26.099
250	gi 9948881	PA2798	probable two- component response regulator	22	2.01	20.958

Supplementary table A7. Complete list of *P. aeruginosa* proteins identified by SILAC. Reproduced with permission from (Chua et al 2016). Due to the large amount of data, please refer to the weblink:

<http://www.nature.com/article->

[assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s3.xlsx](http://www.nature.com/article-assets/npg/ncomms/2016/160219/ncomms10750/extref/ncomms10750-s3.xlsx)

7.2 Appendix B

Supplementary table B1. Mapping statistics of the RNA sequencing raw reads.

	2 dpi_1	2 dpi_2	2 dpi_3	7 dpi_1	7 dpi_2	7 dpi_3
Total fragments	20,446,081	20,239,128	21,051,896	21,291,172	27,074,040	21,948,163
non-rRNA fragments	12,084,954	6,323,790	7,811,760	10,337,454	13,076,972	9,161,384
% of Total fragments non-rRNA	59.11	31.25	37.11	48.55	48.30	41.74
Total fragments map to Mouse	8,748,329	4,017,810	5,038,700	7,375,206	9,399,425	6,369,275
% of Total fragments map to Mouse	42.79	19.85	23.93	34.64	34.72	29.02
Counted fragments map to PA	45,346	51,723	44,850	11,172	6,289	6,356
% of Total fragments map to <i>P. aeruginosa</i>	0.22	0.26	0.21	0.05	0.02	0.03

Supplementary figure B1. The uninfected scratched corneas viewed under bright field microscopy and were observed under CLSM for green fluorescence expression. The results were presented as representative CLSM image of each condition from 3 individual experiments. Scale bar, 20 μm .

Supplementary table B2. Upregulated genes in *P. aeruginosa* 2 dpi *in vivo* cells compared to *in vitro* biofilm cells. The data showed top 200 genes listed were sorted according to most upregulated to least upregulated gene expression.

	Feature ID	Log ₂ Fold change	P value	Annotations
1	PA3600	8.898463671	1.56E-26	
2	PA3601	8.601984914	4.574E-73	
3	PA3283	8.278150791	5.166E-65	
4	PA3284	8.118181036	3.104E-57	
5	PA4837	7.534356609	6.079E-51	
6	PA4836	7.279696559	3.736E-33	
7	PA3282	7.051244342	1.689E-27	
8	PA5540	6.705222103	3.319E-16	
9	PA4277.3	6.594843197	7.912E-11	
10	hasAp	6.512854031	1.1E-33	heme acquisition protein HasAp
11	norB	6.432478052	1.448E-46	nitric-oxide reductase subunit B
12	pcrH	6.411862499	5.167E-23	regulatory protein PcrH
13	rpmJ	6.374991922	2.369E-21	50S ribosomal protein L36
14	PA5536	6.33909202	1.472E-17	
15	PA0905.1	6.170230518	7.88E-10	
16	PA1922	6.166167579	3.354E-18	
17	exoS	5.99040692	2.703E-42	
18	PA5534	5.988647869	2.879E-19	
19	PA5535	5.951852871	9.103E-37	
20	nirS	5.906526405	7.085E-49	nitrite reductase precursor
21	amiA	5.888645636	3.007E-25	N-acetylmuramoyl-L-alanine amidase
22	popD	5.864647728	1.187E-32	Translocator outer membrane protein PopD precursor
23	PA2883	5.8284867	1.905E-26	
24	PA5539	5.751374805	6.138E-16	
25	PA4170	5.717298986	5.728E-16	
26	popB	5.707913292	2.085E-42	translocator protein PopB
27	pcrV	5.588589351	1.587E-27	type III secretion protein PcrV
28	PA0879	5.588130583	3.448E-23	
29	norC	5.56945343	8.82E-19	nitric-oxide reductase subunit C
30	PA3281	5.506114575	2.383E-20	
31	nirM	5.488145909	1.023E-13	cytochrome c-551 precursor
32	PA3432	5.470623287	6.675E-15	
33	PA1673	5.432231979	5.141E-30	
34	PA0781	5.408195728	1.261E-30	

35	PA3431	5.319746842	3.44E-21	
36	rpmH	5.292310357	5.138E-36	50S ribosomal protein L34
37	PA2753	5.287538511	1.103E-13	
38	pscF	5.255602665	6.497E-15	type III export protein PscF
39	nosZ	5.248553727	2.961E-37	nitrous-oxide reductase precursor
40	PA0526	5.215031281	8.74E-09	
41	PA0714	5.191911773	2.404E-19	
42	nosR	5.153501475	8.522E-22	regulatory protein NosR
43	PA3599	5.151902235	4.683E-68	
44	PA3530	5.122831186	6.741E-39	
45	PA4884	5.111712122	3.482E-14	
46	PA0525	5.099743603	5.995E-19	
47	exsE	5.07854773	5.602E-11	ExsE
48	popN	5.06662696	3.63E-14	Type III secretion outer membrane protein PopN precursor
49	narK1	5.041925376	3.425E-24	nitrite extrusion protein 1
50	PA4063	5.034661539	4.171E-21	
51	PA1699	5.03394834	2.649E-08	
52	PA4835	4.99478659	3.611E-23	
53	PA4883	4.990990097	3.987E-10	
54	narI	4.973075505	9.807E-13	respiratory nitrate reductase gamma chain
55	pscG	4.969904564	9.133E-12	type III export protein PscF
56	exsC	4.933494785	1.419E-39	"ExsC, exoenzyme S synthesis protein C precursor."
57	PA1195	4.911605074	3.233E-19	
58	PA1925	4.89472172	3.043E-08	
59	PA3370	4.878462281	1.014E-08	
60	PA0878	4.85753965	7.113E-17	
61	exsA	4.834932688	4.465E-43	transcriptional regulator ExsA
62	exoT	4.833965157	3.404E-30	
63	PA1697	4.831209151	2.664E-18	ATP synthase in type III secretion system
64	PA3412	4.789259542	2.136E-10	
65	cifR	4.757998425	2.225E-26	
66	narK2	4.748514716	9.738E-17	nitrite extrusion protein 2
67	PA0887.1	4.747734456	8.969E-05	
68	pscB	4.70102722	9.265E-12	type III export apparatus protein
69	exoY	4.684944227	2.446E-21	
70	PA4882	4.681239618	2.551E-12	
71	PA3871	4.646900332	8.219E-08	
72	PA3842	4.641508683	1.685E-22	
73	hasD	4.627548619	5.061E-09	transport protein HasD
74	pscD	4.567682443	6.672E-14	type III export protein PscD

75	PA0532	4.547586538	1.853E-12	
76	hasR	4.525721113	1.434E-17	Haem uptake outer membrane receptor HasR precursor
77	ohr	4.520668857	6.235E-26	organic hydroperoxide resistance protein
78	armR	4.452799524	1.023E-05	
79	PA1924	4.447281597	2.196E-06	
80	PA0713	4.410102732	3.036E-28	
81	pscC	4.409652844	1.599E-25	Type III secretion outer membrane protein PscC precursor
82	PA0880	4.407959988	1.542E-05	
83	PA4570	4.406462221	7.408E-51	
84	moaB1	4.398624122	9.965E-09	molybdopterin biosynthetic protein B1
85	narG	4.395097964	1.879E-23	respiratory nitrate reductase alpha chain
86	ibpA	4.382469762	1.659E-16	heat-shock protein IbpA
87	PA4348	4.365607448	5.137E-26	
88	PA1746	4.337978724	1.414E-24	
89	PA1560	4.303239497	7.052E-16	
90	PA5482	4.293213805	7.049E-17	
91	PA3278	4.253617782	1.235E-21	
92	adhA	4.221226128	2.247E-34	alcohol dehydrogenase
93	pcrG	4.211159429	3.522E-07	regulator in type III secretion
94	narH	4.165330294	1.815E-19	respiratory nitrate reductase beta chain
95	PA3231	4.148484206	6.363E-05	
96	PA1923	4.145914264	5.401E-17	
97	PA3913	4.139913075	1.199E-09	
98	morB	4.12942242	1.33E-09	morphinone reductase
99	PA4577	4.119610199	8.449E-11	
100	PA1747	4.114003471	3.754E-10	
101	PA3144	4.099122512	0.0005895	
102	nosD	4.051904427	1.726E-11	NosD protein
103	PA0512	4.044338808	4.959E-09	
104	exsD	4.040410061	1.248E-30	ExsD
105	PA5475	4.040092285	3.007E-25	
106	PA0883	4.03822029	1.412E-07	
107	PA2783	4.036824572	4.302E-15	
108	PA4896	4.021631473	8.685E-25	
109	PA1414	3.957141771	3.68E-26	
110	PA4692	3.947111134	2.764E-55	
111	PA4674	3.936003882	4.358E-12	
112	pscJ	3.934505753	1.505E-13	type III export protein PscJ
113	pilA	3.93251645	2.978E-22	type 4 fimbrial precursor PilA
114	ppyR	3.928816082	6.44E-08	
115	pscH	3.920621615	1.173E-08	type III export protein PscH
116	PA4364	3.919659837	1.031E-05	

117	exsB	3.896223032	2.642E-17	exoenzyme S synthesis protein B
118	rpmI	3.88857968	9.616E-97	50S ribosomal protein L35
119	PA4937.1	3.879343328	0.003199	
120	PA4523	3.873051253	3.473E-69	
121	betB	3.83776881	1.403E-31	betaine aldehyde dehydrogenase
122	pscK	3.774134632	3.753E-05	type III export protein PscK
123	hasE	3.742935274	3.207E-07	metalloprotease secretion protein
124	betI	3.722596081	7.811E-45	transcriptional regulator BetI
125	pelC	3.722311304	0.0019109	
126	cdhC	3.706097266	0.0010029	
127	phrS	3.704504812	8.674E-17	
128	PA4406.1	3.702148435	0.0006873	
129	pcrD	3.682350211	2.154E-20	type III secretory apparatus protein PcrD
130	PA0884	3.672343735	3.039E-13	
131	PA3840	3.653009535	2.511E-14	
132	PA4065	3.644247007	1.346E-22	
133	fhp	3.638442478	5.07E-20	flavoheomoprotein
134	PA0161	3.635164691	0.000132	
135	PA4838	3.621780819	3.31E-16	
136	pscL	3.601510669	5.391E-07	type III export protein PscL
137	PA0907	3.577661177	4.989E-08	
138	PA1692	3.571327804	0.0001204	
139	PA2146	3.565719598	9.188E-05	
140	PA3762	3.565710033	3.843E-12	
141	pyrQ	3.561108452	8.837E-17	dihydroorotase
142	PA0929	3.544639063	4.038E-27	two-component response regulator
143	PA0545	3.544274962	4.365E-08	
144	PA2453	3.532111284	1.164E-13	
145	moeA1	3.525170274	3.363E-07	
146	cif	3.499438218	1.474E-05	
147	PA2759	3.484445363	2.371E-12	
148	PA3911	3.482024444	5.356E-05	
149	PA0882	3.469686655	1.261E-06	
150	PA4834	3.465150463	3.839E-15	
151	PA1429	3.451387541	2.817E-21	
152	PA0738	3.44761102	0.0008459	
153	kdpB	3.430388656	2.24E-05	"potassium-transporting ATPase, B chain"
154	PA3390	3.424519785	0.0001556	
155	narJ	3.391566676	4.718E-05	respiratory nitrate reductase delta chain
156	PA1194	3.389575855	1.582E-09	
157	PA0435	3.361484441	2.777E-07	
158	betA	3.335829159	8.455E-18	choline dehydrogenase

159	PA3912	3.330884855	3.128E-08	
160	PA0346	3.32946341	3.168E-07	
161	pscI	3.328628225	2.373E-07	type III export protein PscI
162	cupA2	3.325915871	0.0075861	chaperone CupA2
163	PA2763	3.321873141	0.0044184	
164	PA4575	3.320579436	4.48E-11	
165	PA5530	3.319332988	5.105E-13	
166	gcl	3.314649963	6.669E-09	glyoxylate carboligase
167	PA0513	3.298056908	1.754E-05	
168	pscO	3.293190411	0.0008179	translocation protein in type III secretion
169	PA1700	3.281660742	0.0006321	
170	PA1602	3.267074475	0.0001781	
171	PA0510	3.265978679	2.266E-07	
172	pilM	3.259859134	7.139E-29	type 4 fimbrial biogenesis protein PilM
173	PA0952	3.259594582	7.737E-15	
174	arcA	3.259483666	2.406E-41	arginine deiminase
175	PA3843	3.255912449	1.308E-08	
176	PA4156	3.249117551	1.618E-18	
177	PA4881	3.248625582	8.539E-10	
178	PA3952	3.248315723	3.618E-10	
179	PA1559	3.227022405	3.322E-20	
180	PA2486	3.226336888	1.286E-05	
181	PA4169	3.22082681	6.907E-05	
182	PA3133	3.202556147	1.89E-08	
183	PA4832	3.197408971	1.956E-09	
184	pilG	3.191223248	4.712E-38	twitching motility protein PilG
185	PA1540	3.187002666	0.000131	
186	pfpI	3.159604478	4.384E-09	protease PfpI
187	PA4358	3.140930557	5.046E-39	
188	PA0531	3.108012338	3.237E-08	
189	toxA	3.096542159	2.987E-06	
190	PA1921	3.089534872	1.57E-06	
191	PA1541	3.066343521	1.335E-07	
192	clpB	3.064555834	3.2E-13	ClpB protein
193	PA2377	3.060643476	1.133E-09	
194	PA4616	3.054255817	1.406E-10	
195	PA3573	3.043990988	2.705E-09	
196	mucA	3.037524514	1.436E-14	anti-sigma factor MucA
197	PA0881	3.036360364	7.088E-06	
198	PA0665	3.034326157	3.497E-19	
199	PA1702	3.032827997	0.0391845	
200	PA3458	3.026146975	7.418E-10	

Supplementary table B3. Downregulated genes in *P. aeruginosa* 2 dpi *in vivo* cells compared to *in vitro* biofilm cells. The data showed top 200 genes listed were sorted according to most downregulated to least downregulated gene expression.

	Feature ID	Log ₂ Fold change	P value	Annotations
1	chiC	-8.705835283	1.86753E-52	chitinase
2	lasB	-8.227833494	7.81214E-92	elastase LasB
3	PA2069	-7.607678626	1.1357E-30	
4	phzB1	-7.240657401	3.12834E-35	
5	PA3331	-7.13688107	1.52821E-33	cytochrome P450
6	phzF1	-6.633771543	4.32198E-11	
7	phzB2	-6.501256997	4.9115E-20	
8	PA3328	-6.451315436	4.72322E-19	
9	fabH2	-6.442957872	1.58623E-27	3-oxoacyl-[acyl-carrier-protein] synthase III
10	phzF2	-6.370019952	4.82769E-10	
11	phzD1	-6.337360935	1.22065E-11	
12	PA3330	-6.303186075	1.08794E-19	
13	PA4133	-6.262305532	2.70825E-66	cytochrome c oxidase subunit (cbb3-type)
14	PA2068	-6.174544422	2.58021E-26	
15	PA3329	-6.13311505	7.91253E-35	
16	opmD	-6.124101974	1.18398E-21	probable outer membrane protein precursor
17	xylL	-6.000213029	1.03105E-08	"cis-1,2-dihydroxycyclohexa-3,4-diene carboxylate dehydrogenase"
18	PA3332	-5.96512002	1.45947E-22	
19	phzA2	-5.947210592	5.87583E-09	
20	antA	-5.943217177	3.12794E-33	anthranilate dioxygenase large subunit
21	PA0122	-5.923396905	9.80283E-88	
22	PA4638	-5.922640172	2.66138E-63	
23	PA4129	-5.898018478	7.40391E-14	
24	phzD2	-5.897166326	2.01026E-12	
25	phzE2	-5.868829998	1.4199E-08	
26	antC	-5.844158782	7.95074E-17	anthranilate dioxygenase reductase
27	hcnB	-5.836882799	1.22626E-33	hydrogen cyanide synthase HcnB
28	PA4131	-5.804080694	9.9242E-40	
29	phzE1	-5.710111836	1.91429E-08	
30	PA3327	-5.683542315	1.4118E-57	

31	PA2111	-5.655713939	4.97418E-11	
32	PA3365	-5.63862211	2.60693E-25	
33	PA3922	-5.634873227	2.56881E-49	
34	opdO	-5.61676936	2.92129E-14	
35	phzA1	-5.515730178	4.56897E-09	
36	PA2116	-5.496143308	1.92667E-11	
37	PA0049	-5.390802906	2.80104E-16	
38	hcnA	-5.380474833	1.67351E-08	hydrogen cyanide synthase HcnA
39	PA4651	-5.373774152	5.60994E-07	
40	hcnC	-5.367001112	1.0482E-35	hydrogen cyanide synthase HcnC
41	PA3190	-5.325381748	2.22286E-54	
42	phzC2	-5.262969133	4.37604E-08	
43	phzG2	-5.174237205	8.84109E-07	
44	PA4078	-5.119177652	6.20171E-27	
45	PA4130	-5.079120244	2.42288E-32	
46	rhlB	-5.064429715	1.91328E-48	
47	PA2367	-5.013915315	1.43448E-17	
48	hcpB	-4.977940761	4.3326E-07	
49	PA5220	-4.95373608	5.90472E-37	
50	phzC1	-4.939322416	5.31426E-07	
51	mexH	-4.933683136	8.44856E-27	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor
52	PA3923	-4.924112585	2.80683E-67	
53	amiC	-4.922855056	1.52506E-22	aliphatic amidase expression-regulating protein
54	PA4648	-4.90957069	2.99975E-13	
55	antB	-4.904663036	4.41831E-14	anthranilate dioxygenase small subunit
56	oprB	-4.896246805	8.68487E-25	Glucose/carbohydrate outer membrane porin OprB precursor
57	PA4141	-4.891395189	4.97101E-88	
58	PA4134	-4.864139615	2.95888E-09	
59	PA2365	-4.81821331	2.6271E-21	
60	phzM	-4.793047428	6.9248E-25	probable phenazine-specific methyltransferase
61	PA2067	-4.791494013	2.8139E-10	
62	mexI	-4.787488926	1.4775E-30	probable Resistance-Nodulation-Cell Division (RND) efflux transporter
63	PA4128	-4.74185827	5.10048E-07	
64	flp	-4.72601663	2.56526E-19	
65	liuC	-4.696156934	3.81894E-24	
66	PA2066	-4.681183503	1.47997E-16	
67	PA2939	-4.648026264	1.8498E-44	

68	lasA	-4.604667749	2.05585E-43	LasA protease precursor
69	PA1894	-4.575598928	9.1523E-10	
70	snr1	-4.573443234	2.0828E-38	cytochrome c Snr1
71	PA4132	-4.545519022	1.14707E-30	
72	amiE	-4.512455022	8.566E-41	aliphatic amidase
73	lpdV	-4.468096544	8.12157E-26	lipoamide dehydrogenase-Val
74	PA2366	-4.463612442	5.5462E-27	
75	PA2114	-4.399541604	1.89398E-28	
76	bkdA1	-4.384546342	1.03185E-46	2-oxoisovalerate dehydrogenase (alpha subunit)
77	phzH	-4.32431111	1.73579E-11	potential phenazine-modifying enzyme
78	PA1869	-4.317001557	2.18562E-18	
79	PA1665	-4.282345128	3.04037E-08	
80	PA1874	-4.255971153	4.6834E-68	
81	maiA	-4.226995564	3.0701E-20	maleylacetoacetate isomerase
82	PA5181	-4.212400306	2.01268E-23	
83	rhlC	-4.164670056	7.6507E-19	rhamnosyltransferase 2
84	rhlA	-4.15213433	1.36625E-36	
85	PA3189	-4.083439737	2.49021E-09	
86	ambC	-4.048022292	4.61806E-17	
87	bkdB	-4.047648826	3.02018E-19	branched-chain alpha-keto acid dehydrogenase (lipoamide component)
88	PA3335	-4.012236488	5.94469E-14	
89	PA2361	-4.006057501	3.83658E-16	
90	catB	-3.984896674	4.68549E-05	muconate cycloisomerase I
91	bkdA2	-3.971616155	5.61051E-33	2-oxoisovalerate dehydrogenase (beta subunit)
92	hpd	-3.97100878	2.05437E-39	4-hydroxyphenylpyruvate dioxygenase
93	PA3187	-3.968782962	1.05484E-15	
94	lecB	-3.965162504	1.78988E-16	fucose-binding lectin PA-III
95	PA1658	-3.901780648	2.14904E-21	
96	hmgA	-3.878475947	1.34403E-24	"homogentisate 1,2-dioxygenase"
97	liuD	-3.857337635	5.45582E-34	
98	catC	-3.854709775	0.0007615	muconolactone delta-isomerase
99	phaC2	-3.802907528	1.50851E-33	poly(3-hydroxyalkanoic acid) synthase 2
100	PA1875	-3.801941767	1.41839E-10	
101	PA3905	-3.793020114	7.03423E-06	
102	PA1663	-3.78174535	1.62334E-09	
103	PA4144	-3.755690122	6.59094E-07	
104	arsC	-3.755527187	0.000849955	ArsC protein
105	napF	-3.754167183	2.21805E-05	ferredoxin protein NapF

106	ambD	-3.745713542	4.29365E-14	
107	mgtA	-3.738427986	6.20841E-11	"Mg(2+) transport ATPase, P-type 2"
108	PA3535	-3.721461746	1.72754E-11	
109	PA4824	-3.707478898	0.001473289	
110	amiR	-3.703662956	3.24199E-10	aliphatic amidase regulator
111	PA0744	-3.650739144	4.36503E-16	
112	PA2112	-3.640675519	3.25198E-05	
113	PA5348	-3.639182306	2.73462E-48	
114	PA3924	-3.633273772	6.98182E-22	
115	PA3326	-3.622208505	4.04322E-50	
116	PA2031	-3.620162958	3.07308E-06	
117	fahA	-3.603197616	1.35947E-26	fumarylacetoacetase
118	PA2370	-3.583184562	1.1657E-05	
119	PA2554	-3.571444424	5.69076E-12	
120	PA1212	-3.538477094	0.001306349	
121	PA2369	-3.52701562	2.85488E-11	
122	PA2566	-3.525184764	4.59105E-18	
123	PA1657	-3.483924981	4.35765E-10	
124	PA1668	-3.479431209	7.03423E-06	
125	PA2448	-3.470396139	2.70954E-11	
126	PA1906	-3.465332849	4.31927E-08	
127	PA4649	-3.462525042	3.19331E-05	
128	lecA	-3.44054929	5.84121E-08	
129	PA2592	-3.427794825	1.13412E-44	
130	mexG	-3.418704504	2.43456E-09	hypothetical protein
131	PA5059	-3.415201887	1.83069E-10	
132	PA3347	-3.414848347	2.15651E-15	
133	PA2682	-3.40040846	3.20862E-11	
134	PA1659	-3.394594641	8.17113E-06	
135	PA3334	-3.391424949	1.31159E-05	
136	cbpD	-3.382753771	3.67716E-13	chitin-binding protein CbpD precursor
137	PA2589	-3.381722455	6.90735E-06	
138	PA4625	-3.37500654	1.34237E-25	#N/A
139	ambB	-3.361484985	2.02458E-27	
140	PA2006	-3.356993527	5.78921E-08	
141	liuB	-3.351685909	8.98161E-24	
142	kynB	-3.338596281	2.54138E-14	"kynurenine formamidase, KynB"
143	mmsB	-3.321360933	1.84321E-12	3-hydroxyisobutyrate dehydrogenase
144	PA2030	-3.306692216	5.86203E-07	
145	ambE	-3.299900184	9.39172E-44	
146	PA1876	-3.288456616	2.34086E-10	

147	PA2329	-3.287934049	9.76037E-08	
148	PA3921	-3.282863757	5.70127E-19	
149	PA3907	-3.268441012	1.39628E-06	
150	PA0227	-3.238987652	0.0009535	
151	xylZ	-3.230709379	0.009184252	"toluate 1,2-dioxygenase electron transfer component"
152	aroP2	-3.204083801	1.11214E-23	aromatic amino acid transport protein AroP2
153	PA4112	-3.17237854	7.46667E-23	
154	PA2110	-3.165975006	0.003949139	
155	hcpA	-3.163087829	0.004617907	
156	PA0177	-3.155071931	1.44093E-06	
157	PA1220	-3.144101489	0.009017539	
158	kynU	-3.129160284	7.22599E-09	
159	dhcB	-3.124879584	2.24219E-15	
160	PA1877	-3.121413456	3.58637E-06	
161	PA3294	-3.114876719	0.000335336	
162	PA4632	-3.113945176	3.34665E-13	
163	PA2330	-3.107504183	9.17614E-09	
164	PA1131	-3.105103054	2.98328E-10	
165	PA4650	-3.104368722	0.000152809	
166	tadB	-3.096643042	0.001173753	
167	PA0052	-3.083760654	9.29436E-06	
168	phhC	-3.06333842	3.31384E-42	aromatic amino acid aminotransferase
169	PA5219	-3.048531902	2.04859E-12	
170	PA5180	-3.047360452	1.79463E-05	
171	PA1216	-3.03890318	0.000552916	
172	ercS	-3.022204405	8.91473E-06	
173	PA2553	-3.019371222	6.78458E-15	
174	pprB	-3.015026461	1.97465E-21	
175	phzS	-3.011790165	1.29966E-15	flavin-containing monooxygenase
176	atoB	-2.997811557	5.60292E-14	acetyl-CoA acetyltransferase
177	aspA	-2.99360687	3.68686E-15	aspartate ammonia-lyase
178	phhB	-2.984526289	1.60806E-28	pterin-4- α -carbinolamine dehydratase
179	PA4497	-2.981855958	1.04447E-17	
180	PA1662	-2.981521683	3.24274E-11	
181	aer2	-2.97971659	2.44304E-12	
182	PA2557	-2.970093672	2.39589E-12	
183	catA	-2.943604592	0.000864502	"catechol 1,2-dioxygenase"
184	phzG1	-2.928260557	0.004618286	
185	napD	-2.9262536	3.02756E-05	NapD protein of periplasmic nitrate reductase

186	PA5101	-2.923394734	0.000327493	
187	PA0123	-2.90637568	4.58953E-14	
188	phhA	-2.903354343	1.76974E-33	phenylalanine-4-hydroxylase
189	PA1215	-2.892616791	0.005240128	
190	atuE	-2.882099294	0.016239421	
191	PA2552	-2.879568031	1.31409E-15	
192	rbsC	-2.873841786	2.72016E-08	membrane protein component of ABC ribose transporter
193	PA0178	-2.86227293	4.77761E-11	
194	PA4591	-2.860918495	0.000395144	
195	PA3488	-2.85462118	7.48495E-06	
196	rsmZ	-2.850032439	1.8666E-06	
197	rhlI	-2.849284698	3.01848E-18	autoinducer synthesis protein RhlI
198	PA4152	-2.829837296	0.021446739	
199	tadZ	-2.82144394	9.81743E-06	
200	PA2360	-2.816658781	2.97088E-05	

Supplementary table B4. Upregulated genes in *P. aeruginosa* 2 dpi *in vivo* cells compared to *in vitro* planktonic cells. The data showed top 200 genes listed were sorted according to the most upregulated to the least upregulated gene expression.

	Feature ID	Log₂ Fold change	P value	Annotations
1	nirS	6.990419561	5.818E-50	nitrite reductase precursor
2	rpmH	6.608988086	1.351E-25	50S ribosomal protein L34
3	rpmJ	6.586254251	1.047E-15	50S ribosomal protein L36
4	norB	6.526512896	8.568E-34	nitric-oxide reductase subunit B
5	PA3283	6.223241634	1.396E-70	
6	PA4277.3	6.170672667	3.188E-09	
7	ccoO2	5.990354221	2.388E-32	
8	PA3284	5.786594989	8.25E-56	
9	ibpA	5.780012399	1.767E-26	heat-shock protein IbpA
10	PA1195	5.772558016	2.471E-18	
11	PA0905.1	5.740603988	2.842E-08	
12	nirM	5.667775807	4.833E-11	cytochrome c-551 precursor
13	PA3281	5.611293566	1.205E-15	
14	PA3370	5.524331152	1.374E-07	
15	PA3871	5.318956951	5.603E-07	
16	PA2913	5.295265304	7.136E-08	
17	PA1673	5.287532305	1.304E-26	
18	PA3282	5.23678782	2.322E-27	
19	norC	5.227166803	5.263E-15	nitric-oxide reductase subunit C
20	ccoQ2	5.221830865	3.335E-14	
21	PA3600	5.056394842	4.751E-35	
22	narI	5.046879407	3.965E-10	respiratory nitrate reductase gamma chain
23	PA5482	5.025908334	4.386E-17	
24	PA5536	4.946959114	6.303E-14	
25	PA4836	4.911684674	1.479E-27	
26	PA1194	4.8936726	2.246E-11	
27	PA0714	4.888781209	9.437E-16	
28	PA1123	4.863388053	1.858E-16	
29	PA1168	4.841090595	4.174E-10	
30	PA4883	4.818485926	2.452E-08	
31	rpmG	4.784219548	1.356E-21	50S ribosomal protein L33
32	ccoP2	4.778471117	1.1E-32	
33	infA	4.777012878	7.354E-20	initiation factor
34	PA0525	4.721088403	9.707E-15	
35	ahpF	4.676301089	5.885E-29	alkyl hydroperoxide reductase subunit F

36	amiA	4.657789732	2.234E-20	N-acetylmuramoyl-L-alanine amidase
37	katB	4.657611221	6.296E-22	catalase
38	PA4837	4.64066881	1.55E-27	
39	PA4170	4.637075119	6.565E-12	
40	PA1746	4.616077836	2.193E-24	
41	ccoN2	4.593329347	9.496E-36	
42	narG	4.578792796	3.113E-22	respiratory nitrate reductase alpha chain
43	PA4365	4.550576113	4.398E-05	
44	PA1922	4.495295591	2.002E-14	
45	PA4348	4.437876796	2.304E-22	
46	PA5534	4.410077686	3.401E-15	
47	PA2630	4.385455141	1.168E-14	
48	PA0887.1	4.384230224	0.0003384	
49	rpsT	4.383824389	4.814E-30	30S ribosomal protein S20
50	PA0512	4.379264389	1.519E-07	
51	PA0781	4.367564549	6.067E-23	
52	PA4881	4.3654276	2.306E-10	
53	narK1	4.346499289	8.659E-19	nitrite extrusion protein 1
54	PA5535	4.318314479	7.546E-25	
55	PA5540	4.310908674	2.084E-13	
56	PA0878	4.294948781	3.9E-13	
57	PA0738	4.288336229	0.0003203	
58	nirF	4.287430347	2.6E-12	heme d1 biosynthesis protein NirF
59	PA0526	4.273188881	6.852E-07	
60	htpG	4.256201899	2.868E-14	heat shock protein HtpG
61	PA2883	4.238167749	1.209E-19	
62	PA0128	4.211898271	8.648E-09	
63	rpsU	4.142971894	1.909E-28	30S ribosomal protein S21
64	clpB	4.131923476	5.653E-23	ClpB protein
65	PA4429	4.122688808	1.697E-34	
66	grpE	4.12024343	1.492E-10	heat shock protein GrpE
67	PA0132	4.103335306	1.797E-16	beta-alanine--pyruvate transaminase
68	PA0515	4.096099301	8.141E-07	
69	betI	4.055648393	1.887E-41	transcriptional regulator BetI
70	rpsP	4.028785567	4.755E-29	30S ribosomal protein S16
71	PA0848	4.02135646	6.642E-15	
72	narJ	4.014230349	5.612E-05	respiratory nitrate reductase delta chain
73	PA4835	4.012690215	5.371E-17	
74	PA4884	4.001252798	1.242E-10	
75	cdhC	3.989270449	0.0010206	
76	cifR	3.982139016	4.649E-20	

77	nosZ	3.933358609	1.89E-25	nitrous-oxide reductase precursor
78	nirC	3.91319959	6.516E-05	
79	rpmE	3.898641543	6.447E-26	50S ribosomal protein L31
80	PA0545	3.874447647	1.699E-08	
81	adhA	3.861582426	2.088E-28	alcohol dehydrogenase
82	PA2753	3.834013897	2.373E-09	
83	PA3913	3.823362144	1.334E-07	
84	betB	3.815857307	7.598E-30	betaine aldehyde dehydrogenase
85	groES	3.810610565	3.16E-13	GroES protein
86	rpmF	3.797739684	1.15E-33	50S ribosomal protein L32
87	PA3575	3.794379223	7.235E-06	
88	PA3912	3.792091126	1.939E-07	
89	glyA2	3.76271745	9.562E-15	serine hydroxymethyltransferase
90	folE2	3.725685685	5.778E-21	GTP cyclohydrolase I precursor
91	PA5539	3.721972983	2.305E-11	
92	narH	3.687472712	4.403E-15	respiratory nitrate reductase beta chain
93	PA4139	3.677785445	6.299E-30	
94	cupA2	3.676333066	0.0047177	chaperone CupA2
95	PA4065	3.675598461	1.979E-19	
96	hslU	3.645375067	2.869E-11	heat shock protein HslU
97	rpsI	3.641132373	7.477E-52	30S ribosomal protein S9
98	PA2826	3.639743501	7.57E-20	
99	PA0532	3.621707254	1.354E-08	
100	PA1925	3.604991217	4.218E-06	
101	PA4182	3.56723978	3.201E-12	
102	PA4832	3.555109124	1.655E-08	
103	rpsL	3.544738327	2.236E-92	30S ribosomal protein S12
104	PA0320	3.544012599	0.0019613	
105	atpE	3.537758413	2.698E-61	atp synthase C chain
106	PA0141	3.530556107	3.777E-17	
107	PA0883	3.52950733	6.919E-06	
108	PA4937.1	3.515844514	0.0076858	
109	rplL	3.513694036	4.745E-67	50S ribosomal protein L7 / L12
110	nirL	3.509066533	0.0001902	heme d1 biosynthesis protein NirL
111	PA0513	3.499429505	4.664E-05	
112	PA1924	3.491576767	9.615E-05	
113	PA1864	3.478710164	0.0018551	
114	PA0510	3.46420676	2.529E-06	
115	PA0534	3.449606284	8.582E-18	
116	nosR	3.438884778	1.205E-13	regulatory protein NosR
117	PA5401	3.437237956	0.00574	
118	PA4692	3.428487741	1.523E-40	

119	pyrQ	3.418333622	2.63E-14	dihydroorotase
120	PA4430	3.402338197	6.588E-41	
121	PA0760	3.39294213	1.385E-06	
122	narK2	3.378839426	1.733E-11	nitrite extrusion protein 2
123	PA5480	3.36687349	1.358E-05	
124	faoA	3.364522493	1.625E-09	fatty-acid oxidation complex alpha-subunit
125	PA2783	3.363063569	5.26E-11	
126	morB	3.343147331	4.638E-07	morphinone reductase
127	PA3573	3.308699813	4.225E-09	
128	estX	3.308524322	0.0001341	EstX
129	hslV	3.288345739	9.32E-08	heat shock protein HslV
130	PA0879	3.277854802	6.04E-16	
131	PA2819	3.262971845	2.866E-06	
132	rpsJ	3.257161746	7.619E-43	30S ribosomal protein S10
133	PA3601	3.223950708	1.169E-24	
134	PA1923	3.208828788	1.15E-11	
135	PA4364	3.196950994	0.0003112	
136	PA1699	3.194137645	1.433E-05	
137	nirN	3.177666048	1.755E-09	
138	betA	3.176213859	1.571E-15	choline dehydrogenase
139	phrS	3.159988676	1.637E-12	
140	PA2983	3.157176188	2.003E-12	
141	dnaK	3.156509664	7.912E-11	DnaK protein
142	PA0535	3.131352382	3.316E-05	
143	prs	3.125553858	6.366E-23	ribose-phosphate pyrophosphokinase
144	nirJ	3.121277202	9.474E-07	heme d1 biosynthesis protein NirJ
145	PA3133	3.112727675	4.373E-07	
146	oprG	3.099806683	2.239E-27	Outer membrane protein OprG precursor
147	rubA1	3.097167601	0.0157286	Rubredoxin 1
148	PA4834	3.087146279	1.437E-11	
149	argS	3.081635997	7.261E-29	arginyl-tRNA synthetase
150	mreD	3.076110857	0.0001568	rod shape-determining protein MreD
151	PA1845	3.073422387	0.0175858	
152	PA1748	3.064054363	3.621E-16	
153	PA2715	3.061267641	0.0108675	
154	PA4882	3.060286568	1.512E-07	
155	armR	3.059791168	0.0007798	
156	gcvH2	3.055901043	9.394E-10	glycine cleavage system protein H2
157	PA2767	3.054149143	0.0008192	
158	PA2910	3.043274639	0.0192442	
159	PA0880	3.0204303	0.0011056	

160	PA4838	3.010542584	1.623E-11	
161	PA2221	3.008413464	9.438E-09	
162	PA5530	3.008277603	1.829E-10	
163	rpmC	2.988336754	1.22E-24	50S ribosomal protein L29
164	rplD	2.972702053	8.271E-64	50S ribosomal protein L4
165	PA3237	2.971768713	0.0002292	
166	eco	2.968833004	7.169E-17	ecotin precursor
167	rplM	2.965805744	8.494E-35	50S ribosomal protein L13
168	rpsH	2.961033345	6.978E-48	30S ribosomal protein S8
169	adk	2.95913479	8.007E-21	adenylate kinase
170	PA3911	2.944971209	0.0008174	
171	pscK	2.942263808	0.0011165	type III export protein PscK
172	PA5139	2.936000696	2.861E-09	
173	PA0508	2.935249042	8.556E-08	
174	PA3144	2.927766374	0.0113046	
175	PA3586	2.925536213	0.0053855	
176	rpsB	2.914975173	5.922E-60	30S ribosomal protein S2
177	secF	2.902824133	1.736E-16	secretion protein SecF
178	PA2763	2.898497687	0.0159917	
179	PA1697	2.894285854	5.23E-11	ATP synthase in type III secretion system
180	PA3577	2.889788748	0.043027	
181	PA5397	2.884727994	0.0381911	
182	PA3952	2.877937036	6.917E-08	
183	secD	2.875226134	7.161E-26	secretion protein SecD
184	PA0881	2.870883033	9.855E-05	
185	PA2982	2.845701005	1.284E-12	
186	PA5568	2.839886482	9.704E-39	
187	pscG	2.838899261	1.561E-06	type III export protein PscF
188	pelB	2.820575672	1.017E-07	
189	PA0989	2.817644996	0.0013053	
190	pscO	2.817546671	0.0054992	translocation protein in type III secretion
191	aceA	2.810885092	1.849E-19	
192	PA0169	2.809224526	0.0007784	
193	ccpR	2.78803753	8.132E-16	cytochrome c551 peroxidase precursor
194	nosD	2.78525846	3.846E-07	NosD protein
195	lpxB	2.780287286	2.903E-07	lipid A-disaccharide synthase
196	PA1700	2.779841428	0.0049562	
197	PA3057	2.776353584	0.0080949	
198	PA1429	2.776158925	2.594E-14	
199	PA4574	2.773347945	1.789E-07	
200	PA2453	2.767014857	3.64E-09	

Supplementary table B5. Downregulated genes in *P. aeruginosa* 2 dpi *in vivo* cells compared to *in vitro* planktonic cells. The data showed top 200 genes listed were sorted according to the most downregulated to least downregulated gene expression.

	Feature ID	Log ₂ Fold change	P value	Annotations
1	PA4638	-8.370150836	2.6E-127	
2	chiC	-8.245003879	2.47E-47	chitinase
3	PA0122	-7.969816058	3E-160	
4	rhlB	-7.296337838	3.4E-101	
5	rhlA	-6.948356106	8.1E-103	
6	PA4141	-6.891640502	3.3E-176	
7	PA3939	-6.598794948	3.81E-14	
8	PA3441	-6.398512661	1.93E-74	
9	PA3190	-6.263729695	2.03E-75	
10	PA4917	-6.231353622	7.19E-38	
11	PA0178	-6.1529389	7.3E-51	
12	PA3442	-6.11155894	2.39E-22	
13	PA2091	-6.075589216	8.59E-09	
14	PA2069	-5.960667048	4.33E-19	
15	PA2310	-5.899705439	6.1E-11	
16	PA3444	-5.896111037	4.38E-18	
17	aprA	-5.757709538	8.9E-121	
18	lasB	-5.724609648	7.2E-45	elastase LasB
19	aer2	-5.678870723	2.97E-44	
20	piv	-5.546342432	1.54E-29	
21	PA2086	-5.535427538	7.65E-07	
22	PA0177	-5.512034747	4.45E-19	
23	PA2308	-5.493918431	1.65E-09	
24	PA3447	-5.479601568	3.84E-06	
25	PA0179	-5.479320228	1.09E-60	
26	PA3443	-5.405909308	1.23E-14	
27	phzF2	-5.344360704	2.67E-07	
28	phzF1	-5.219729878	3.74E-07	
29	PA2090	-5.158668248	3.53E-08	
30	lecA	-5.104663388	6.99E-17	
31	PA3445	-5.05710602	9.31E-20	
32	PA2309	-5.009653329	7.89E-11	
33	aprD	-5.005119619	2.46E-50	alkaline protease secretion protein AprD
34	rhlC	-4.983950045	7.61E-27	rhamnosyltransferase 2

35	PA4916	-4.963524909	7.16E-26	
36	lecB	-4.915417801	3.64E-25	fucose-binding lectin PA-III
37	PA3307	-4.865341877	7.47E-15	
38	PA3448	-4.831303198	1.13E-06	
39	PA3932	-4.824585216	2.14E-15	
40	PA5460	-4.820866551	1.45E-27	
41	PA4142	-4.71799605	2.35E-26	
42	PA0983	-4.712972414	3.56E-29	
43	lasA	-4.703983921	2.22E-45	LasA protease precursor
44	PA3446	-4.664808624	6.26E-16	
45	PA2263	-4.655487648	3.07E-07	
46	PA3331	-4.654483594	1.8E-14	cytochrome P450
47	PA3347	-4.608092626	7.17E-28	
48	pgl	-4.578784854	1.75E-24	6-phosphogluconolactonase
49	PA0209	-4.568593035	0.000176	
50	PA0052	-4.550503828	8.44E-12	
51	PA2939	-4.529148036	3.41E-42	
52	PA2367	-4.514102066	2.84E-14	
53	PA0175	-4.511518806	6.86E-16	
54	PA0744	-4.510071255	2.27E-24	
55	PA0110	-4.492806529	2.3E-08	
56	PA1245	-4.467306277	2.06E-50	
57	PA2068	-4.452520369	7.59E-14	
58	PA3449	-4.436415508	3.82E-06	
59	PA4156	-4.406207129	5.09E-72	
60	PA3340	-4.358580405	3.22E-32	
61	PA2370	-4.335101808	5.69E-08	
62	PA2307	-4.326180438	3.44E-07	
63	oprB	-4.287228108	5.22E-19	Glucose/carbohydrate outer membrane porin OprB precursor
64	phzG2	-4.277496595	7.4E-05	
65	PA2359	-4.260298886	3.05E-08	
66	PA4144	-4.233528013	1.34E-08	
67	PA1784	-4.166676384	2.94E-23	
68	PA2597	-4.163151278	1.85E-08	
69	aprF	-4.145085386	7.49E-35	Alkaline protease secretion outer membrane protein AprF precursor
70	PA3520	-4.099559586	2.95E-07	
71	PA2573	-4.080384089	2.87E-54	
72	PA3922	-4.04545509	1.49E-25	
73	fabH2	-4.032311176	5.49E-11	3-oxoacyl-[acyl-carrier-protein] synthase III
74	napF	-4.008998748	4.95E-06	ferredoxin protein NapF
75	PA4112	-4.006138731	2.58E-36	

76	PA1131	-4.004761208	9.57E-17	
77	PA4889	-3.99776351	1.49E-06	
78	PA0201	-3.993911456	4.59E-11	
79	ambE	-3.989521398	5.93E-64	
80	PA2366	-3.961626892	2.3E-21	
81	ambC	-3.937164191	4.61E-16	
82	aprE	-3.936789038	9.4E-31	alkaline protease secretion protein AprE
83	PA1041	-3.931674135	1.87E-31	
84	opmD	-3.931420502	3.38E-09	probable outer membrane protein precursor
85	gapA	-3.912376636	5.54E-65	glyceraldehyde 3-phosphate dehydrogenase
86	PA2360	-3.903830721	1.71E-09	
87	PA2291	-3.894767765	3.24E-29	
88	PA4843	-3.838537939	1.4E-57	
89	PA2369	-3.792850126	6.56E-13	
90	PA3181	-3.786911713	3.38E-31	2-keto-3-deoxy-6- phosphogluconate aldolase
91	PA3330	-3.777983685	2.28E-07	
92	PA0806	-3.776922721	0.000763	
93	PA2598	-3.766419761	5.26E-08	
94	PA2572	-3.760648377	1.64E-42	
95	PA3450	-3.760276538	4.94E-15	
96	thiC	-3.758599739	4.56E-31	thiamin biosynthesis protein ThiC
97	PA2365	-3.744735053	6.87E-13	
98	PA2092	-3.740403782	5.11E-05	
99	pprB	-3.730709403	1.29E-32	
100	PA4090	-3.730591241	1.17E-50	
101	PA3734	-3.725857514	1.76E-13	
102	PA4336	-3.715164086	3.78E-16	
103	PA2089	-3.713812509	8.93E-24	
104	PA2094	-3.711754604	0.000188	
105	PA3986	-3.711120483	8.58E-32	
106	PA3923	-3.692728749	1.17E-37	
107	flp	-3.687669474	8.01E-12	
108	femR	-3.665739696	5.69E-15	
109	PA2312	-3.661120268	3.34E-06	
110	PA0575	-3.646955578	7.27E-33	
111	bphP	-3.646727878	1.64E-52	
112	PA3723	-3.639016786	4.66E-42	
113	phzE1	-3.635733984	0.000862	
114	PA3329	-3.61974966	3.36E-12	
115	zwf	-3.619364491	1.49E-24	glucose-6-phosphate 1-

				dehydrogenase
116	PA2588	-3.615319831	1.24E-22	
117	PA2024	-3.613007089	7.25E-23	
118	phzE2	-3.596094165	0.001229	
119	PA3089	-3.570375039	5.69E-14	
120	PA2368	-3.565130668	0.008107	
121	rhlR	-3.551024526	3.34E-57	transcriptional regulator RhlR
122	PA2361	-3.54955329	1.23E-12	
123	PA2937	-3.538733135	1.49E-24	
124	PA3938	-3.530793888	1.47E-07	
125	PA2448	-3.525236025	1.5E-11	
126	PA4359	-3.522607488	6.93E-13	
127	PA4573	-3.516703882	3E-15	
128	PA4324	-3.512084536	3.1E-19	
129	mmsB	-3.499620096	9.6E-14	3-hydroxyisobutyrate dehydrogenase
130	PA4155	-3.494556716	2.74E-29	
131	PA1298	-3.492126685	0.003925	
132	PA2371	-3.491723987	5.79E-26	
133	xdhA	-3.491000086	3.2E-12	xanthine dehydrogenase
134	PA0174	-3.483377577	0.001639	
135	PA2174	-3.470250957	1.15E-16	
136	PA4925	-3.462375332	5.21E-14	
137	PA4223	-3.457444355	5.69E-14	
138	mdcA	-3.456683498	6.79E-21	malonate decarboxylase alpha subunit
139	fepD	-3.447341238	3.33E-06	ferric enterobactin transport protein FepD
140	PA3328	-3.4393785	8.6E-06	
141	tonB2	-3.424764446	0.000241	
142	PA2161	-3.421533559	0.014099	
143	PA2088	-3.420449461	4.36E-06	
144	fepB	-3.41811919	8.4E-19	ferrienterobactin-binding periplasmic protein precursor FepB
145	PA1906	-3.415031802	8.37E-08	
146	PA0123	-3.40902627	4.25E-19	
147	PA4220	-3.408121476	4.67E-12	
148	ambD	-3.408085287	1.04E-11	
149	PA3667	-3.406664641	1.52E-22	
150	PA3924	-3.403799192	4.74E-19	
151	phzB2	-3.374335858	7.63E-06	
152	PA5101	-3.368198508	2.51E-05	
153	PA0745	-3.363503973	3.16E-20	
154	pchF	-3.354851095	6.43E-16	pyochelin synthetase

155	PA2600	-3.347263568	3.75E-06	
156	arnE	-3.334088481	0.000734	
157	PA2162	-3.333111861	1.43E-10	
158	ptxS	-3.326115071	1.51E-23	transcriptional regulator PtxS
159	PA1930	-3.325545989	6E-25	
160	PA2084	-3.323525927	1.31E-08	
161	coxB	-3.322487756	7.64E-40	"cytochrome c oxidase, subunit II"
162	PA3332	-3.316320315	3.09E-07	
163	PA1745	-3.312859047	6.43E-10	
164	tauD	-3.311947309	8.39E-05	taurine dioxygenase
165	gcd	-3.308087234	8.18E-59	glucose dehydrogenase
166	PA1728	-3.300247469	1.64E-28	
167	PA3662	-3.293658014	4.51E-26	
168	PA0483	-3.289868017	1.09E-15	
169	crfX	-3.286693944	1.87E-15	
170	PA1356	-3.282984099	2.32E-05	
171	xdhB	-3.277936243	3.56E-28	xanthine dehydrogenase
172	PA2228	-3.274458078	9.4E-12	
173	PA4929	-3.273530461	1.35E-14	
174	PA3740	-3.269930511	2.24E-13	
175	PA2920	-3.259590183	7.93E-32	
176	pvdL	-3.258348048	2.68E-25	PvdL
177	PA2062	-3.249379297	3.23E-09	
178	atsA	-3.248171779	5.63E-07	arylsulfatase
179	fepG	-3.242578677	8.53E-06	ferric enterobactin transport protein FepG
180	PA1760	-3.241094624	1.03E-27	
181	PA4111	-3.228238925	8.87E-06	
182	PA2087	-3.227194536	0.009531	
183	PA4915	-3.226149043	7.58E-29	
184	PA3187	-3.222823092	1.83E-10	
185	PA3786	-3.214680219	2.34E-14	
186	PA0505	-3.214656844	2.02E-29	
187	PA1107	-3.208359548	3.76E-10	
188	PA4471	-3.206482365	4.44E-58	
189	PA5020	-3.185764447	2.97E-09	
190	mdcE	-3.183471395	0.001094	malonate decarboxylase gamma subunit
191	PA0788	-3.182044386	2.79E-27	
192	PA2423	-3.166318726	9.43E-21	
193	PA2260	-3.165941639	2.22E-07	
194	PA2475	-3.159889222	1.67E-08	
195	edd	-3.157345883	5.59E-34	phosphogluconate dehydratase
196	PA4130	-3.152957408	1.01E-12	

197	cyoC	-3.152582576	7.32E-18	cytochrome o ubiquinol oxidase subunit III
198	ldh	-3.143124698	4.22E-36	leucine dehydrogenase
199	PA2689	-3.136690886	0.005924	
200	PA2067	-3.13654084	9.91E-05	

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