c-di-GMP distribution across single and mixed species biofilms

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NANYANG
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**C -DI-GMP DISTRIBUTION ACROSS
SINGLE AND MIXED SPECIES BIOFILMS**

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ABBREVIATION

°C	Degree Celsius
ASV	Unstable Variant
BCA	Bicinchoninic acid assay
C-di-GMP	bis- (3'-5')-cyclic dimeric guanosine monophosphate
CFP	Cyan Fluorescent Protein
cfu	Colony forming units
CID	collision induced dissociation
CSLM	Confocal Scanning Laser Microscope
d	Day
DsRed	Red Fluorescent Protein
ESI+	Electrospray ionization
FRET	Förster resonance energy transfer
<i>g</i>	Gravitational force
G	Gauge
g	Gram
GFP	Green Fluorescent Protein
Gmr	Gentamycin resistance
h	Hours
HPLC	High pressure liquid chromatography
KHz	kilohertz
KDa	Kilodalton
Korr	Correction Ring
l	Litter

LB	Luria Bertani medium
LC-MS	Liquid chromatography coupled with Mass spectrometer
LUT	Look Up Table
M	Molar
M9	M9 minimal medium
min	Minutes
ml	Millilitres
mm	Millimetres
mM	Millmolar
MS/MS	Tandem mass spectrometry
nm	Nanometres
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pGpG	5'-phosphoguanylyl-(3'-5')-guanosine
pM	Pico molar
PMT	Photomultiplier tube
(p)ppGpp	Guanosine pentaphosphate
qRT-PCR	Quantitative Reverse Transcription PCR
rpm	Revolutions per minute
RSCV	Rugose Small colony variant
s	Second
SCV	Small colony variant
SDS	Sodium dodecyl sulphate
SIM	Selected ion monitoring

SNP	Sodium nitroprusside
SNV	Single nucleotide variant
TEAB	Triethylammonium bicarbonate buffer
TeO ₂	Telurite
V	Volt
YFP	Yellow Fluorescent Protein
μF	Microfarad
μg	Micrograms
μl	Microliters
μM	Micro molar
μm	Micrometres

ABSTRACT

Bacteria in nature preferentially grow as a surface associated, matrix encapsulated structures, called biofilms. Biofilms play a major role in society, such as degrading waste and in the recycling of used water. At the same time, biofilms are responsible for many chronic, infectious diseases, biocorrosion of engineered structures and membrane fouling of water treatment plants. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is an intracellular signaling molecule that plays a central role in the biofilm life cycle. While the molecular mechanisms of the c-di-GMP signaling pathway have been well studied, the differential distribution of c-di-GMP across biofilms, which commonly display physiological heterogeneity, is less well understood. This is partially due to the limitation of current methodologies for the quantification of c-di-GMP, which are based on chemical extraction. Chemical methodologies also fail to take the physiological heterogeneity of biofilm into consideration and thus represent an average c-di-GMP concentration across the entire biofilm. To address these issues, a ratiometric, image-based quantification method was developed here based on expression of the green fluorescence protein under the control of the c-di-GMP responsive *cdrA* promoter (Rybtke., *et al.* 2012). The approach has been successfully applied to biofilms at different developmental stages and as well as during dispersal. Using this dynamic, real-time monitor, a transient state of increased c-di-GMP (up to 2 h) before the expected decrease in c-di-GMP was observed for biofilms under starvation conditions. The observation has been validated by comparison with chemical measurements of c-di-GMP with increased temporal resolution. Transcriptomic analysis of starved biofilms and planktonic cultures indicated that 50 out of 52 signal transduction genes were induced upon starvation, including three diguanylate cyclase and three phosphodiesterase genes.

Additionally, it was observed that c-di-GMP was localized to the outer boundary of mature colonies (diameter >50 μm) rather than showing a uniform distribution in smaller colonies (diameter <50 μm). After continuous starvation for up to 3 days, some of the biofilm remained in the flow cell and that biomass displayed high reporter fluorescence, suggesting those cells contained high concentrations of c-di-GMP. Isolation and genetic characterization of these cells lead to the conclusion that these colonies that failed to disperse upon carbon starvation were mutants.

Ratiometric imaging was applied to a model mixed species biofilm consisting of *Pseudomonas aeruginosa* PAO1, *Pseudomonas protegens* Pf5 and *Klebsiella pneumoniae* KP1 to understand the distribution of c-di-GMP during mixed species biofilm growth. The c-di-GMP localization patterns of *P. aeruginosa* in the mixed species community were generally similar to that of monospecies biofilm although the total c-di-GMP level of *P. aeruginosa* was much less than that of the monospecies biofilm. Interestingly, the total c-di-GMP measured for the mixed species biofilm using LC-MS was in fact higher than that of any of the mono species biofilms. Starvation of the mixed species biofilm leads to dispersal and a transient increase of c-di-GMP similar to the monospecies biofilm. While some of the mixed species biofilm did not disperse, no morphotypic variants were detected, which contrasted with the monospecies biofilms.

In conclusion, a method for the visualization and semi-quantification of c-di-GMP *in situ*, in real time was developed and validated. Using this method, c-di-GMP was found to be localized at the boundary of mature colonies. Under the improved temporal and spatial resolution, a transient increase in c-di-GMP was observed during starvation-induced dispersal and the reporter system was equally applicable to investigate c-di-GMP distributions in a mixed species biofilm system.

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Microbiology, being the study of microscopic organisms, has traditionally dealt with isolated, single-species cultures grown under batch conditions (Kaslow & Moser, 2000). This classic approach has significantly improved our understanding of the role of microbes in disease and food production as well as having led to discoveries of life saving antibiotics and therapeutic compounds, enzymes and metabolic products that have been used in a wide range of biotechnologies (Bud, 1991). While the use of monocultures for the investigation of microbial processes has been invaluable for such discoveries, it is increasingly appreciated that bacteria grown in isolation under batch cultures do not represent the natural environments in which those microorganisms normally exist (Zobell, 1943). Indeed, most microorganisms exist in multispecies consortia, where they experience cooperation and competition for resources. Furthermore, these environments are highly dynamic, with fluctuations in nutrient concentrations and types, as well as changes in water content, temperature and light for examples. These processes drive the evolution of such communities and their cognate population members. One mechanism that has evolved, which is associated with the enhanced capacity of microbes to persist in natural environments or hosts, is the formation of a surface associated, matrix encapsulated structure, called a biofilm (Costerton *et al.*, 1995). It is currently understood that most microbes preferentially grow as biofilms under natural conditions, highlighting the importance of biofilm formation. Biofilm formation is responsible for many chronic, infectious diseases, but also is beneficial to mankind in several ways such as degrading waste and in the recycling of used water (Costerton *et al.*, 1999, Nicoletta *et al.*, 2000,

Biffinger *et al.*, 2007). In the past two decades, the need to better understand biofilms and their applications has primarily been responsible for a paradigm shift from the conventional single species planktonic cultures to studies of communities and their interactions.

Much of the existing microbiology literature, where microorganisms were treated as planktonic, free-living bacteria may not be always applicable to biofilms, since the multicellular structured organization of biofilms is different from the planktonic counterparts. For example, biofilm bacteria are 10-1000 times more resistant to antimicrobial agents than their planktonic equivalent (Mah *et al.*, 2003). This is in part due to the fact that biofilm bacteria are protected in a biofilm matrix and show differential gene expression in different regions of the biofilm (Stewart & Costerton, 2001).

Irrespective of whether it is composed of a single or mixed species, the development of a biofilm typically follows a series of loosely defined stages and can be considered to represent a rudimentary life-cycle. As part of this process, free swimming planktonic bacteria reversibly attach to a surface, followed by irreversible attachment coupled with exopolysaccharide production and biofilm microcolony formation. Finally, when the biofilms are mature, the biofilm matrix is at least partly broken down to allow for the dispersal of cells back into the planktonic phase to continue the lifecycle. The notion that the biofilm represents a type of life-cycle is supported by observations indicating that biofilm formation, development and dispersal are genetically regulated. Further, the bacterial second messenger bis-(3'5')-cyclic dimeric guanosine monophosphate (c-di-GMP) plays a central role in the regulation of the genetic pathways involved in the biofilm life cycle. Indeed, many of the environmental signals sensed by the bacteria such as starvation, oxidative stress or

some chemical cues are sensed and transmitted through the c-di-GMP signalling network, leading to specific changes in gene expression (Jenal & Malone, 2006). For example, exogenously added nitric oxide can induce biofilm dispersal by altering the intracellular c-di-GMP concentration. The discovery that biofilm dispersal can be triggered by mimicking specific environmental signals also has a range of potential biotechnological applications where there is a need to control biofilm formation. While it is possible to induce biofilm dispersal artificially, it is commonly observed that not all biofilms can be dispersed. For example, while nitric oxide induced dispersal in drinking water biofilms, 53% of the biofilm biomass remained after treatment. One possible explanation for incomplete dispersal is that the biofilm is heterogeneous in terms of the physiological status of the bacteria, and this stratification is partly dependent on their location in the biofilm. For example, cells near the biofilm surface will have better access to nutrients and oxygen than cells in the centre of microcolonies. The observations that biofilms cannot be completely dispersed strongly argues that there is a need for an improved understanding of the molecular pathways that underpin dispersal responses, such as NO induced dispersal, to improve their efficacy as biofilm control strategies.

One factor that may strongly impact the response of the biofilm to dispersal cues is directly related to the inherent heterogeneity of the biofilm. Specifically, this heterogeneity may be reflected in an uneven distribution of c-di-GMP across the biofilm and thus could result in different levels of response from the cells within the biofilm. Even within planktonic cells, it has been suggested that c-di-GMP is not evenly distributed (Christen *et al.*, 2010). However, the spatial and temporal distribution of c-di-GMP in a biofilm in relation to this heterogeneity is poorly explored currently. This is in part due the current method of quantifying c-di-GMP

based on liquid chromatography–mass spectrometry (LC-MS) that requires extraction and homogenization of the biofilm. Thus, concentrations reported are an average from the whole biofilm and do not take into account this spatial variation in the biofilm as a consequence of metabolic gradients. In addition, many bacteria express multiple enzymes capable of modifying c-di-GMP (*Pseudomonas aeruginosa* has 47 c-di-GMP metabolising proteins, and *Escherichia coli* encodes 29 (Römling *et al.*, 2013)) and it is not clear how these interact to trigger a meaningful response to environmental cues. One hypothesis has been that c-di-GMP modifying proteins are closely associated with their targets and that total cellular pools do not matter as much as localized c-di-GMP concentrations within the cell (Römling *et al.*, 2013). To address whether there are localized c-di-GMP pools in the cell, Christen *et al.* (2010) constructed a Förster resonance energy transfer (FRET)–based biosensor to monitor the c-di-GMP concentration and demonstrated the asymmetrical distribution of the c-di-GMP inside planktonic *P. aeruginosa* cells. Therefore, such reporters, which can be calibrated to the c-di-GMP concentration, can potentially be used to address the c-di-GMP concentration in different parts of the biofilm. Chua *et al.* (2013) studied the relationship between c-di-GMP and antibiotic resistance using a fluorescence based reporter (Rybtke *et al.*, 2012). They found that the distinct intracellular levels of c-di-GMP expressed in biofilm cells versus cells dispersed from biofilms were responsible for the differential expression of proteins causing an enhanced antibiotic resistance of the dispersal cells.

In contrast to monospecies biofilms, natural biofilms are comprised of a diverse range of species, which may include eukaryotes, protozoa and viruses in addition to bacteria. For example, a metagenomic study of biofilms formed on concrete waste water pipelines revealed a total of 629,161 operational taxonomic units (OTUs) at the

front end of the pipeline and 641,853 161 OTUs at the back end of the pipeline (Gomez-Alvarez *et al.*, 2012). The complexity of these natural systems, along with the difficulty of reproducibility, have made studies of natural communities challenging and hence, the desire for better controlled community studies has led to the use of defined model mixed species systems. One such model system for studying bacteria that are the early colonizers of the oral cavity includes *Streptococcus gordonii*, *Streptococcus oralis* and *Actinomyces naeslundii*. Using these three organisms, it was demonstrated that *S. oralis* and *A. naeslundii* displayed a mutualistic interaction, where neither can grow on their own (Palmer *et al.*, 2001). Another defined mixed species system developed in our laboratory involves *P. aeruginosa* PAO1, *Pseudomonas protegens* Pf5 and *Klebsiella pneumoniae* KP1 that have been genetically tagged with the yellow fluorescent protein (YFP), the cyan fluorescent protein (CFP) or the *Discosoma sp.* red fluorescent protein (DsRed), respectively. This model mixed community has been used to study the spatial organization of mixed species biofilms in a laboratory environment with high reproducibility (Lee *et al.*, 2013). Studies of this model community led to the observation that the community was more tolerant to antimicrobial and anionic surfactant stress compared to single species biofilms. The discovery that the biofilm community displays such unexpected phenotypes argues that there is much yet to be learned about biofilm development, especially for mixed species systems.

1.2 Development of a biofilm

A mature biofilm, consisting of surface-attached colonies surrounded by extracellular matrix and separated by fluid-filled channels, can have a relatively complex architecture (Wood *et al.*, 2000). Such complex architecture formation may require a developmental process rather than the simple, stochastic aggregation of

microbes at one place. Molecular studies of single species biofilms suggest that there are multiple stages of development and it is possible to identify specific genes that are expressed during these different stages or that are required for the biofilm to progress from one stage to the next. For example, previous studies showed that the various stages of development differed by approximately 35% in terms of changes in protein expression for *P. aeruginosa* biofilms (Stoodley *et al.*, 2002). The biofilm lifecycle typically begins with the reversible attachment of planktonic cells on a surface, followed by irreversible attachment and binary division. The dividing cells form microcolonies and macrocolonies followed by biofilm maturation. Finally, the biofilm cells escape from the biofilm matrix to colonise new habitats during the dispersal phase (Figure 1.1). Each of these steps involves several key molecular events and are described in the following sections.

Figure 1.1: The biofilm life cycle. a) initial, reversible attachment, b) irreversible attachment and binary division, c) early development and microcolony formation, d) macrocolony formation and biofilm maturation and e) biofilm dispersal. Reproduced from (Davies, 2003).

1.2.1 Reversible attachment

Free-floating planktonic bacteria from the bulk phase approach a surface to initiate biofilm formation, which is typically driven by flagella and pili. At this stage, attachment is strongly influenced by the physicochemical forces of the surface, which are a combination of the surface material's properties and the bacterial cell wall (van Loosdrecht *et al.*, 1989). For example, the bacterial surface is typically negatively charged due to the phosphate group in the lipopolysaccharide. Depending on the surface, several long-range forces such as electrostatic repulsion and van der Waals forces come into play. The most critical parameter involved in the van der Waals force is the *Hamaker* constant, which is a material property defined by the distance between the bacterium and the substratum as well as the geometry of the surface. The strength of the Van der Waals interaction with a bacterium with regard to different surface types has been calculated and ranked in terms of relative strength of attraction as: glass > polystyrene > Teflon (Hermansson, 1999). Alterations in the cell surface can also lead to changes in surface attraction. For example, it has been shown that loss of B-band lipopolysaccharides in *P. aeruginosa* reduces attachment to hydrophilic surfaces and increases attachment to hydrophobic surfaces (Makin & Beveridge, 1996).

Once bacteria make contact with the surface, specific reversible molecular interactions involving flagella or pili, enable them to explore the surface. Bacteria can swarm using polar flagella, or can use type IV pili based twitching motility, which is 100 fold slower than swarming. Recently, a new type “walking” motion in *P. aeruginosa* has been characterized, which is caused by movement of polarly attached bacteria that are vertical to the surface (Jin *et al.*, 2011). This polar attachment allows bacteria to efficiently steer and position themselves in the direction of the fluid flow

by release of type IV pili and this is referred to as the “slingshot” motion. During exploration of a surface, *P. aeruginosa* deposits a trail of the polysaccharide Psl, which influences the surface motility of subsequent cells and enhances attachment (Zhao *et al.*, 2013). This deposition of Psl on the surface can be considered to be a preconditioning film that is necessary for microcolony formation. Similarly, during the initial development of dental biofilms, the surface is coated with a layer of proteins and glycoproteins of salivary origin and this helps to enhance attachment of bacteria (Schilling & Bowen, 1992). The bacteria finally slows down its surface exploration due to reduced flagellar movement and this is considered as the completion of the first phase of biofilm development and culminates in commitment of the bacteria to the surface, resulting in irreversible attachment (Ha & O'Toole, 2015).

1.2.2 Irreversible attachment

After initial attachment to the surface and surface exploration, bacteria commit to the surface by irreversibly attaching along their longitudinal axis. In addition to repressing surface motility, irreversible attachment also appears to require the production of exopolysaccharides. Even though *P. aeruginosa* produces three types of polysaccharides, Pel, Psl and alginate, only Psl has been shown to be important for early stage biofilm development (Giraud *et al.*, 2010). Using defined mutants, it was demonstrated that the *P. aeruginosa* $\Delta pslAB$ mutant failed to attach to glass or mucin-coated surfaces (Ma *et al.*, 2006). In contrast, *pel* mutants in a non-piliated *P. aeruginosa* PAK strain, failed to develop a biofilm; however the lack of pili is known to result in poor or no attachment and hence, failure of the *pel* mutant to attach in those studies was most likely due to the lack of pili rather than Pel (Giraud *et al.*, 2010). Different polysaccharide components in the biofilm matrix can be functionally

redundant, where it has been shown that deletion of Psl results in increased production of Pel, while deletion of Pel enhanced the production of alginate. Similarly, the overexpression of alginate caused a 30% decrease in Psl promoter activity. Deletion of both Pel and Psl lead to a complete loss of biofilm formation in *P. aeruginosa* PAO1 (Ghafoor *et al.*, 2011). Another important component that facilitates firm attachment between the cells and the surface is believed to be extracellular DNA (eDNA). For example, treatment of an initial stage biofilm with DNase-I was found to dissolve the microcolonies and to prevent biofilm formation (Whitchurch *et al.*, 2002).

1.2.3 Microcolony formation

The irreversible attachment stage is typically followed by the formation of complex multicellular aggregates called microcolonies. Several factors such as iron concentration, alginate, Pel, Psl etc. have been shown important for microcolony formation by *P. aeruginosa*. Even though the *P. aeruginosa* PAO1 $\Delta pelA$ mutant formed microcolonies, it did not develop into mushroom shaped structures in mature biofilms after 96 h cultivation when compared to the isogenic wild type. The *P. aeruginosa* PAO1 $\Delta pslBCD$ mutant formed smaller microcolonies than those of both the wild type and the *P. aeruginosa* $\Delta pelA$ mutant. The *P. aeruginosa* PAO1 $\Delta pelA \Delta pslBCD$ double mutant formed only a monolayer biofilm (Yang *et al.*, 2011). Thus, polysaccharide production plays a significant role in biofilm microcolony formation.

1.2.4 Macrocolony formation

The subsequent development of macrocolonies from microcolonies is still poorly understood. Some researchers have suggested that macrocolony formation is the continuation of microcolony development. However, this stage represents the biofilm

maturation phase, which typically involves the generation of a complex architecture by the redistribution of cells from the substratum to the top of the colony as well as channel formation for the effective transport of nutrients and waste products. During this phase, the bacteria produce additional matrix materials that enhance the biofilm matrix for better strength. One particular feature of maturation is the establishment and interaction of subpopulations of the developing biofilm.

Reinforcement of the biofilm matrix is a key process in macro colony formation. Carbohydrate binding proteins such as lectins are known to bind to polysaccharides in the biofilm matrix providing extra strength (Jenninngs *et al.* 2015). Similarly, glucan-binding proteins of *Streptococcus mutans* are essential for shaping the biofilm architecture (Lynch *et al.*, 2007). In addition, the crosslinking of negatively charged eDNA with the positively charged Pel reinforces the biofilm matrix further. Using a fluorescently labelled *Wisteria floribunda* lectin specific for the Pel polysaccharide, Pel or Psl were localized to the periphery of microcolonies (Jennings *et al.*, 2015). The secreted protein CdrA was also found to bind to Psl, further reinforcing the matrix (Rybtke *et al.*, 2015). Biofilm matrix is also reinforced by structural proteins such as TasA (Spore coat-associated protein N) of *Bacillus subtilis* and amyloid proteins (Romero *et al.*, 2010).

Apart from proteins, some enzymes also secreted into, and entrapped within the biofilm matrix, and may include enzymes such as cellulase, protease, lipase, alginate lyase etc. These enzymes help biofilms to assimilate nutrients by acting as an external digestive system (Flemming & Wingender, 2010). Extracellular enzymes can be efficiently retained in the biofilm matrix suggesting advantages to production of industrial scale biocatalysts and in wastewater treatment process.

The sharing of resources and coordination between populations is necessary for complex structure formation in biofilms. For example, the formation of the classic mushroom shaped structure of *P. aeruginosa* biofilms requires migration of a motile subpopulation over a non-motile, immature microcolony, using twitching motility (Yang *et al.*, 2009). The *P. aeruginosa pilA* mutant (type 4 fimbrial precursor), defective in twitching motility, can only form stalks of the biofilm, and is unable to form fully differentiated mushroom structures. Similarly a *pqsA* (*Pseudomonas* quinolone signal) mutant can only form a flat biofilm. When the *pqsA* mutant and *pilA* mutant were co-cultured, the *pilA* mutant formed the stalk of the biofilm and the *pqsA* mutant formed the cap by migrating on top of the *pilA* mutant (Yang *et al.*, 2009). It has been suggested that the PQS controlled DNA release in the stalk subpopulation of the mushroom-shaped structures is necessary for development of the cap-forming subpopulation that does not express PQS. These observations highlight the requirement of specific bacterial functions, likely expressed in specific subpopulations, in forming the mature macrocolonies of a biofilm.

Biofilm maturation is also associated with the formation of channels and voids within the biofilm. For *P. aeruginosa*, surfactants, such as rhamnolipids have been shown to play an important role in this process. While biofilms formed by the *P. aeruginosa* rhamnolipid mutant *rhlA* were identical to the wild type for the first 5 d of development, biofilms of the *rhlA* mutant failed to form these characteristic channels or open areas after 6 d. Thus, it has been suggested that surfactants maintain open channels in the biofilm by interacting with and modifying the physical properties of the substratum (Davey *et al.*, 2003). Biosurfactants achieve this by modifying the electric charge of the surface and thus altering hydrophilicity (Neu, 1996).

1.2.5 Dispersal

Dispersal is considered to be the last stage of the biofilm lifecycle, when bacterial cells return to their planktonic form, which allows bacteria to spread to and colonize new sites. Dispersal can be categorized into passive and active processes. Biofilms continually release a small number of cells into the environment, mostly due to physical abrasion, called erosion dispersal. In contrast, when a microcolony reaches a certain population size, the cells undergo dispersal, which is an active, regulated process that is distinct from the passive, erosion of cells from the biofilm surface (Kaplan, 2010, McDougald *et al.*, 2012). Several environmental conditions such as nutrient concentration, oxygen, light and signals such as nitric oxide are known to act as dispersal cues (Gjermansen *et al.*, 2005, Thormann *et al.*, 2005, Barraud *et al.*, 2009). These environmental cues are known to trigger a series of molecular pathways that led to biofilm dispersal. One such mechanism is mediated by the RNA binding global regulatory protein CsrA (carbon storage regulator) of *E. coli*, an activator of biofilm dispersal. Deletion of *csrA* enhanced biofilm formation and caused defects in biofilm dispersal (Jackson *et al.*, 2002). Similarly, Singh *et al.* (2005) observed that rhamnolipids produced by *P. aeruginosa* cause rapid detachment of mature biofilms and hollowing of the centre of the mushroom-shaped mature biofilm structure. Further, the exogenous addition of rhamnolipids caused biofilm dispersal and reduced attachment to surface (Boles *et al.*, 2005). It was also shown that exogenously added rhamnolipids caused dispersal of *Bordetella bronchiseptica* biofilms, suggesting that surfactants may disrupt biofilms of some organisms (Irie *et al.*, 2005).

Since biofilms are embedded in an EPS matrix, dispersal requires dissolution of the matrix. A variety of matrix degrading agents are known to cause dissolution of the matrix, triggering dispersal (Kaplan, 2010). For example, the exogenous addition of

alginate lyase to a biofilm was shown to disperse *P. aeruginosa* biofilms (Boyd & Chakrabarty, 1994).

Exposure of *P. aeruginosa* biofilms to 500 nM of the nitric oxide releasing compound sodium nitro prusside (SNP) resulted in a 68% reduction in biofilm biomass (Barraud *et al.*, 2009). The dispersal in response to nitric oxide is c-di-GMP mediated and molecular mechanisms are described in more detail below. Flow cell biofilms of *Pseudomonas putida* biofilms were observed to disperse immediately in response to the stop of nutrient flow and this could be due to the rapid depletion of either oxygen or the carbon source. *P. putida* has been shown to disperse upon carbon starvation and this response was associated with a GGDEF/EAL domain protein PP0164, that is involved in modifying the intracellular concentration of c-di-GMP (Gjermansen *et al.*, 2005). Similarly, up to 80% of the biomass of *Shewanella oneidensis* MR-1 biofilms were found to detach in response to sudden decrease in oxygen.

Recent data suggests that dispersed cells represent an intermediate stage in the biofilm lifecycle that is distinct from both biofilm and planktonic cells (Chua *et al.*, 2014). The transcriptomes of dispersed cells were distinct from biofilm cells and planktonic cells and the dispersed cells were more virulent against immune cells such as macrophages. In addition the newly dispersed cells were more sensitive to iron stress and other stress factors than the biofilm (Chua *et al.*, 2014). This transient stage lasted approximately 2 h after the dispersal event before the bacteria fully converted to a planktonic phenotype.

1.3 Biofilm heterogeneity

Bacteria within different parts of the biofilm may experience extreme differences in their chemical and physical environment. This is partly due to the high density of

cells present within the biofilm as well as due to diffusional limitations as a function of the matrix. For example, bacterial metabolic activity results in a concentration gradient of oxygen, nutrients, waste products and signalling compounds. These chemical microenvironments can affect bacterial activity, giving rise to local physiological heterogeneity in a biofilm. In order to fully understand the biology of biofilm bacteria, it is important that the activities in microcolonies are resolved spatially and temporally (Stewart & Franklin, 2008).

1.3.1 Chemical heterogeneity in biofilms

Oxygen gradients are one of the most studied forms of heterogeneity within biofilms (De Beer *et al.*, 1994, Staal *et al.*, 2011). Using a planar optode, the dynamic nature of oxygen concentrations within a biofilm in relation to media flow rate and time were demonstrated. The interior of the microcolony is typically oxygen deprived at low flow rates but with high flow rates and a diffusion time of 150 s, the interior of the colony also became saturated with oxygen (Kühl *et al.*, 2007, Staal *et al.*, 2011).

The relationship between oxygen availability and physiological heterogeneity has also been investigated for *P. aeruginosa* biofilms grown in a drip flow reactor. In this system, attached bacteria receive nutrients and oxygen from the flowing media at the top surface. Alkaline phosphatase activity, as an indicator of aerobic metabolism, was observed as a 30 µm layer from the surface under normal growth conditions, below which no activity could be detected. However, when the ambient air was replaced with pure nitrogen, the active zone was reduced to 2 µm, whereas supplementation with oxygen restored the active zone to approximately 46 µm (Xu *et al.*, 1998).

As described above, mature biofilms are often found to have water filled micro channels, which have been hypothesized to play a key role in delivering nutrients throughout the biofilm. Using a combination of microsensors and imaging techniques,

researchers have plotted oxygen concentration across a microcolony. The comparison of the oxygen contour with the cell clusters showed that the oxygen concentration was in fact correlated with biofilm structure, with the concentration reducing from the exterior biofilm surface to the interior core. However, oxygen pockets were sometimes observed even at the base of the microcolony and inside the cell clusters, suggesting oxygen transport through microchannels. While the microchannels appear to deliver oxygen, and presumably other compounds, to different parts of the biofilm, the rate of transfer for the microchannels was significantly lower than the rate of bulk transport to the biofilm surface layer (De Beer *et al.*, 1994).

Oxygen is not the only compound that can become stratified within a biofilm. Nutrients, waste products, metabolic intermediates, signalling compounds etc. can also become differentially distributed across the biofilm. In natural biofilms, the association of different species introduces another level of heterogeneity. The metabolic waste product of one organism might be the food source of the other organism, resulting in microgradients within the biofilm based upon communal metabolism. This is particularly well exemplified in microbial mats, based on the spatial orientation of sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB). SRBs, obligate anaerobes, show high metabolic activity during the daytime when the mats are super saturated by oxygen as a function of photosynthetic activity in the mats. The SOBs protect SRBs from toxic oxygen concentrations in return for reduced sulfite (Decho *et al.*, 2010).

It is generally assumed that the cells at the surface of the biofilm might be metabolically more active since they have greater access to nutrients and oxygen, whereas cells inside the microcolony may be less active due to limited access to energy sources. This was indeed shown to be the case, where it was observed that the

cells at the surface of the microcolony were metabolically more active compared to those in the base of the biofilm (Davies, 2003). While this may suggest the cells in the interior of the biofilm are at a disadvantage, slow growth can sometimes be beneficial. For example, antimicrobials such as ciprofloxacin and tetracycline target metabolically active cells. In biofilms, these antibiotics mostly kill the cells on the outside of the microcolonies, whereas the cells in the interior, being metabolically dormant, are less sensitive. In contrast, when biofilms were treated with an antimicrobial peptide, colistin, which targets metabolically inactive cells, cells in the interior of biofilm were preferentially killed (Pamp *et al.*, 2008). This suggests that combinations of antibiotics targeting both metabolically active and non-active cells are more likely to kill almost all the biofilm and could be a novel therapeutic strategy (Herrmann *et al.*, 2010).

1.3.2 Genetic heterogeneity in biofilms

In contrast to transient changes in gene expression, some cells acquire mutations, leading to subpopulations of bacterial cells with different genotypes even within a monospecies biofilm. For example *Serratia marcescens* MG1 produces six different morphological colony variants when grown as a biofilm under flow conditions. These morphotypic variants show significant differences from the wild type in attachment, biofilm formation and cell structure properties. These phenotypic variants tend to be genetically stable and are called morphotypic variants (Koh *et al.*, 2007). Genetic diversification has been hypothesized to protect the population from variety of stresses and adverse environmental conditions. This phenomenon, generally known as “insurance effects” in bacteria (Boles *et al.*, 2004), is based on the observation that some variants are individually better adapted to tolerate specific stressors, such as

hydrogen peroxide. In this way, the population may gain the benefits provided by a single variant.

One of the colony variants produced by the *P. aeruginosa* overproduces both of the Pel and Psl polysaccharides and appears wrinkly and small with an elevated capacity to form biofilms and is thus called a Rugose Small Colony Variant (RSCV) (Starkey *et al.*, 2009). This phenotype is typically due to a frameshift mutation in the *wspF* gene MJK8, which causes elevated levels of c-di-GMP in the cell and thus enhanced biofilm formation. Similarly, *P. protegens* produces four known colony variants with distinctive morphologies such as the cauliflower colony variant (CCV), the rough colony variant (RCV), the small colony variant (SCV) and the wrinkled colony variant (WCV). *K. pneumoniae* also produces a non-mucoid colony variant during biofilm development (Lee *et al.*, 2015). Interestingly, while these variants are associated with increased stress tolerance of biofilm populations, the formation of a mixed species biofilm with *P. aeruginosa*, *P. protegens* and *K. pneumoniae*, results in a biofilm that either does not produce variants (*P. aeruginosa* and *K. pneumoniae*) or a significantly reduced number of variants (*P. protegens*). Thus, variant formation may primarily be a function of populations and not communities, although this remains to be more thoroughly explored (Lee *et al.*, 2015).

1.4 Communication within biofilms

Bacterial communication by means of small signalling molecules is known to be important for the biofilm structure formation and performing group tasks such as the bioluminescence of *Vibrio harveyi*, antibiotic production by *Streptomyces* species etc. Two types of bacterial communication involving small signalling molecules include, intracellular communication by means of c-di-GMP and extracellular, cell-to-cell signalling by quorum sensing (Kolenbrander *et al.*, 2002). Signalling can be enhanced

during biofilm formation where such signals interact with the matrix polymers and achieve higher concentrations than are present in the bulk liquid phase (Seviour *et al.*, 2015).

1.4.1 Cell to cell signaling- Quorum sensing

Quorum sensing (QS) is a cell-density dependent mechanism by which bacteria “talk” to each other (Miller & Bassler, 2001). Bacteria in a biofilm are present at a relatively high density and are able to sense neighbouring cells by such chemical cues, allowing them to coordinate gene expression and the associated phenotypes at the population level, including bioluminescence, virulence factor production or biofilm development. *P. aeruginosa* has three QS systems, LasI/LasR, RhlI/RhlR and the Pseudomonas Quinolone Signal (PQS) system. LasI and RhlI synthesise the autoinducers N- (3-oxododecanoyl)-homoserine lactone (HSL) and N-(butyryl)-homoserine lactone, respectively. PQS synthesises 2-heptyl-3-hydroxy-4-quinolone (Miller & Bassler, 2001), (Wade *et al.*, 2005). At high cell density, LasR binds its associated HSL autoinducer and binds to specific promoters to activate virulence gene expression. LasR, when bound to its cognate signal, also activates *lasI* expression, which establishes a positive feedback loop. The RhlR-autoinducer complex also regulates production of rhamnolipids, which are surfactants that play an important role in biofilm development (described in section 1.2.4). The PQS system is composed of the *pqsABCDE* operon as well as *phnAB*, *pqsH* and *PqsR* (Wade *et al.*, 2005). The PQS system works in conjugation with the Las and Rhl systems. The Las system can indirectly downregulate the formation of PQS, and also directly upregulate the expression of the regulator *pqsR* (Bacalso *et al.*, 2011). While the general model indicates that QS activation occurs for cells at high density, recent studies have shown that this is not the case for all bacteria that utilise QS. For example, single cell

analysis of planktonic *V. harveyi* cultures revealed that only 69% of the population respond to quorum sensing to produce bioluminescence (Anetzberger *et al.*, 2009). This implies that there is functional heterogeneity even for QS based gene expression. Such heterogeneity is likely to reflect the interaction of QS regulators with other global regulatory systems, as has been shown previously for QS genes in *P. aeruginosa*. The QS genes in *P. aeruginosa* are divided into 4 classes depending on their pattern of regulation. For example class I responds to the QS molecule 3OC₁₂-HSL immediately, class II respond late where as class III responded to both signals early, and class IV responded to both signals late. These classes of genes respond differently depending on the phase of growth (Whiteley *et al.*, 1999). Because of the inherent gradients and physiological heterogeneity of the biofilm, it is likely that cells in different parts of biofilm might be in different phases of growth and hence activation of the QS system may also differ across the biofilm.

1.4.2 Intracellular signaling: c-di-GMP

C-di-GMP was initially identified as an allosteric activator of cellulose synthesis in *Acetobacter xylinum* (Ross *et al.*, 1987). Subsequently, the c-di-GMP pathway has been identified in several bacterial species and controls a range of diverse functions (Amikam & Benziman, 1989) such as motility, virulence, stress resistance, colony morphology, secondary metabolite secretion, cell cycle regulation etc (Figure 1.2). Bacteria synthesize c-di-GMP from two molecules of guanosine-5'-triphosphate (GTP) by an enzyme, diguanylate cyclase (DGC). The DGC enzymes contain conserved amino acid sequence motifs “GGDEF” (Gly-Gly-Asp-Glu-Phe). C-di-GMP is broken down into two molecules of linear 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) by phosphodiesterases enzyme (PDEs) that contain conserved motifs, called EAL domains. pGpG is then subsequently degraded into two molecules of guanosine

monophosphate (GMP) by oligoribonuclease (Orn), a newly identified phosphodiesterases (Orr *et al.*, 2015). In addition to EAL domain phosphodiesterases that break down c-di-GMP in two steps, another group of PDEs breakdown c-di-GMP directly into 2 molecules of GMP, and contain the conserved HD-GYP amino acid domain.

Using these functional domains as the basis for a bioinformatics analysis indicated that these enzymes are broadly distributed throughout much of the bacterial kingdom, although it is not present in Archaeobacteria (Tal *et al.*, 1998). Interestingly, some bacteria encode multiple enzymes with either DGC or PDE activity. For example, *P. aeruginosa* has 47 c-di-GMP metabolising proteins, *E. coli* encodes 29 and *Vibrio fischeri* has more than 50 GGDEF or EAL domain proteins (Römling *et al.*, 2013). If the cellular pool of c-di-GMP is dependent on the sum activity of those of multiple DGCs and PDEs, and if the level of c-di-GMP in the cell is the determinant of the associated phenotypes, there would be a single phenotypic response. However, many of these enzymes are associated with controlling a specific phenotype, against the background of other such enzymes present in the cell. For example RoeA and SadC are two DGCs in *P. aeruginosa* with distinct and independent functions. RoeA controls polysaccharide production while SadC controls flagellar motility (Merritt *et al.*, 2010). This would therefore argue that the cells have mechanisms to control the expression of individual c-di-GMP regulated phenotypes. In part, this is achieved by differences in affinity of different c-di-GMP binding proteins for c-di-GMP. In *E. coli* the dissociation constant (K_d) of the PilZ domain protein YcgR is 0.84 μ M (Ryjenkov *et al.*, 2006), whereas the affinity of PelD is 0.5 μ M (Li *et al.*, 2012). Thus, the two c-di-GMP responsive proteins are activated at different concentrations of c-di-GMP.

Figure 1.2: Cyclic di-GMP signalling. C-di-GMP is synthesized from two molecules of guanosine triphosphate (GTP) by a cellular enzyme containing a DGC domain. It is broken down into its linear form, pGpG (5'-phosphoguanylyl-(3'-5')- guanosine), by enzymes containing PDE domains. This pGpG is further broken down into two molecules of GMP by non-specific PDEs. Phosphodiesterases containing the HD-GYP domain directly breakdown c-di-GMP in to GMP. High and low levels of c-di-GMP decide whether bacteria will undergo biofilm formation or planktonic growth. Figure adopted from (Hengge, 2009).

Furthermore, not all of the GGDEF and EAL enzymes are active at the same time or sub-cellular location. In *E. coli*, only a few GGDEF and EAL domain genes are expressed during active growth, whereas other such genes, e.g. those required for curli and cellulose synthesis, are under the control of the stationary phase sigma factor, RpoS, (Weber *et al.*, 2006). It has also been suggested that there might be 'local pools' of c-di-GMP acting directly with the effector proteins (Andrade *et al.*, 2006). The Lpl0329 protein of *Legionella pneumophila* contains both GGDEF and

EAL domains and both domains have been demonstrated to be enzymatically active, which could potentially lead to the simultaneous synthesis and degradation of c-di-GMP. The activity of Lpl0329 is controlled by phosphorylation by the atypical histidine kinase Lpl0330 that catalyses the phosphotransfer to Lpl0329, resulting in a reduction in DGC activity, whereas phosphodiesterase activity remains unaffected (Levet-Paulo *et al.*, 2011). In some cases, one of the two domains is enzymatically inactive or catalytically incompetent, but rather may function as an allosteric activation or inhibition site.

1.5 C-di-GMP signalling during biofilm development and dispersal

P. aeruginosa uses flagellar mediated swimming motility to overcome the repulsive force exerted by the substratum to attach to the surface during reversible attachment (refer to section 1.2.1) and this flagellar motility is tightly controlled by FleQ, a transcriptional regulator of flagellar expression. Binding of c-di-GMP inhibits the ATPase activity of FleQ rendering it incapable of regulating the expression of flagellar genes (Baraquet & Harwood, 2013). Similarly, the “bacterial clutch protein”, YcgR, contains a PilZ domain making it as a primary target for c-di-GMP regulation. Under conditions of high intracellular c-di-GMP, YcgR causes a reduction in flagellar motility by interaction of other flagellar proteins (FliG) and the stator (MotA) and thus, YcgR forms a complex with c-di-GMP that modulates flagellar rotation.

During reversible attachment, *P. aeruginosa* exhibits swarming and twitching motility for surface exploration. *P. aeruginosa* uses type IV pilli (Tfp) for twitching motility. The protein FimX, involved in the assembly of Tfp, has both GGDEF and EAL domains, making it as a primary target for c-di-GMP binding (Kazmierczak *et al.*, 2006). The DGC SadC and the PDE BifA both regulate swarming motility and hence biofilm formation. The $\Delta sadC$ mutant, which produces reduced amounts of c-

di-GMP, was found to be defective in attachment and showed a hyper swarming phenotype whereas the $\Delta bifA$ mutant, with increased intracellular concentrations of c-di-GMP, showed increased attachment and is a hyper biofilm former.

Polysaccharide production and biofilm matrix formation is one of the important events during biofilm microcolony formation. C-di-GMP is also important for polysaccharide production, especially the Pel polysaccharide. Deletion of the DGC RoeA resulted in the complete loss of Pel production as well as impaired early stage biofilm formation (Merritt *et al.*, 2010). The PEL exopolysaccharide is under the regulation of PelD, which has a degenerate, inactive GGDEF domain (Whitney *et al.*, 2012). Alginate, which gives structural integrity to biofilm microcolonies (Chang *et al.*, 2007), is under the regulation of Alg44, which has a high c-di-GMP affinity PilZ domain protein. Increasing intracellular levels of c-di-GMP levels by overexpression of this DGC enhanced the formation of alginate in mucoid strains (Merighi *et al.*, 2007). The chemosensory system WspR-WspF regulates EPS production and biofilm maturation. WspR contains a GGDEF domain and WspF contains an EAL domain. Deletion of the *wspF* gene caused an increase in intracellular levels of c-di-GMP and resulted in a wrinkly morphology usually associated with high production of Pel and Psl. The *wspF* mutant is a hyper biofilm former and shows a 2 fold increase in the expression of *psl* and a 13 fold increase in *pel* expression compared to the isogenic wild type (Hickman *et al.*, 2005). Another important component for the biofilm matrix is a 220 KDa protein CdrA. The *cdrA* transcripts were 27 fold up regulated in the high c-di-GMP mutant *wspF*, compared to the wild type. CdrA was found to be localized with the Psl polysaccharide, enhancing the structural integrity of the biofilm matrix (Borlee *et al.*, 2010).

Figure 1.3: The role of c-di-GMP signalling in biofilm dispersal. External cues such as oxygen concentration, nitric oxide, light, nutrient concentration etc. are perceived by cell surface sensory receptors and result in alteration of c-di-GMP pool, which in turn react with specific receptors that co-ordinately activates a cascade of proteins results in cell surface modifications and secretion of extracellular enzymes causing dispersal. Figure adopted from (McDougald et al., 2012).

Active dispersal occurs when cells within the biofilm begin to turn on genes associated with planktonic growth and escape the biofilm. In many cases, dispersal is induced in response to specific environmental signals or cues that are transmitted intracellularly through the c-di-GMP signalling network. Upon sensing environmental cues, such as low oxygen concentration, nitric oxide (NO), nutrient levels, temperature or light, or quorum sensing signals, DGCs or PDEs are stimulated (Figure 1.3). For example NO induces the reduction of the intracellular c-di-GMP concentration in *P. aeruginosa* through stimulation of PDEs including DipA, NbdA and MucR. The chemotaxis transducer protein BdlA, which harbors two PAS domains, was found to be involved in NO-induced biofilm dispersal. During nutrient

induced dispersal, specifically glutamate induced dispersal, glutamate is sensed by NicD resulting in NicD phosphorylation and subsequent activation of BdlA and the phosphodiesterase DipA. Active NicD contributes to the non-processive proteolysis and activation of BdlA via phosphorylation and temporarily elevated c-di-GMP levels. BdlA, in turn, activates DipA, a phosphodiesterase and this results in the overall reduction of c-di-GMP levels (Basu Roy *et al.*, 2014). The intracellular c-di-GMP in turn interacts with specific receptors that activate a cascade of proteins at the transcriptional, translational or post-translational levels to induce changes in the cell surface that promote either biofilm formation (high levels of c-di-GMP) or dispersal (low levels of c-di-GMP). The regulatory pathways may operate either via a global intracellular pool of c-di-GMP or localized subcellular pools of c-di-GMP (Christian *et al.*, 2010) or via direct interactions of the DGCs or PDEs (McDougald *et al.*, 2012). For example, exposure of a wild type biofilm to the nitric oxide releasing compound, sodium nitroprusside (SNP), resulted in a 50% drop in intracellular c-di-GMP and resulted in dispersal of the biofilm (Barraud *et al.*, 2009).

1.6 Multispecies biofilms

Even though there is a large body of research aimed at determining the mechanisms and consequences of biofilm formation, most of that work has focused on single species systems and comparatively less is known regarding such processes in regard to multi-species biofilms. In nature, biofilms can exist as consortia of hundreds or thousands of organisms; it has been estimated that a healthy human oral cavity is colonised by more than 500 individual bacterial species (Egija *et al.*, 2009). Because of the very nature of the biofilm structure, localized interactions within these communities are predicted to affect the coexistence of the component species,

community structure and function. These bacteria interact with each other in many ways, such as using the metabolic end products that other species produce, or communicating by releasing specific molecules. The biology of these multi-species systems is potentially quite different from that of a single species system due to the potentially expanded metabolic capacity of such a community. In addition to metabolic interactions, microorganisms in high density biofilm communities may compete through the secretion of antagonistic compounds to increase their individual fitness. One example is found in dental plaque, where *Streptococcus* sp. and *Veillonellae* sp. are known to be early colonizers of dental biofilms. Studies of the dual species biofilms formed by *S. mutans* and *V. parvula* have shown that the growth of *S. mutans* is enhanced those dual-species biofilms compared to single species biofilms. It is thought that this is a consequence of metabolic sharing as Streptococci produce lactate during growth on sugars, and *Veillonellae* can utilize short-chain organic acids, especially lactate for growth (Hansen *et al.*, 2007). Dental biofilms undergo periodic nutrient fluctuations, temperature fluxes, pH changes as well as exposure to detergents. A freshly cleaned tooth surface is soon covered with a layer of glycoproteins of salivary origin, called salivary pellicle. Early colonizers such as *Streptococcus mitis*, *Streptococcus gordonii*, *Streptococcus sanguinis* and *Streptococcus oralis* bind to this preconditioned surface mediated by salivary receptors, α -amylase, sialylated mucins, proline-rich protein and cell fragments. Initial colonization is followed by late colonizers, e.g. *F. nucleatum*, *Treponema denticola*, *Porphyromonas gingivalis* and *Prevotella intermedia*, that attach to the early stage bacteria. While initial colonisation is somewhat stochastic, secondary colonisation tends to be species specific. For example *F. nucleatum* co-aggregates with many other species of the community to act as a bridging or nucleating organism

during biofilm development. The coaggregation by *F. nucleatum* is mediated by a 350 kDa cell surface protein called RadD, which facilitates cell to cell attachment (Kolenbrander *et al.*, 2010).

It is also interesting to consider infections of Cystic Fibrosis (CF) patients, where at the start of infection the lungs are typically colonized by many bacterial species, *Staphylococcus aureus*, *Haemophilus influenzae*, *P. aeruginosa* and *Burkholderia cepacia* being the most abundant (Kirov *et al.*, 2007). Subsequently, the bacterial community composition changes and *P. aeruginosa* becomes the most dominant species. This community rearrangement occurs due to inter-species interactions and human intervention through drugs and antibiotic treatments. *S. aureus* is known to ‘sensitize’ the lungs for subsequent infection by *P. aeruginosa* (Burns *et al.*, 1998). Once established, *P. aeruginosa* lyses the cells of *S. aureus* and other Gram positive bacteria and uses the iron released by lysis to support its own growth. Similarly, alginate production by *P. aeruginosa* can cause local hypoxia leading to elimination of other speices (Harrison, 2007).

Advances in metacommunity sequencing and analysis has been applied to a range of complex communities such as effluent treatment plants, biofilms on reverse osmosis membranes, the human intestine etc., which has led to the realisation that the microbial consortia in natural environments is much more rich than previously understood (Chen *et al.*, 2004, Claesson *et al.*, 2009). While knowing what organisms are present, it is probably more important to understand the spatial organisation of such communities as well as their functional roles to better define the functional interactions between these organisms (Harmsen *et al.*, 1996). Fluorescent *in situ* hybridization (FISH) is a technique that has been used to study the spatial organisation of mixed bacterial communities without disrupting their natural

organisation. However, one must know what organisms are present in order to select or design probes, which are normally based on the 16s rRNA gene. Thus, FISH is not generally an ideal tool for community level discovery of what organisms are present, but rather is an excellent tool for the targeted determination of the presence and abundance of key organisms of interest. The development of laboratory model systems with combinations of bacterial species that have been fluorescently tagged is another useful approach to track and study individual species in a mixed species environment *in situ* using imaging techniques. One such model community, consisting of green fluorescent protein (GFP) tagged *P. aeruginosa* and yellow fluorescent protein (YFP) tagged *A. tumefaciens*, showed that quorum sensing dependent motility was an advantage for *P. aeruginosa*, where *P. aeruginosa* blanketed the *A. tumefaciens* when grown as mixed species biofilm (An *et al.*, 2006). Similarly, a model system with *P. aeruginosa* PAO1, *P. fluorescens* Pf5, *K. pneumoniae* KP1 tagged separately with the cyan fluorescent protein, yellow fluorescent protein (YFP) and *Discosoma sp.* red fluorescent protein (DsRed), respectively, showed enhanced growth and increased tolerance to SDS and antibiotic treatment. At the same time this system allowed for the spatial monitoring of each species (Figure 1.4), where both spatial and temporal localizations of different species can be tracked and quantified. This system will be used in this study for exploring the molecular mechanisms of mixed species biofilm development

Figure 1.4: Confocal laser scanning image of a model microbial multispecies biofilm system. The blue color represents *P. Protegens* Pf5, yellow represents *P. aeruginosa* PAO1 and red represents *K. pneumoniae* KP1. Orthogonal view showing top pannel x - z plane, right y - z and middle corresponding x - y plane. Magnification 20x. Scale bar: 50 μ m.

1.7 C-di-GMP signalling in biofilm - current challenges

Even though the molecular mechanism of c-di-GMP signalling is well understood, studies regarding the implications of temporal and spatial dynamics of this molecule during biofilm have development been challenging due to lack of tools and techniques. The heterogeneity within the biofilm could possibly lead to localized high or low c-di-GMP pools, which might be the underlying reasons behind incomplete biofilm dispersal when treated dispersal inducing chemicals. Being an intracellular secondary messenger whose concentration varies quickly, a real time measurement system is well suited to undertake studies of the temporal and spatial changes in c-di-GMP production and degradation. Current methods for the quantification of c-di-GMP use liquid chromatography–mass spectrometry (LC-MS), which is laborious

and does not give a real-time measurement. Further, this approach typically requires extraction of the second messenger leading to an average concentration across the entire biofilm. Christen *et al.* (2010) constructed a fluorescence resonance energy transfer (FRET) based biosensor to monitor the c-di-GMP concentration. Even though the sensor is quite applicable to planktonic single cell cultures, where the authors demonstrated an asymmetrical distribution of the c-di-GMP inside the cell, its basic principle of inverse relation between c-di-GMP to the FRET signal reduces its usefulness for studies of biofilms, where the cells inherently have higher c-di-GMP concentrations than planktonic cells. At low power magnification, the signal intensity is quite low and hence is unsuitable for the magnifications that would be used for biofilm imaging (unpublished data; this study).

Rybtke *et al.* (2012) constructed a reporter for c-di-GMP, where the fluorescence intensity is directly proportional to the concentration of intracellular c-di-GMP, and quite applicable in biofilm studies. The c-di-GMP reporter used here is a promoter fusion of the c-di-GMP responsive *cdrA* gene (PA4624) with green fluorescent protein (GFP) (Rybtke *et al.*, 2012). The mechanism of regulation of *cdrA* transcription by c-di-GMP is not known; however, the expression levels of *cdrA* strongly correlate with c-di-GMP levels. CdrA is a 220 kDa adhesion like protein encoded by gene PA4624 and is responsible for cell-cell aggregation and biofilm maturation in *P. aeruginosa* and is transcriptionally regulated by c-di-GMP. The levels of relative *cdrA* transcript were 27 fold higher in the c-di-GMP overproducing *wspF* strain in comparison to the wild type (Borlee *et al.*, 2010). In addition to CdrA, there are other potential candidates for c-di-GMP reporters such as the promoters of the c-di-GMP responsive genes *pelA*, *pslA*, PA2441, PA4621 etc (Hickman *et al.*, 2008). Converting the fluorescence intensity into a quantifiable concentration of c-di-

GMP in standard units (pM/ μ g of protein) would allow one to compare different biofilm systems which wide applications.

1.8 Objectives and scope

Biofilm formation is the primary lifestyle of bacteria and the molecular mechanisms of its development are under intense investigation for many organisms to understand how biofilm development is regulated and the consequences of biofilm formation. While c-di-GMP signalling is now known to play a central role in biofilm development for many bacteria, including *P. aeruginosa*, heterogeneity of intracellular c-di-GMP concentration in various sub-populations within the biofilm has yet to be fully explored. Additionally, the distribution of c-di-GMP signalling in mixed species biofilms is as yet largely unexplored. Therefore, the objective of this study is to develop and validate an imaging based method to understand the spatial and temporal changes in c-di-GMP within biofilms during development and dispersal for single and mixed species biofilms.

This objective will be met by addressing the following Aims:

- Develop a method for the *in situ* visualization and quantification of c-di-GMP in cells and biofilms.
- Study the distribution, and dynamics of c-di-GMP during biofilm development using the newly developed technique.
- Study the change in c-di-GMP in response to dispersal cues with improved temporal and spatial resolution.
- Study the distribution and change in c-di-GMP during multi-species biofilm development and dispersal.

CHAPTER 2. DISTRIBUTION OF C-di-GMP DURING MONO SPECIES BIOFILM DEVELOPMENT

2.1 Introduction

The bacterial secondary messenger bis-(3'5')-cyclic dimeric guanosine monophosphate (c-di-GMP) plays a central role in cell cycle, motility, virulence, biofilm formation and dispersal (Jenal & Malone, 2006). The molecular pathways through which c-di-GMP operates have been intensely studied in recent years, some of which are now well understood (Mills *et al.*, 2011). C-di-GMP is synthesized from two molecules of guanosine triphosphate (GTP) by diguanylate cyclases (DGC), and it is broken down into a linear form, phosphate-guanine-phosphate (pGpG), by phosphodiesterase enzymes (PDE). The proteins involved in c-di-GMP turnover can be identified by the presence of conserved domains, where DGCs contain a highly conserved amino acid sequence motif GGDEF, and PDEs contain EAL or HD-GYP domains. Most of these proteins also contain sensory input domains, such as PAS or GAF, on its N-terminus allowing them sense various environmental cues. Proteins encoding putative or experimentally demonstrated DGC or PDE activity have been identified in all major bacterial phyla, making it a universal bacterial signaling molecule (Jenal & Malone, 2006). The model organism *Pseudomonas aeruginosa* encodes 41 predicted proteins that may be involved in modifying intracellular c-di-GMP concentrations, some of which have been experimentally validated. The concentration of c-di-GMP in bacteria is often associated with controlling whether they grow as planktonic cells or as biofilms. Essentially, low c-di-GMP levels are associated with planktonic growth and high concentrations lead to biofilm development (Römling *et al.*, 2013).

The intracellular concentration of c-di-GMP appears to play a role in the regulation of multiple stages of the biofilm life cycle. For example, the first step of biofilm development, reversible attachment, is tightly regulated by flagellar movement mediated by FleQ, a transcriptional regulator of flagellar expression. Binding of c-di-GMP to FleQ results in a conformational change, leading to downstream gene activation. FleN, the antiactivator of FleQ and c-di-GMP can inhibit the ATPase activity of FleQ rendering FleQ incapable of regulating downstream flagellar genes. Other flagellar motility associated proteins such as YcgR and PilZ also have c-di-GMP binding sites. Thus, increased c-di-GMP results in reduced motility and the initiation of biofilm formation in *P. aeruginosa* (Baraquet & Harwood, 2013). During biofilm maturation, bacteria produce extracellular polysaccharides and other biofilm matrix biopolymers, which is also regulated by c-di-GMP. For example, the production of the polysaccharides Pel and Psl is positively regulated by c-di-GMP (Franklin *et al.*, 2011). C-di-GMP is also intimately involved in regulating biofilm dispersal. It was recently shown that the dispersed cells contain low levels of c-di-GMP compared to the biofilm cells (Chua *et al.*, 2014). This change in c-di-GMP is likely to be a consequence of the cells perceiving particular signals or cues associated with the initiation of dispersal. For example, exposure of biofilms to nitric oxide releasing compounds causes dispersal of *P. aeruginosa* biofilms, where exposure to NO is directly linked to the induction of PDE activity, resulting in decreased concentrations of c-di-GMP (Barraud *et al.*, 2009). Similarly, glutamate induced dispersal, also known as nutrient induced dispersal, has been shown to be mediated through *dipA*, a known phosphodiesterase.

Given that biofilms display physiological heterogeneity (Stewart & Franklin, 2008) and that c-di-GMP modifying enzymes also respond to changes in oxygen,

nutrients and other environmental signals, it is likely that the c-di-GMP concentration may differ in distinct regions of the biofilm, where bacteria are experiencing different concentrations of nutrients or signals. However, this heterogeneity in the distribution of c-di-GMP has not been demonstrated to date. This is in part due to the fact that the current method of quantifying c-di-GMP is based on liquid chromatography–mass spectrometry (LC-MS), requiring extraction and homogenization of the biofilm. Thus, concentrations are an average of the whole biofilm and do not take into account spatial variation in the biofilm as a consequence of metabolic gradients. To address whether there are localized c-di-GMP pools in the cell, Christen *et al.* (2010) constructed a fluorescence resonance energy transfer (FRET)–based biosensor to monitor the c-di-GMP concentration (Christen *et al.*, 2010) and demonstrated the asymmetrical distribution of the c-di-GMP inside the planktonic cell. Therefore, such reporters, which can be calibrated to the c-di-GMP concentration, can potentially be used to address the c-di-GMP concentration in different parts of the biofilm. Similarly, Rybtke *et al.*, 2012 constructed a reporter for c-di-GMP, where the fluorescence intensity is directly proportional to the concentration of intracellular c-di-GMP and has been used for biofilm studies (Rybtke *et al.*, 2012).

Here, an image-based methodology has been developed to visualize and quantify c-di-GMP *in situ* in real time using the above-mentioned reporter. The methodology allows for the visualization of c-di-GMP across the biofilm during its development and allows for investigation of c-di-GMP in different regions of the biofilm. Using this reporter, it was observed that there was a striking difference in c-di-GMP concentrations across the biofilm where some parts of microcolonies were found to have relatively high concentrations of high c-di-GMP. Most importantly c-di-GMP

concentrations were found to be localized at the outer boundary of mature colonies, while smaller, less mature colonies showed a more uniform distribution of c-di-GMP.

2.2 Materials and methods

2.2.1 C-di-GMP reporter

Four constructs of the fluorescence-based reporter for c-di-GMP (Table 2.1) were obtained (Rybtke *et al.*, 2012) and tested for their suitability in the current study.

Table 2.1: C-di- GMP reporter constructs used in this study.

Reporter construct	Description	Reference
Tn7 <i>cdrA::gfp</i>	Genomic insertion-stable GFP	(Rybtke <i>et al.</i> , 2012)
Tn7 <i>cdrA::gfp</i> (ASV)	Genomic insertion-unstable GFP	(Rybtke <i>et al.</i> , 2012)
<i>pcdrA::gfp</i>	Plasmid- stable GFP	(Rybtke <i>et al.</i> , 2012)
<i>pcdrA::gfp</i> (ASV)	Plasmid-unstable GFP	(Rybtke <i>et al.</i> , 2012)

2.2.2 Transformation

Electro competent PAO1 was prepared as described (Choi & Schweizer, 2005) with some modifications. Briefly, 50 ml of overnight culture of bacteria were concentrated by centrifugation (1000 g, for 5 min at room temperature) washed twice with 300 mM sucrose to remove residual salts and resuspended in 1 ml of 300 mM sucrose. One hundred microliters of this concentrated bacterial solution were mixed with 400 ng of plasmid and transferred to a 2 mm pulse cuvette and electroporated (Bio-Rad Gene Pulser Xcell™ Electroporation Systems) using 2500 V, 25 μ F and 200 Ω resistance. Immediately after electroporation, 1 ml of LB broth (10 g/l Tryptone, 5 g/l Yeast extract and 5 g/l NaCl) was added, the cultures were transferred to a 15 ml test tube and incubated in 37°C for 1 h with shaking at 180 rpm. Transformants were

selected by plating onto LB agar plates supplemented with 300 µg/ml carbenicillin and incubated overnight at 37°C.

2.2.3 Bacterial strains and culture media

Bacteria (Table 2.2) were either cultured on LB agar or M9 minimal medium (48 mM Na₂HPO₄; 22 mM KH₂PO₄; 9 mM NaCl; 19 mM NH₄Cl; 2 mM MgSO₄; 0.1 mM CaCl₂) with 20 mM glucose.

Table 2.2: Bacterial strains used in this study

Strain	Description	Application	Reference
PAO1 $\Delta wspF$ <i>pcdrA::gfp</i> (ASV)	Mutant expressing high c-di-GMP with c-di-GMP reporter; Biofilm forming	Positive control for biofilm studies, c-di-GMP monitoring	(Rybtke <i>et al.</i> , 2012)
PAO1 $\Delta wspF$ $\Delta pel \Delta psl$ <i>pcdrA::gfp</i> (ASV)	Mutant expressing high c-di-GMP with c-di-GMP reporter; Non biofilm forming	Positive control for planktonic studies, c-di-GMP monitoring	(Rybtke <i>et al.</i> , 2012)
PAO1 Tn7::gfp	Wild type PAO1 with stable GFP	Positive control for stable GFP	This study
PAO1 <i>pcdrA::gfp</i> (ASV)	Wild type PAO1 with c-di-GMP reporter	For dynamic c-di-GMP studies in planktonic	This study
PAO1 Tn7- Gm- eCFP <i>pcdrA::gfp</i> (ASV)	Wild type PAO1 stably expressing CFP with c-di-GMP reporter	For c-di-GMP quantification in biofilm.	This study

2.2.4 Biofilm development in flow cells

Biofilms were cultivated in three-channel flow cells (channel dimensions, 1 x 4 x 40 mm) (Tolker - Nielsen & Sternberg, 2011). The flow cells were fed with M9

minimal medium with 20 mM glucose as carbon source at a flow rate of 9 ml/h. Each channel was inoculated with 0.5 ml of diluted overnight culture containing approximately 1×10^8 cfu/ml.

2.2.5 C-di-GMP measurement in planktonic culture

A TECAN Infinite 200 PRO plate reader was used to measure c-di-GMP in planktonic cells based on *cdrA::gfp* fluorescence (Excitation 485 nm, Emission 520 nm, excitation band width 9 nm and emission band width 20 nm; manual gain of 80) and growth was monitored by quantifying the absorbance at OD₆₀₀. Measurements were recorded in 10 min intervals. The relative fluorescence, GFP/OD₆₀₀, was calculated and was used as a measure of c-di-GMP per cell.

2.2.6 Microscopy and image analysis

Biofilm images were acquired using a Carl Zeiss Confocal Laser Scanning Microscope CLSM 780 (Carl Zeiss, Germany) using 20x objective (LD plan-Neofluar 20x / 0.4 Korr M27). Two separate optical channels were used to image the biofilm biomass and c-di-GMP reporter. A stably expressed CFP, inserted into the genome, was used to quantify the biomass and GFP was quantified as a reporter for c-di-GMP. Laser voltage was measured using a Sanwa™ CD800a digital multimeter and kept at 0.587 V throughout the experiment and pinholes of 50 µm and 58 µm were used for CFP and GFP, respectively. An emission bandwidth of 440-503 nm was used for CFP and 497-598 nm was used for GFP while the master gain was set to 700 and the digital gain of 1.00 was used for all of the images. Images were processed using ImageJ Version 1.47 (Abràmoff *et al.*, 2004).

2.2.7 Chemical quantification of c-di-GMP by LC-MS

Biomass extraction from flow cell biofilms

The planktonic phase of the flow cell biofilms were carefully decanted by tilting the flowcell to one end. A syringe containing 2 ml of 1 mM ice-cold ammonium acetate was attached to one end of the flow cell and another empty syringe was connected at the other end. The biomass inside the flow cell could then be removed through back and forth (10 times) washing using the syringes. To avoid leakage, only negative pressure was applied to the syringe.

C-di-GMP extraction

Two milliliters of planktonic culture or 1.5 ml of extracted biofilm were centrifuged (10000 g for 10 min at 4°C) and the pellets were washed with 2 ml of ice-cold 1 mM ammonium acetate. The pellets were then resuspended in 2 ml of ice-cold Acetonitrile, Methanol, Water (40%: 40%: 20%) and homogenized by passing it five times through a 20 G needle. The solution was then lysed for 30 min (Power 100%, frequency 37 kHz) in a water bath sonicator (Elmasonic P, Elma, Singen, Germany) filled with ice water. The cell debris was then centrifuged at 10000 g for 10 min at 4°C and the supernatant was retained and kept in ice. The pellet was again resuspended in 2 ml of ice-cold Acetonitrile, Methanol, Water (40%: 40%: 20%) and repeated once more as above. The supernatants were pooled and stored in a -20°C freezer until further processing. The extract was further evaporated at 4°C in a cooling speed-vac (CentriVap[®] Benchtop Vacuum Concentrators) coupled with a CentriVap Cold Traps (Labconco Corporation USA, 73850 Series) until the volume reached approximately 500 µl. The extracted mixture was then cooled to -80°C and then

freeze-dried overnight (Labconco FreeZone 4.5 Liter Benchtop Freeze dry system). The powdered extract was dissolved in 200 μ l of 1 mM ice-cold ammonium acetate, and centrifuged at 10000 *g* for 10 min at 4°C. The supernatant was then transferred to HPLC vials and sent to NERI (NUS - Environmental Research Institute), National University of Singapore for analysis.

LC-MS

Quantification of c-di-GMP was performed in an EQuan MAX™ LC-MS system, using an Accela 1250 UHPLC pump connected to a Thermo Velos Pro Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Chromatographic separation was achieved using a Nucleodur C18 Pyramid column (2 mm x 50 mm, 3 μ m) (Macherey-Nagel GmbH, Düren, Germany), held at 40°C, with a solvent flow rate of 0.3 ml/min and an injection volume of 10 μ l. The mobile phase A was 10 mM ammonium acetate buffer, containing 0.1% formic acid, and the mobile phase B was acetonitrile, containing 0.1% formic acid. The solvent gradient conditions were as follows: 0% B from 0 min to 10% at 3 min, 90% at 4 min, hold for 5 min, then return to 0% at 5.5 min and equilibrated for 4.5 min, with a total run time of 10 min.

Quantification was performed by positive ion electrospray ionization (ESI+), using selected ion monitoring (SIM) scan type with center mass *m/z* 691.1021 at high-resolution (60,000). For qualitative analysis, MS/MS was performed using collision induced dissociation (CID) with normalized collision energy 20 (of maximum), with an isolation width of 1 Da and activation time of 30 min. The MS conditions were as follows: the source voltage was 3.6 kV, heater and capillary temperature were 300°C, sheath, auxiliary and sweeper gas flows were 40, 15 and 1 (arbitrary units), respectively.

Protein quantification

Protein concentrations were determined using the Bicinchoninic acid assay (BCA) protocol, where 1 ml of planktonic culture or 500 μ l of biofilm extract was pelleted by centrifugation (10000 g for 10 min at 4°C) and washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄). The pellets were then resuspended in 1 ml of lysis buffer (0.1% SDS, 0.5 M triethylammonium hydrogen carbonate buffer, Sigma-Aldrich Co) and homogenized by passing five times through a 20 G needle. The solution was then lysed for 30 min (Power 100%, frequency 37 kHz) in a water bath sonicator filled with ice water. The extracted material was then centrifuged at 1000 g for 10 min at 4°C. A standard curve was generated using 25 μ l of BSA standards (2000 μ g/ml to 0 μ g/ml) to quantify protein concentrations in 25 μ l of samples in triplicate. Briefly, samples were added to 96 well microplates and 200 μ l of freshly prepared bicinchoninic acid and copper sulphate (50:1 ratio) were added and then incubated at 37°C for 30 min. The absorbance was measured at 562 nm in a plate reader.

2.3 Results

2.3.1 Evaluation of different c-di-GMP reporter constructs

To determine which constructs of the *cdrA::gfp* reporter were the most appropriate for real time quantification of c-di-GMP concentrations, each was evaluated using confocal laser scanning microscope. Overnight planktonic cultures were imaged and the mean intensity of the image was calculated as the total intensity of the image divided by total cell count. The reporter carrying the plasmid *pcdrA::gfp* was the one that showed the highest fluorescence, 0.19 ± 0.07 , while the least fluorescence, 0.013 ± 0.006 , was observed for the strain where the construct was

inserted into the genome and that uses an unstable variant of the GFP protein Tn7-MTR2 (Tn7-*cdrA::gfp* (ASV)). The *P. aeruginosa* strain carrying the plasmid based copy of the reporter with the unstable GFP and the strain with the reporter construct with the stable GFP inserted into the genome showed similar, very low levels of fluorescence 0.09 ± 0.04 and 0.07 ± 0.04 , respectively (Figure 2.1). While the *P. aeruginosa* strain carrying the plasmid encoded reporter with the stable GFP gave the highest levels of fluorescence, it is unsuitable for the study dynamic changes in c-di-GMP levels here since the GFP protein half-life is 26 h (Corish and Tyler-Smith 1999). Therefore, the strain carrying the unstable GFP based reporter on a plasmid was selected for further studies.

Figure 2.1: Comparison of different *cdrA* reporter constructs in *P. aeruginosa*. Overnight grown, planktonic cultures of *P. aeruginosa* PAO1 with different versions of the *cdrA::gfp* reporter construct were compared for fluorescence intensity. Reporter activity was determined based on analysis of confocal images, where the mean intensity was calculated as the total image intensity per cell.

2.3.2 Plasmid stability

The *pcdrA::gfp* (ASV) construct was determined above to be best suited for the visual mapping of c-di-GMP across the biofilm. However, antibiotics, necessary for

the selective maintenance of the plasmid could introduce artifacts in interpretation of the data since it has been shown that sub-lethal concentrations of antibiotics can drive biofilm formation or interfere with signaling processes (Hoffman *et al.*, 2005). Therefore, to avoid such confounding influences, it was important to determine the stability of the plasmid in the absence of antibiotic selection. *P. aeruginosa* PAO1 *pcdrA::gfp* (ASV) was grown in a conical flask with and without gentamycin selection pressure and was subcultured every day for 3 d. At the end of the third day, cells were stained with 4', 6-diamidino-2- phenylindole (DAPI) and three random images were captured using confocal laser scanning microscope using the blue and green channels to image total cells and the c-di-GMP reporter simultaneously. The results showed that more than 90% of the cells maintained c-di-GMP associated (green) fluorescence after 72 h and that there was no difference in the percentage of cells expressing GFP for the populations grown in the presence or absence of gentamicin selection (Figure 2.2).

Figure 2.2: C-di-GMP reporter plasmid stability. Percentages of bacteria expressing the *pcdrA::gfp* (ASV) (green fluorescence) after 72 h with and without gentamicin selection. Cells were counterstained with DAPI and Confocal images were captured with green and blue channel. Cells fluorescing in blue without green fluorescence is considered as plasmid lost. Data points are average of 15 images from three independent experiments. Error bars represent the standard deviation (n = 15).

2.3.3 C-di-GMP reporter validation

In order to test the c-di-GMP reporter, and to use it in planktonic studies, where the sensitivity of plate reader is low, the *pcdrA::gfp* (ASV) reporter was transformed into a *P. aeruginosa* *wspF* deletion mutant. The deletion of the *wspF*, methylesterase whose mutation results in high intercellular c-di-GMP. However, the high c-di-GMP also results in aggregate formation making the quantification of c-di-GMP unreliable. Therefore, the polysaccharide genes *pel* and *psl* were also deleted in the same strain resulting in the PAO1 mutant $\Delta wspF \Delta psl \Delta pel$ and the reporter *pcdrA::gfp* (ASV) was subsequently introduced into this strain. Based on quantitative image analysis, it was observed that the mutants, *P. aeruginosa* $\Delta wspF$ and *P. aeruginosa* $\Delta wspF \Delta psl \Delta pel$ produced more fluorescence than the wild type *P. aeruginosa* (Figure 2.3 A - D). The normalized GFP expression, and hence, c-di-GMP concentration, was the highest for the $\Delta wspF$ mutant (7324.7 gray value/cell), followed by the $\Delta wspF \Delta psl \Delta pel$ mutant (4552.4 gray value/cell) and the least fluorescence was observed for the wild type *P. aeruginosa* (264.8 gray value/cell).

2.3.4 Calibration of the reporter strain

To demonstrate that the reporter produces fluorescence in proportion to c-di-GMP concentrations, treatments such as sodium nitroprusside (SNP), that are known to either result in decreased c-di-GMP (Barraud *et al.*, 2009) or to increase c-di-GMP, e.g. Telurite (TeO₂) (Chua *et al.*, 2015) were added to planktonic cultures and the fluorescence responses were measured over time. It was found that the Tecan plate reader was not sensitive enough to detect the fluorescence produced from wild type *P. aeruginosa* carrying the *pcdrA::gfp* (ASV) (data not shown). Therefore, the PAO1 $\Delta wspF \Delta psl \Delta pel$ mutant was used for the SNP addition studies while the wild type

strain carrying the *pcdrA::gf p*(ASV) reporter was used for the c-di-GMP induction experiments using tellurite.

Figure 2.3: Comparison of c-di-GMP reporter based fluorescence of high c-di-GMP producing mutant versus wild type *P. aeruginosa*. Confocal images of (A) wild type *P. aeruginosa*. (B) *P. aeruginosa* Δ *wspF* (C) *P. aeruginosa* Δ *wspF* Δ *psl* Δ *pel* (D) Fluorescence intensity per cell. Error bars represent the standard deviation (n = 3). Magnification: 20x. Scale bar: 10 μ m.

Since the fluorescence intensity can be a factor of the number of cells present at the time of measurement, the growth of the organisms was also measured and fluorescence was normalized to the OD₆₀₀. Exposure of the cells to the nitric oxide

(NO) generating compound-SNP resulted in an increase in growth as measured by an increase in OD₆₀₀ (Figure 2.4). Exposure of bacteria (20 h) to 125 μ M SNP showed the highest growth, OD₆₀₀ 1.071, followed by 250 μ M SNP reaching a maximum OD₆₀₀ of 0.909. *P. aeruginosa* PAO1 $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) with no added SNP only achieved a maximum OD₆₀₀ of 0.801. At the same time, exposure of *P. aeruginosa* *pcdrA::gfp* (ASV) to 20 μ g/ml Telurite (TeO₂) showed a reduced growth for the wild type strain, which achieved approximately 50% (OD₆₀₀ 0.49) of the control *P. aeruginosa* *pcdrA::gfp* (ASV) culture growth within 20 h, while exposure to 50 μ g/ml Telurite completely inhibited the growth of *P. aeruginosa* *pcdrA::gfp* (ASV).

Figure 2.4: Growth of *P. aeruginosa* in the presence of c-di-GMP altering compounds. Optical density (OD₆₀₀) was measured every 10 min in a Tecan plate reader with continuous shaking at 37°C. Each data point is the average of three biological replicates. ●, *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) 125 μ M SNP; ■, *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) 250 μ M SNP; ▲, *P. aeruginosa* *pcdrA::gfp* (ASV); ◆, *P. aeruginosa* *pcdrA::gfp* (ASV) TeO₂ 20 μ g/ml; ▼, *P. aeruginosa* *pcdrA::gfp* (ASV) TeO₂ 50 μ g/ml.

When the GFP production, an indication of the intracellular c-di-GMP concentration, was measured and normalized against growth (Figure 2.5), it was

observed that for the NO exposed cells, the c-di-GMP concentration was reduced in a dose dependent manner. *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) exposed to 125 μ M of SNP showed a relative fluorescent intensity drop of 85% within the first 30 min. There was a 59% decrease in fluorescence for the *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) mutant in the presence of 250 μ M of SNP. At the same time, the wild type *P. aeruginosa* *pcdrA::gfp* (ASV) with 20 μ M Tellurite added showed a slight increase in RFU after 6 h of exposure while no difference was observed at 50 μ M Tellurite. The fluorescence of the wild type PAO1 *pcdrA::gfp* (ASV) control was below the detection limit for Tecan plate reader.

Figure 2.5: The effect of c-di-GMP modulating compounds on expression of the *pcdrA::gfp* (ASV) in *P. aeruginosa*. Bacteria were grown in 24 well plates in a Tecan plate reader with continuous shaking and at 37°C. Optical density (OD₆₀₀), and fluorescence intensity was measured every 10 min and fluorescence intensity is normalised with the optical density and is represented as relative fluorescence units (RFU). ●, *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) 125 μ M SNP; ■, *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ *pcdrA::gfp* (ASV) 250 μ M SNP; ▲, *P. aeruginosa* *pcdrA::gfp* (ASV); ▼, *P. aeruginosa* *pcdrA::gfp* (ASV) TeO₂ 20 μ g/ml; ◆, *P. aeruginosa* *pcdrA::gfp* (ASV) TeO₂ 50 μ g/ml. Representative data from three biological replicates. Error bars represent the standard deviation (n = 3).

2.3.5 *In situ* ratio metric imaging

The planktonic data demonstrate that the *pcdrA::gfp* (ASV) responded as expected to the addition of compounds that are known to either reduce (NO) or increase (telurite) the intracellular concentration of c-di-GMP and was stable in the absence of antibiotic selection. Therefore, this strain was subsequently investigated for use in mapping the spatial distribution of c-di-GMP across the biofilm. To achieve this goal, it was necessary to develop a method that would allow for normalization of the c-di-GMP related GFP expression, with the total cell numbers. Thus, Z-stack images were captured by confocal microscopy and the images were then separated into channels corresponding to either CFP, representing the biomass, or GFP, representing c-di-GMP. Dividing the reporter channel by the biomass channel gives a ratio for each image, and this ratio is presented, as a pseudo-color image represents the effective amount of c-di-GMP per biomass. This causes problems for those areas where there is no biofilm or where there is some background fluorescence, since the division of a small numerical (i.e. the GFP channel) by another small number (background CFP) artificially inflates the normalized data. To address this problem, a mask was created from the biomass channel by adjusting the threshold. The mask was then converted to binary by dividing by 255 (the maximum pixel value of an 8 bit image $2^8-1=255$). The resulting binary mask contains values of either zero or one, where one represents the biomass and zero is the background. Multiplying the mask image with the ratio image gives masked ratio image, masking out the background. The masked ratio image was then segmented into 500 bins from zero to one to create a histogram. This histogram can be used for quantifying images or comparison of different images. In the histogram, the higher ratio (numbers closer to 1) represents a higher c-di-GMP concentration and the lower ratio represents (numbers closer to 0) a lower

concentration. Counts of how many pixels are active with a particular ratio (values ranging from 0 to 1 in 0.002 increment) were designated as the pixel frequency. The pixel frequency is multiplied by the ratio, where the higher ratio represents an increase in concentration of c-di-GMP. The masked ratio image was then converted to a look up table “16 colors” for visualizing the c-di-GMP distribution. The ratio of GFP/CFP of 1 is colored as white and that of 0 is black. In Figure 2.6, the work flow and a representative image is given as an example of masked ratio image using a 16 color LUT. The areas with red color correlate with microcolonies having high c-di-GMP, while the yellow region represents parts of the biofilm with lower concentrations of c-di-GMP. The black areas are areas with very low c-di-GMP or background areas with no biomass.

Figure 2.6: Work flow of masked ratio metric imaging and quantification. The raw images containing CFP for biomass and GFP for c-di-GMP were split to respective channels. Dividing the GFP image by the corresponding CFP image results in a ratio image, representing the c-di-GMP amount per biomass. To address the issue of low background, a binary mask was created from the CFP image. Multiplying the mask image by ratio image yields a masked ratio image. The masked ratio image can then be converted into 16 color LUT for visualization or can be binned to a set scale of 0-1 in 500 bins.

2.3.6 Reporter calibration

The fluorescence response of the *pcdrA::gfp* (ASV) reporter was calibrated against the intracellular concentration of c-di-GMP, as determined by LC-MS/MS based quantification. This allows the arbitrary relative fluorescence units (RFU) to be correlated with cellular concentrations of c-di-GMP and thus, the RFU can be used to estimate c-di-GMP concentration. *P. aeruginosa pcdrA::gfp* (ASV) and *P. aeruginosa ΔwspF pcdrA::gfp* (ASV) were imaged using the ratiometric imaging method described above and subsequently the same cultures were extracted for c-di-GMP quantification using LC-MS. As expected, planktonic cells of *P. aeruginosa pcdrA::gfp*(ASV) (Figure 2.7 A) had the lowest c-di-GMP concentration as measured by LC-MS (0.025 nM/μg of protein) and the lowest average weighted pixel frequency of 157507. The planktonic *P. aeruginosa ΔwspF* mutant had a higher c-di-GMP concentration of 0.36 nM/μg of protein than the *P. aeruginosa pcdrA::gfp* (ASV) biofilm, 0.25 nM/μg of protein. The same trend was also reflected in the pixel frequency, confirming that the ratiometric based imaging correlated with chemical measurements. Planktonic cultures of the *P. aeruginosa ΔwspF pcdrA::gfp* (ASV) had a pixel frequency of 450073 compared to 226652 for biofilms of the *P. aeruginosa pcdrA::gfp* (ASV). The planktonic culture of the *P. aeruginosa ΔwspF pcdrA::gfp* (ASV) (Figure 2.7 C) exhibited pellicle formation and within the pellicles, there were some areas with very high or very low c-di-GMP, demonstrating a non-uniform distribution of c-di-GMP. The *P. aeruginosa ΔwspF pcdrA::gfp* (ASV) mutant biofilm had the highest measured c-di-GMP (0.85 nM/μg of protein) and the highest average weighted pixel frequency (630083). A standard curve was generated, where the average weighted pixel frequency was plotted against the c-di-GMP concentration (Figure 2.7 F). A color scale bar was generated from the standard graph with the highest c-di-GMP

concentration of 75 pM/ μ g of protein represented by the white color and the lowest concentration of protein, 0.38 pM/ μ g, represented by the blue color.

Figure 2.7: C-di-GMP reporter *pcdA::gfp* (ASV) calibration. Ratiometric image of: (A) planktonic *P. aeruginosa pcdA::gfp* (ASV); (B) a biofilm of *P. aeruginosa pcdA::gfp* (ASV); (C) a planktonic culture of *P. aeruginosa Δ wspF pcdA::gfp* (ASV) and (D) a biofilm of *P. aeruginosa Δ wspF pcdA::gfp* (ASV). (E) A comparison of image quantification versus chemical quantification (F) where the c-di-

GMP concentration was plotted versus the average weighted pixel frequency. R-squared value=0.90827, x-intercept 0.07099, y-intercept 0.00527 and slope = -0.70655. Magnification 20x. Scale bar represents 50 μm . The colour scale represents the c-di-GMP concentration normalised to protein concentration in 16 colour LUT, white represents approximately 75 pM/ μg of protein and black represents 0.38 pM/ μg of protein.

2.3.7 The distribution of c-di-GMP in a developing biofilm

Based on the validation studies above, the spatial distribution of c-di-GMP across the different regions of a developing biofilm as well the relative concentration of c-di-GMP was determined for multiple stages of biofilm development. For these experiments, a flow cell biofilm was grown in the presence of M9-Glucose, z-stack images were taken at 24 h intervals and ratiometric imaging was applied for each time point at multiple positions across the biofilm.

Microcolonies were found to have a uniform concentration of c-di-GMP on day 2 (Figure 2.8 A). In contrast, the distribution of c-di-GMP on day 4 appeared to be more localized, revealing areas of high and low c-di-GMP. Mature (diameter > 50 μm) colonies were found to have more c-di-GMP on the boundary of the microcolony than on the interior (Figure 2.8 C). At the same time, small colonies (< 50 μm) had a uniform concentration of c-di-GMP and there was no pattern of spatial differentiation. This may be an indication that mature microcolonies, before dispersal, express higher c-di-GMP at the outside of the microcolonies than on the inside.

The pixel frequency on day 2 was 424,138, increased to 525,174 on day 3 and subsequently dropped by three fold to 162,321 on day 4, with a further decrease to 57,272 on day 5 (Figure 2.8 F). Previous data has shown that *P. aeruginosa* biofilms grown in flow cells fed with M9 minimal medium with glucose undergo dispersal

around days 3-4 (Lee *et al.*, 2013), suggesting that this decrease in c-di-GMP concentration might be due to dispersal on day 4.

Figure 2.8: C-di-GMP distribution across a developing, flow-cell biofilm. (A) Day 2, (B) Day 3, (C) Day 4, (D) Day 5 and (E) Histogram of biofilm development on days 2, 3, 4 and 5. (F) Average weighted pixel intensity as determined by the ratiometric method. The colour scale represents the c-di-GMP concentration normalised to protein concentration, where white represents approximately 75 pM/ μg of protein and black represents 0.38 pM/ μg of protein. Magnification 20X. Scale bar 50 μm .

2.4 Discussion

A methodology for visualizing and quantifying c-di-GMP was developed here to address the distribution of c-di-GMP in relation to the heterogeneity of biofilms. The methodology is based on a fluorescence reporter *pcdrA::gfp*, already proven to be effective in planktonic cultures (Rybtke *et al.*, 2012). However, the application of this reporter for the quantification of c-di-GMP during biofilm development encountered several challenges such as background fluorescence and biomass normalization. A ratiometric imaging methodology was developed here to solve those issues. The technique of ratiometric imaging is commonly used, especially for imaging intracellular levels of Zinc, Calcium and pH (Kikuchi *et al.*, 2004, Taki *et al.*, 2004). The fluorophores used here were CFP and GFP and these were selected based on a number of criteria. The ideal fluorophore for the background biomass indicator should be easy to separate from the GFP signal associated with the c-di-GMP reporter, should have high quantum yield, should show strong fluorescence and must be nontoxic to the cells (Kikuchi *et al.*, 2004). Based on these selection criteria, dsRed and RFP were eliminated, where the spectrum of dsRed is broad and hence cross talks with the GFP channel. Furthermore, RFP was suitable with respect to the quantum yield and fluorescent intensity, but was toxic to *P. aeruginosa*. Even though CFP cross-talks with the GFP channel, the emission of GFP does not cause CFP excitation. Thus, excitation of GFP only yields a GFP signal (output). Since the constitutively expressed CFP signal is much stronger than the GFP reporter signal, cross talk from the CFP channel into the GFP channel can be eliminated by using a narrow pass band filter for the CFP channel.

It is important to understand the potential pitfalls of ratiometric imaging such as chromatic shift and spherical aberrations (North, 2006) before any detailed analysis

can be undertaken since ratio images can produce false results if these factors are ignored. Ratio imaging uses two different color for imaging, with light of two different wavelengths. Chromatic aberration is the property of light to be focused at different points in space based on the wavelength. This can result in failure of the images to be superimposable despite being from the same source. This issue is well recognized in the industry and microscope manufactures generally design corrective lenses compensate for the chromatic shift. While the lenses used in this study (Plan-Apochromat) are claimed to correct the chromatic shift, testing of the objectives by using a 6 μm CFP/GFP fluorescent bead showed that both the CFP and GFP superimpose with near perfection on the XY-axis (See Figure A.1 image C), but the chromatic shift in the Z-axis was significant. This can result in the ratio image giving inaccurate readings for 3-D images (See Figure A.1 image D). Therefore, the chromatic shift was measured and corrected by adjusting the individual slices (x-y images) in ImageJ software (see Appendix B for detailed protocol). The ratio image of the corrected image showed perfect superimposition (See Figure A.1 image E). In this way, all of the ratio images used in the study were corrected for the chromatic shift.

While the chromatic shift can be corrected, other errors could also occur during imaging such as spherical aberration, which is the property of light bending differentially in different parts of a lenses such as at the center or around the edges (North, 2006). This effect depends on the refractive index of the medium, thickness of coverslip and the refractive index of the sample. In part, use of the correct coverslip can partly address the spherical aberration and the lenses are typically adjusted by the manufacturer to the correct aberration. None the less, spherical aberration due to the change in refractive index remains unanswered there is not currently an approach to

correct for this factor. For example, the fluid channels in the microcolony have a different refractive index than that of the biomass, so it is possible to have a spherical aberration when imaging the biofilm microcolony. It may be possible to directly measure these effects using an artificial biofilm made from a fluorescent material, that replicates the microstructure of a biofilm as well as the fluid channels. Other factors that can impact the output or quantification based on ratiometric or other imaging techniques include settings for laser voltage, power, gain, offset etc. Therefore, these parameters were quantified and kept constant for all imaging in this study.

In order to use the reporter system and the ratiometric imaging developed here in a semi-quantitative fashion the method was extended by binning the images into a fixed scale (Figure 2.8). The scale used here was a ratio of 0-1 in 500 bins and this was based on comparison of multiple images from our own system. The approach also involved the implementation of a binary mask, as has been used widely in computational auditory sensing and background noise cancellation (Wang, 2005). The corrected masked ratio image could be used for *in situ*, non-destructive live imaging, suitable for dynamic intracellular signaling molecules such as c-di-GMP. The ratio images for the visualization of c-di-GMP distribution in biofilm were correlated with chemical measurements of c-di-GMP. One of the strengths of the reporter used here is that the fluorescent intensity was calibrated against the amount of intracellular c-di-GMP in four strains that inherently displayed high or low c-di-GMP concentrations in biofilms and planktonic samples. Clearly, the confidence in the calibration could be improved by including additional strains known to have different levels of c-di-GMP or to use more chemical treatments to achieve a broader range of c-di-GMP and fluorescent outputs. Indeed, a fifth strain encoding the phosphodiesterase YhjH, which has a low c-di-GMP content, was available but not used since this strain does

not form sufficient biofilm for testing in the system used here. Nonetheless, the calibration studies presented here were sufficient to address the key question of this chapter, which was to define the spatial and temporal distribution of c-di-GMP during biofilm development

C-di-GMP concentrations were expressed here in pM/ μ g of protein. Even though it is possible to express the concentration per bacterial cell, this would require an approximation of the number of cells per unit volume in the biofilm, which was not possible given the level of resolution (20X magnification) used here. This was partly constrained because the biofilm is highly dense, with many cells overlapping each other. Therefore, based on the level of resolution achievable, the quantification was based on protein content, which allowed for a comparison of planktonic and biofilm cells. It may be possible in the future to relate fluorescence intensity and c-di-GMP concentration at the level of individual cells with improved image resolution. Indeed, with sufficiently increased resolution, it would be of interest in the future to determine if this reporter can be used to show sub-cellular localisation of c-di-GMP within individual cells

The ratio imaging of developing biofilm showed that the distribution of c-di-GMP changes with time and has localized hotspots of high c-di-GMP across the biofilm (Figure 2.8). These areas with relatively high c-di-GMP in comparison to the neighboring areas are first report of this kind. Similar hot spots have been observed in variety of biological situation such as calcium hotspots in neuroblastoma cells, copper hotspots in vineyard soils (Silver *et al.*, 1990) (Jacobson *et al.*, 2007). These hot spots could either represent high c-di-GMP expression in those cells in response to local physiological conditions or they could be the result of mutations in the bacteria in that

region, leading to the constitutive overproduction of c-di-GMP. It has been shown that there is 100 fold increase in the mutation rate in biofilms compared to planktonic cells (Conibear *et al.*, 2009). Such spontaneous mutations could result in elevated c-di-GMP if the mutations are in genes such as *wspF*, that are known to modify the c-di-GMP pool. Indeed, such mutants are commonly isolated from biofilm (Koh *et al.*, 2007, Lee *et al.*, 2015) and this will be discussed in more detail in Chapter 3 and 4.

The ratiometric imaging of a developing biofilm showed a differential distribution of c-di-GMP across different parts of the biofilm (Figure 2.8 A - D). A pattern of high c-di-GMP localized on the outside of the mature colony ($> 50 \mu\text{m}$) with a much lower concentration in the interior of the microcolonies (day 4), may suggest that those colonies are in the process of transitioning to the dispersal phase, which is associated with low c-di-GMP. This would be consistent with the observation that dispersal initiates from the interior of the microcolony (Stewart *et al.*, 2007) or that can be observed as motile cells within the microcolony, called seething dispersal (Hunt *et al.*, 2004). This difference in c-di-GMP is likely to be due to the gradients of electron donors and acceptors that are established within the biofilm, resulting in physiological stratification of the cells embedded within the matrix (Stewart & Franklin, 2008). Thus, under such conditions, the bacteria at the outside of the colony may experience higher nutrient and oxygen compared to the bacteria inside of the microcolonies. Thus, the application of a real time c-di-GMP reporter can provide essential information on physiological status of cells within the biofilm, which enables an improved understanding of the response of those cells to dispersal agents as well as biocides that target different populations of cells.

CHAPTER 3. C-di-GMP DISTRIBUTION ACROSS BIOFILMS DURING INDUCED DISPERSAL

3.1 Introduction

Biofilm microcolonies upon reaching maturation, break open and release cells, allowing the bacteria to escape and seek out new areas for colonization, and this process is called biofilm dispersal (Harmsen *et al.*, 2010). Bacteria typically disperse from the biofilm in response to environmental cues such as starvation, oxygen limitation and some signal molecules such as nitric oxide (Gjermansen *et al.*, 2005, Barraud *et al.*, 2009, McDougald *et al.*, 2012). Biofilm dispersal is an active, energy requiring process and has been shown to be regulated by through several molecular pathways (Morgan *et al.*, 2006) (Purevdorj-Gage *et al.*, 2005, McDougald *et al.*, 2012). Studies of biofilm dispersal are of broad interest, especially where manipulation of the dispersal pathway can be exploited to control biofilms for clinical and industrial applications. For example, nitric oxide (NO) releasing hydrophobic polymers are known to cause less adhesion of bacteria on blood-contacting biomedical devices such as catheters and arteriovenous grafts (Reynolds *et al.*, 2004). The perception of these environmental signals or cues typically translates into changes in the intracellular concentration of the second messenger c-di-GMP, where decreasing concentrations of c-di-GMP results in dispersal. This results in dispersal cells that are sensitive to phagocytosis and traditional antibiotics (Chua *et al.*, 2014)

While it is clear that these cues or pathways can be manipulated for biofilm control, e.g. by exposing biofilms to exogenously added NO donors, exposure to dispersing conditions rarely results in complete biofilm dispersal and this is especially true under natural conditions where biofilms exist as mixed species consortia.

Treatment of biofilms formed on PVC coupons from a drinking water system using the NO donor SNP resulted in 47.3% removal of the biofilm, but did not disperse the entire biofilm (Barraud *et al.*, 2009). This might be due to the fact that biofilm is heterogeneous. For example, even a mono species biofilm growing on a single carbon substrate can develop three different zones depending on oxygen availability such as oxygenic, limited oxygenic and anoxic zones since oxygen diffuses from the medium into biofilm, from the liquid-biofilm interface. A facultative aerobic bacterial biofilm thus grows aerobically in the oxygenic zone and through fermentation in the anoxic zone (Xu *et al.*, 1998, Stewart & Franklin, 2008). Hence, dispersal as a consequence of these environmental cues is a reflection of cellular physiology, it is likely that incomplete dispersal is a reflection of physiological heterogeneity across the biofilm.

Since c-di-GMP is one of the key intracellular signals responsible for the control of dispersal, it is likely that the concentration of c-di-GMP varies across the biofilm. This suggestion, which would directly be a reflection of the cells' perception and response to changing oxygen, carbon source concentration or NO accumulation, could potentially explain why dispersal is also heterogeneous across the biofilm. It is also unclear from where within the biofilm dispersal initiates, although there is evidence that at least for some cues such as starvation dispersal happens in the interior of the microcolonies (Hunt *et al.*, 2004). It is therefore of interest to monitor or quantify the distribution of c-di-GMP in different regions of the biofilm as it develops. As highlighted in the previous chapter, chemical quantification of c-di-GMP in the biofilm only represents an average concentration but does not help to define the heterogeneity of its distribution within the biofilm nor does it allow for dynamic studies of changes in c-di-GMP.

In the previous chapter, a method for real time, *in situ* monitoring and quantification for c-di-GMP was developed. The methodology has been applied here to map the c-di-GMP distribution in a biofilm as it developed and ultimately underwent dispersal to complete the biofilm life-cycle.

3.2 Materials and methods

3.2.1 Bacterial strains and culture medium

Pseudomonas aeruginosa PAO1, chromosomally tagged with a mini-Tn7 encoded cyan fluorescent protein (CFP) and carrying the c-di-GMP reporter plasmid *pcdrA::gfp* (ASV), PAO1 $\Delta wspF$ *pcdrA::gfp* (ASV) and *P. aeruginosa* $\Delta wspF$ Δpel Δpsl *pcdrA::gfp* (ASV) were cultured in M9 minimal medium (48 mM Na₂HPO₄; 22 mM KH₂PO₄; 9 mM NaCl; 19 mM NH₄Cl; 2 mM MgSO₄; 0.1 mM CaCl₂) with 20 mM glucose at room temperature.

3.2.2 Biofilm development in flow cells

Biofilms were cultivated in three-channel flow cells (channel dimensions, 1 x 4 x 40 mm) (Tolker-Nielsen & Sternberg, 2011). The flow cells were fed with M9 minimal medium with 20 mM glucose as the sole carbon source at a flow rate of 9 ml/h. Overnight cultures of *P. aeruginosa* were diluted to OD₆₀₀ of 0.1 in M9 medium, representing approximately 1×10^8 cells/ml and each channel was inoculated with 0.5 ml of this inoculum.

3.2.3 Online effluent monitoring

An online biofilm monitor (custom designed and constructed in the University of Konstanz) was used to measure the biofilm dispersal as a decrease in turbidity (OD₅₈₀

nm) during biofilm starvation (Schleheck *et al.*, 2009). The outlets of the flow cells were connected to glass tubes (inner-diameter 2.8 mm, 6.0 cm in length) using silicon tubing. The optical density was measured and recorded using a data logger (Labjack U12, Meilhaus, Germany) and recorded continuously using DAQfactory-express software (Azeotech, USA).

3.2.4 Starvation treatment

Starvation of planktonic cultures was achieved by transferring overnight grown bacterial cultures into sterile 50 ml Falcon tubes and centrifuged at 10000 g for 5 min at room temperature. Supernatants were discarded and bacteria were resuspended in M9 medium without a carbon source. The bacterial suspensions were then transferred to a new, sterile conical flask and incubated at room temperature with shaking (180 rpm). To initiate starvation in flow cell biofilms, a separate media bottle was connected to the pump containing M9 medium without a carbon source to initiate starvation.

3.2.5 Morphology analysis by colony plating

Effluents from the flow cells were collected under sterile conditions, serially diluted with media (M9-glucose for normal growth, M9-without a carbon source during starvation) and plated onto LB agar plates. The plates are incubated for 3 d at room temperature and colonies were enumerated by identifying colony morphology. A compound microscope (Zeiss Primo Star Compound Microscope) with 4x magnification was used to identify colony variants.

3.2.6 C-di-GMP measurement in planktonic cultures

A TECAN infinite 200 PRO plate reader coupled with i-controlTM software was used to measure c-di-GMP in planktonic cells based on *pcdrA::gfp* fluorescence (Excitation 485 nm, Emission 520 nm, excitation band width 9 nm and emission band width 20 nm; manual gain of 80) and growth was monitored by quantifying the OD₆₀₀ in 10 min intervals. The relative fluorescence, GFP/OD₆₀₀ was calculated and was used as a measure of the c-di-GMP concentration per cell.

3.2.7 Microscopy ratio metric imaging

Biofilm images were acquired using a Carl Zeiss Confocal Laser Scanning Microscope CLSM 780 (Carl Zeiss, Germany) using a 20x objective (LD plan-Neofluar 20x / 0.4 Korr M27). Two separate optical channels were used to image the biofilm biomass and the c-di-GMP reporter. The constitutively expressed, chromosomally integrated CFP was used to determine the biomass and GFP was used for the c-di-GMP reporter. Laser voltage was measured using a SanwaTM CD800a digital multimeter and kept at 0.587 V for all experiments. Pinhole diameters of 50 μ m and 58 μ m were used for CFP and GFP respectively. An emission bandwidth of 440-503 nm was used for CFP and 497-598 nm was used for GFP. The master gain was set to 700 and a digital gain of 1.00 was used for all of the images. Images were processed using ImageJ version 1.47 (Abràmoff *et al.*, 2004).

3.2.8 Chemical quantification of c-di-GMP

C-di-GMP extraction and quantification was performed as described (Chapter 2). Biomass was washed with 2 ml of ice cold 1 mM ammonium acetate and resuspended in an ice cold solution containing acetonitrile (40%), methanol (40%) and water

(20%), and homogenized by passing the suspension five times through a 20 G needle. The cell suspension was then lysed for 30 min (Power 100%, frequency 37 kHz) in a water bath sonicator (Elmasonic P, Elma, Singen, Germany) filled with ice water. The cell debris was then removed by centrifugation at 10000 g for 10 min at 4°C and the supernatants were stored on ice. The pellets were resuspended in 2 ml of ice-cold acetonitrile/methanol/water mixture and process were repeated once more. The supernatants were then pooled and stored in a -20°C freezer. The extracts were further evaporated at 4°C in a cooling speed-vac (CentriVap® Benchtop Vacuum Concentrators) coupled with a CentriVap Cold Trap (Labconco Corporation USA - 73850 Series) until the volume was reduced to approximately 500 µl. The extracted mixture was then cooled to -80°C and then freeze-dried. The powdered extract was dissolved in 200 µl of 1 mM ice-cold ammonium acetate, and then centrifuged at 10000 g for 10 min at 4°C. The supernatant was then transferred to HPLC vials and sent to Metabolites biology laboratory, National University of Singapore, Singapore-117543 for analysis. Quantification of c-di-GMP was performed in an EQUAN MAX™ LC-MS system, using an Accela 1250 UHPLC pump connected to a Thermo Velos Pro Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA).

3.2.9 RNA extraction and transcriptomic analysis

Biomass from the flow cell was extracted using 2 ml of RNA protect cell reagent (Qiagen, USA) by flushing the flow cell 10 times using two 5 ml syringes attached at each end of the flow cell. Biomass from planktonic cultures was spun down (1000 g for 10 min at 4°C) and stabilized using 2 ml of RNA protect before extraction. The manufacture's protocol was used to extract the RNA (RNeasy® Mini Kit; Qiagen, USA) from both biofilm biomass and planktonic cells and subsequent DNase

treatment was carried out using TURBO-DNA-free kit (Ambion, USA) according to the manufacturer's instructions. Isolated RNA was cleaned and concentrated (RNA Clean & Concentrator™-5; Zymo research corporation, USA) before determining its purity using a Qubit® 2.0 Fluorometer (Life Technologies, USA). TapeStation (Agilent 2200 TapeStation System; Santa Clara, CA 95051, United States) was used to verify integrity of the RNA sample and only samples with RNA Integrity Number (RIN) above 9 were sent for library preparation and sequencing. A Truseq RNA sample preparation kit (TruSeq RNA Library Preparation Kit v2; Illumina, Inc. San Diego, USA) was used for library preparation and sequenced on the HiSeq 2500 platform (Illumina, USA) .

3.2.10 Computational analysis-transcriptomics

Sequences were mapped to the *P. aeruginosa* PAO1 reference genome (NCBI Reference Sequence: NC_002516.2) using TopHat. The cufflinks program was used to generate a transcriptome assembly for each experimental condition from the TopHat results. These assemblies were then merged together using Cuffmerge. The merged assemblies were imported into Cuffdiff to calculate differential expression levels and to test the statistical significance of observed changes (Trapnell *et al.*, 2012).

3.2.11 Variant analysis

Planktonic cultures of isolated colony variants were grown overnight and DNA from each sample was extracted using QIAamp DNA Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocol. The quantity of each DNA sample was measured using Qubit DNA kit and the quality was assessed using the TapeStation. Sequencing libraries were prepared using the TruSeq DNA Sample Preparation Kit

(Illumina, USA) and sequenced on the MiSeq (Illumina, USA) platform. The paired-end reads were trimmed to remove adapters and mapped to the reference genome sequence of *P. aeruginosa* (NCBI Reference Sequence: NC_002516.2) using CLC genomic workbench 6 (CLC bio, Denmark).

3.2.12 Pathway analysis

In order to visualize how the gene networks are altered in expression in response to starvation, a pathway analysis has been carried out using the KEGG search & color pathway tool. (http://www.genome.jp/kegg/tool/map_pathway2.html). The list of genes (PA number) with their fold change was uploaded to the KEGG search & color pathway tool in a tabular format, which then retrieves information on the suite of genes that are affected in different pathways.

3.2.13 Quantitative PCR

cDNA was synthesized from respective RNA samples using SuperScript[®] III First-Strand Synthesis System (Invitrogen, Life Technologies Corporation; USA). cDNA concentrations were measured using Nanodrop (Thermo Fisher Scientific Inc; place) and concentrations were normalized using distilled water before proceeding to qPCR. KAPA SYBR[®] FAST qPCR Kits (Kapa Biosystems, Massachusetts) was used for master mix preparation and qPCR was performed using the StepOne[™] System (Applied Biosystems, Thermo Fisher Scientific; USA). NCBI Primer blast was used for the design of primers (Ye *et al.*, 2012). The designed primer pairs were (Table C.1) further tested in IDT Oligo Analyzer (Integrated DNA Technologies) for secondary structures using the Hairpin function.

3.2.14 Statistical analysis

Analysis of variance (ANOVA) was performed with GraphPad Prism software (GraphPad Software, San Diego, CA). P value <0.005 was considered statistically significant

3.3 Results

3.3.1 C-di-GMP levels decrease during starvation of planktonic bacteria

In order to determine if starvation conditions affect c-di-GMP levels in *P. aeruginosa*, overnight grown planktonic bacteria, with the c-di-GMP reporter, were washed and resuspended in medium with or without a carbon source, aliquoted to 24 well plates and the optical density (OD₆₀₀) and GFP expression were measured (Figure 3.1).

Figure 3.1: Measurement of c-di-GMP levels during starvation of planktonic cultures. Bacteria were washed and resuspended in starvation or complete media and resuspended in same volume of respective medium. GFP fluorescence and OD₆₀₀ were monitored at 37°C with intermittent shaking using (continued on next page)

a Tecan plate reader. Measurements were collected every 30 min. Each point represents the average of biological triplicate cultures. Error bars have been removed for clarity. ●, non starved *P. aeruginosa*; ■, starved *P. aeruginosa*; ▲, non starved $\Delta wspF$; ◆, non starved $\Delta wspF\Delta psl\Delta pel$ ▼, starved $\Delta wspF$; ○ starved $\Delta wspF\Delta psl\Delta pel$.

Starvation of the cells resulted in a rapid decrease in c-di-GMP levels, as shown by the reduction in relative fluorescence (Figure 3.1). This reduction in GFP production was most pronounced for the c-di-GMP overproducing strain, *P. aeruginosa* $\Delta wspF\Delta psl\Delta pel$ with a maximal fluorescence intensity difference of 43.5% after 45 min of starvation. The wild type *P. aeruginosa*, showed only a 33.2% reduction in fluorescence intensity after the same duration of starvation and this is likely due to the inherently lower c-di-GMP levels in the wild type cells prior to starvation. To verify the readings from the plate reader, five random CLSM images were acquired and quantified from starved and non-starved planktonic cultures. Quantitative image analysis (Figure 3.2) indicated that starvation for 24 h resulted in a 62.5% reduction in the fluorescent signal for the *P. aeruginosa* wild type and 56.2% for the *P. aeruginosa* $\Delta wspF$ strain.

Figure 3.2: Quantification of c-di-GMP levels during starvation of planktonic cultures using CLSM imaging. Images were acquired using a 63x objective, normalized with corresponding images acquired using photomultiplier. The average grey value of the reporter was normalized with the average grey value of the photomultiplier tube (PMT) channel. A total of 5 images were captured from two independent experiments. Error bars represent standard deviations (n=5).

3.3.2 C-di-GMP distribution during biofilm starvation

The *P. aeruginosa* PAO1 mini-Tn7 CFP *pcdA::gfp* (ASV) biofilm was grown in a flow cell for 4 d, at which time starvation was induced by changing the medium to M9 salts without glucose. An individual microcolony was continuously imaged for 4 h at 15 min intervals to monitor the change in fluorescence signal (Figure 3.3). Ratiometric imaging was applied to the images to visualize the c-di-GMP distribution during starvation.

Figure 3.3: C-di-GMP distribution during biofilm starvation. Time-lapse images of mature *P. aeruginosa* biofilms under flow conditions as visualized by CLSM. Images were recorded in real-time over 240 min. *P. aeruginosa* was tagged with the mini-Tn7 carrying the cyan fluorescent protein (CFP) and c-di-GMP concentration was visualized using the *pcdrA::gfp* (ASV) reporter plasmid. (A) Orthogonal view of the mature biofilm, (B) raw *pcdrA::gfp* (ASV) expression before starvation (Rainbow LUT), (C) raw *pcdrA::gfp* expression after 15 min of starvation, (D) raw *pcdrA::gfp* (ASV) expression after 120 min of starvation, (E) Masked ratiometric image before starvation (16 colours LUT), (F) masked ratiometric image after 15 min of starvation, (G) masked ratiometric image after 60 min starvation and (H) masked ratio metric image after 120 min of starvation. FIRE-LUT colour scale at the top image represents the grey values 0-225, where the blue color represents 0 and red represents 225. The 16 colour LUT in the bottom image represents the c-di-GMP concentration, white is equivalent to approximately 75 pM c-di-GMP / μ g of protein and black is equivalent to approximately 0.38 pM c-di-GMP / μ g of protein.

Raw images (Figure 3.3 B, C and D) were converted using a Rainbow Look Up Table (LUT) format for easy interpretation. The blue color represents a near zero grey value, and green to red represent grey values of 50-255. Shortly after the start of starvation, the total fluorescence intensity increased, indicating an increase in c-di-GMP levels. The average fluorescent intensity of the raw reporter channel before the start of starvation was 16.69 and this increased to 30.93 within 15 min of the start of starvation, a 1.8 fold increase. There was no further increase and indeed, the intensity dropped to 24.28 after 3 h of continuous starvation. The ratiometric images obtained

from the raw images (Figure 3.3 E, F, G and H) also showed a similar pattern with regard to fluorescence intensity levels. Immediately after the start of starvation treatment, the c-di-GMP concentration was found to increase. In addition, there was no decrease in biofilm biovolume during the 3 h experiment (data not shown). This was in contrast to the expected results, where it has previously been shown that starvation rapidly induced dispersal (Huynh *et al.*, 2012).

Figure 3.4: C-di-GMP quantification of *P. aeruginosa* during biofilm starvation using ratiometric imaging. Time lapse imaging was performed in 15 min time intervals at the same location for biofilms formed in a flowcell. Representative data from one of the three biological replicates is presented here.

When the total fluorescence intensity of the ratiometric images or average c-di-GMP concentration per biomass were calculated from the ratiometric images (Figure 3.4), the c-di-GMP concentration was found to increase immediately after the induction of starvation and was then observed to decrease over time. The pixel frequency increased from 329151 to 408498 within the first 15 min of starvation and subsequently decreased to 106659, a 67.5% reduction within 3 h.

3.3.3 C-di-GMP levels show a transient increase during starvation

To verify the observation above, chemical measurements were obtained with increased temporal resolution. The results showed that c-di-GMP increased from 0.025 nM c-di-GMP/ μ g of protein to 0.05 nM c-di-GMP/ μ g of protein within 90 min of the start of starvation (Figure 3.5). After 90 min, the concentration of c-di-GMP started to decrease and within 3 h, was below the initial concentration prior to starvation, of 0.025 nM c-di-GMP/ μ g protein.

Figure 3.5: Chemical quantification of c-di-GMP from planktonic bacteria during starvation. Starvation was induced in an overnight grown *P. aeruginosa* planktonic culture. Samples were withdrawn in triplicate and c-di-GMP was extracted using chemical methods. Biomass was normalized to protein concentration. Error bars represent the standard deviation (n=3). Independent experiments were repeated three times.

3.3.4 Transcriptomic analysis suggests that genes involved in c-di-GMP metabolism increase in expression during starvation

Since it was evident from the results above (Figure 3.5) that c-di-GMP increased transiently during starvation, it was of interest to determine whether genes related to c-di-GMP regulation were altered in their expression. RNA samples were isolated from planktonic cultures as well as from biofilms of wild type *P. aeruginosa* and compared against the same samples after 2 h of starvation, when the c-di-GMP concentration was at its peak. Planktonic bacteria showed a total of 902 genes to be differentially expressed during starvation whereas biofilms showed significant changes in the expression of only 78 genes during starvation. In the planktonic culture (Table 3.1), 51 signal transduction related genes were up regulated and only two were downregulated in response to starvation. None of the genes coding for known c-di-GMP phosphodiesterases or cyclases showed significant changes during starvation of either planktonic bacteria or biofilms. However, PA4781, a gene coding for a putative protein containing both phosphodiesterase (EAL) and cyclase (GGDEFF) domains was induced during starvation. Interestingly, *vfr*, a global virulence response regulator that has been previously shown to be down regulated when the cellular c-di-GMP levels are high, was also shown to be repressed during starvation here (Almblad *et al.*, 2015). This further provides support for the observations here that c-di-GMP was increased in the first 2 h of starvation for planktonic cells, the time at which the transcriptomic analysis was performed.

Table 3.1: Differentially expressed signal transduction genes during the starvation of planktonic cultures.

Pathway	Induced during starvation	Repressed during starvation
Signal transduction	PA0175, <i>aer2</i> , <i>pprB</i> , PA4781, PA0177, PA0178, <i>bfiS</i> , <i>dctQ</i> , PA4036, <i>ercS</i> , <i>pprA</i> , PA1930, PA0179, PA5072, PA4520, PA2133, PA2920, PA2072, PA5442, <i>siaA</i> , PA2867, PA4982, PA1336, <i>kdpD</i> , PA3462, <i>cttP</i> , PA2571, <i>ladS</i> , PA0575, PA1951, <i>ercS</i> , PA4112, PA2573, PA4633, <i>muxA</i> , <i>muxB</i> , <i>muxC</i> , PA1181, <i>bdlA</i> , PA2177, <i>retS</i> , PA2652, PA4108, <i>pctA</i> , <i>pctB</i> , <i>algR</i> , PA2654, PA4915, PA1458, PA3349	<i>dnaA</i> , <i>yfr</i>

3.3.5 Transcriptomic analysis suggests that 3 days old biofilms are similar to carbon starved planktonic cells

To interpret the molecular mechanisms of the starvation response, the global change in gene expression during and after starvation was examined using a global transcriptomics approach. A total of 902 genes were significantly changed during planktonic starvation (Table 3.2), out of which 564 (57%) genes were upregulated. By comparison, only 78 genes were differentially expressed in starved compared to non-starved biofilms and 64% of genes were induced in response to starvation. When the transcriptomes of planktonic and biofilm cells were compared, 507 genes were differently expressed, where 235 of those genes were common to planktonic starvation (Figure 3.6). A detailed list of common genes were shown in Appendix C table C.5. This suggests that biofilms at the time of sampling, day 3, might already be experiencing carbon source limitation. Based on this, subsequent data analysis focused on the starvation response of planktonic cells.

Table 3.2: Differentially expressed genes during starvation

Conditions	Total number of genes significantly altered in expression [§]	Up regulated*	Down regulated*
Planktonic starvation	902	564	320
Biofilm starvation	78	30	43
Biofilm vs. planktonic (unstarved)	507	276	211

[§] False discovery rate of 0.005.

*Log 2 fold change of genes before starvation down regulated vs. after starvation.

Figure 3.6: Venn diagram of differentially expressed genes during transcriptomics. Biofilm starvation, planktonic starvation and a comparison of the biofilm vs. planktonic cells in the presence of a carbon source were represented.

During starvation of planktonic cells, genes involved in adaptation and protection (*typA*, *oprH*, *ahpC*, PA3529, PA3865.1, PA3866, PA2411, *tpx*, *lasI*, *nfuA*, *pvdQ*, *pvdL*, *pvdG*, *pvdJ*, *pvdN*, *pvdO*, *pvdP*, *pvdA*, PA3450 and *sodM*) were down regulated. Many of these genes are quorum-sensing related genes (*lasI*, *pqsA*, *pqsB* and *pqsC*) that are normally expressed in growing populations. The genes responsible for secondary metabolite and siderophore synthesis (PA5161, *rmlA*, *rmlC*, *rmlD*, *pchE*, *pchF*, *pchG*, *pchB* and *pchD*) were also found to be downregulated. Motility and attachment genes (*cupB3*, *cupB4*, *cupA2*, *cupA3*, *cupA4*, *cupA5*, PA3352, *pslH*, *pslI*, *cdrB*, *flgJ*, PA5114, *fimU*, *pilV*, *pilW*, *pilX*, *flgM*, *pslE*, *pslF*, *pslG* and *fimV*) were mostly up regulated, although some of these genes were down regulated (*opgH*, *oprQ*, *wbpH*, *wbpG* and *tonB1*). Genes coding for toxins and secretion systems were also repressed in starved planktonic cells (*ambC*, *ambD*, *aprE*, *lasB*, *cbpD*, *pchC*, *aprA*, *pvdF*, *hsiC2*, *pvdD*, *piv*, *pasP* and *rhIA*). The fold change of genes and specific functions are given in Appendix C Table C.4 . There were a total of 38 starvation specific genes (common to planktonic starvation and biofilm starvation) identified here. Most of these genes were involved in adaptation and protection (*aphC*, *pvdQ*, *pvdL*, *pvdJ*, *pvdA*, PA3450, PA2411, *sodM* etc). Aminoacid metabolism was also found to be down regulated during starvation. A detailed list of common genes is presented in Appendix C, Table C.5.

Table 3.3: List of genes that were differentially expressed during starvation of planktonic cells

Functions/Pat hways	Positive regulation*	Negative regulation*
Amino acid metabolism	PA0164, <i>katA</i> , PA3504, <i>fahA</i> , <i>maiA</i> , <i>gcvP1</i> , PA0743, <i>hmgA</i> , <i>aaaA</i> , PA2084, PA4344, PA3372, PA3374, PA3375, <i>sdaA</i> , <i>gcvT2</i> , PA1217, <i>ldh</i> , PA0704, <i>alr</i> , <i>phhB</i> , <i>phhC</i> , <i>selA</i> , <i>phhA</i> , <i>spuA</i> , PA1997, <i>pauA7</i> , <i>aruB</i> , <i>aruD</i> , PA4163, <i>aruE</i> , <i>ansB</i> , <i>arcA</i> , <i>putA</i> , <i>arcB</i> , <i>soxG</i>	PA3459, <i>gabT</i> , <i>pauC</i> , <i>metG</i> , <i>glnA</i> , <i>ilvE</i> , <i>serA</i> , PA1750, <i>pheA</i> , <i>serC</i> , <i>hisC2</i> , <i>lysC</i> , <i>argB</i> , <i>leuA</i> , PA4774, <i>asd</i> , PA4773, <i>aspA</i> , <i>sahH</i> , <i>ilvI</i> , <i>ilvC</i> , PA2393, <i>metK</i> , <i>pvdH</i> ,
Carbohydrate metabolism	<i>hdhA</i> , <i>exaC</i> , <i>lpdV</i> , PA3415, PA3416, PA3417, PA2265, <i>fdnG</i> , PA3568, <i>fruI</i> , <i>fruK</i> , PA2264, PA2263, <i>glcD</i> , <i>glcE</i> , PA3758, <i>fruA</i> , PA3373, <i>acsB</i> , <i>acnA</i> , <i>aceA</i> ,	<i>gltA</i> , <i>acnB</i> , <i>ppsA</i> , <i>icd</i> , <i>glmM</i> , <i>algC</i> , <i>sdhA</i> , <i>aceF</i> , PA3001, <i>sucB</i> , <i>murA</i> , <i>prs</i> , <i>wbpI</i> , <i>prpD</i> , <i>wbpB</i> , <i>sdhB</i> , <i>pgl</i> , <i>zwf</i> , <i>mqaA</i> , PA3181, <i>sucC</i> , <i>sucD</i> , <i>sucA</i> , <i>edd</i> , <i>idh</i> , <i>aceE</i> , <i>gapA</i> , <i>fumC1</i>
Fatty acid metabolism	PA3924	
Energy Metabolism	PA0113, <i>napA</i> , <i>napD</i> , <i>napF</i> , PA0112, <i>napC</i> , PA0107, <i>coIII</i> , <i>coxA</i> , <i>coxB</i> , PA1400, <i>nosD</i> , <i>nosF</i> , <i>nosZ</i> , <i>snr1</i> , <i>nuoB</i> , <i>nuoE</i> , <i>nuoF</i> , PA2482, <i>cioA</i> , <i>nuoG</i> , <i>nuoH</i> , <i>napB</i>	<i>etfA</i> , <i>etfB</i> , <i>azu</i> , <i>cysP</i> , <i>atpD</i> , <i>atpE</i> , <i>atpF</i> , <i>atpH</i> , <i>sbp</i> , <i>atpA</i> , <i>atpG</i> , <i>cyoA</i> , <i>cyoB</i> , <i>iscU</i>
Lipid metabolism	<i>lipA</i> , PA2553, <i>eutB</i> , PA4025, PA0744, <i>liuE</i> , PA2557, <i>estA</i> , <i>fabF2</i> , <i>gcdH</i> , <i>pcs</i> , <i>atuF</i> , PA0746	<i>faoA</i> , <i>accC</i> , <i>fabH2</i> , <i>foaB</i> , <i>glpD</i>
Metabolism of cofactors and vitamins	<i>hpd</i> , <i>pdxB</i> , <i>pntAA</i> , <i>pntAB</i> , <i>pntB</i> , PA0502, PA0503, <i>bioF</i> , <i>panE</i> , PA1280, <i>cobC</i> , <i>cobD</i> , <i>cobP</i> , <i>cobQ</i> , <i>cobU</i> , PA4880, <i>hemH</i> ,	<i>pdxA</i> , <i>pncB1</i> , PA5539, <i>pchA</i> , <i>iscS</i>
Metabolism of terpenoids and polyketides	PA0746, <i>atuF</i>	<i>pchE</i> , <i>pchF</i> , <i>pchG</i> , <i>pchB</i> , <i>pchD</i>
Nucleotide metabolism	PA3516, PA3517, PA1931, PA1932, PA1933, <i>cyaA</i> , PA0387, <i>arcC</i> , <i>eddB</i>	<i>purA</i> , <i>pyrQ</i> , <i>guaA</i> , <i>cmk</i> , <i>nrdJb</i> , <i>nrdJa</i> , <i>cysN</i> , <i>pnp</i> , <i>adk</i> , <i>dnaN</i> , <i>rpoB</i> , <i>rpoA</i> , <i>rpoC</i> , <i>nrdA</i> , <i>cysD</i> , <i>nrdB</i> (continue on next page)

Adaptation, Protection	-	<i>typA, oprH, ahpC, PA3529, PA3865.1, PA3866, PA2411, tpx, lasI, nfuA, pvdQ, pvdL, pvdG, pvdJ, pvdN, pvdO, pvdP, pvdA, PA3450, sodM</i>
Biosynthesis of cofactors	<i>pqqF, hpd, pdxB, pntAA, pntAB, pntB, PA0502, PA0503, bioF, panE, PA1280, cobC, cobD, cobP, cobQ, cobU, PA4880, hemH</i>	<i>fpr, hemO, pdxA, pncB1, PA5539, pchA, iscS</i>
Biosynthesis secondary metabolites	-	<i>PA5161, rmlA, rmlC, rmlD, pchE, pchF, pchG, pchB,</i>
Cell wall / LPS / capsule	<i>lecA, pslA, pslB, pslC, waaA</i>	<i>lpxC</i>
Chaperones		
Folding, sorting and degradation	<i>dsbG, dipZ</i>	<i>secA, secG, dnaK, rne, rho, secB, secY, groEL</i>
Membrane transport	<i>PA3709, PA5096, PA5097, PA5094, PA5095, PA4913, PA0860, nosY, PA1876, PA4500, PA4909, PA4910, PA4911, modC, PA4143, pcrD, PA2912, PA2913, PA2914, PA2371, PA1386, PA5153, PA1339, PA1340, PA1341, PA1342, PA4505, PA4506, PA3211, PA3212, PA2367, puuR, fhaI, braC, PA2361, PA2362, PA0324, PA0325, PA0326, rbsB, PA3858, PA5266, PA4496</i>	<i>tolB, opr86, ffpvB, PA4514, PA1302, aprD, oprL, oprF, hasR, pvdR, pvdT, PA5505, oprE, oprC, PA4065, PA5530, PA4222, PA4223, PA3190, PA5030, tolR, phuR, PA3931, pvdE, PA4156, hasAp, hcpI, PA2204, PA4837, rhlB, ffpvA</i>

Membrane proteins	<i>tadA, tadB, tadZ, PA1190, tadG, PA3079, PA0109, PA2984, PA2262, PA4821, PA5544, PA3521, PA3522, PA3523, cupE3, pelD, pelE, pelF, mexI, opmD, PA0043, PA2369, PA2858, PA0349, PA0451, PA0452, tgpA, PA0007, PA1108, PA1258, PA0809, PA2477, PA2478, PA3340, PA0334, PA1735, PA3920, potA, PA2938, arcD, PA3893, PA3894, PA0811, kup, PA0397, PA2439, pra, PA3311, PA4011, mexF, oprN, PA0476, PA3526, PA3465, PA5113, PA3690, PA5265, PA1041, triB, PA1497</i>	<i>ostA, tolA, pyoS5, icmP, cmpX, PA4833, PA4933, PA4467, fptA</i>
Motility & Attachment	<i>cupB3, cupB4, cupA2, cupA3, cupA4, cupA5, PA3352, pslH, pslI, cdrB, flgJ, PA5114, fimU, pilV, pilW, pilX, flgM, pslE, pslF, pslG, fimV</i>	<i>opgH, oprQ, wbpH, wbpG, tonB1</i>
Non-coding RNA	<i>phrS, P30, PA3760, crcZ, rgsA</i>	<i>ssrS, prrF1, prrF2</i>
Protein secretion	<i>PA1875, PA1877,</i>	<i>tssC1</i>
Putative enzymes	<i>PA3957, PA0745, PA2597, PA1860, PA3723, PA1617, PA5048, pqqH, PA3534, PA3667, PA3759, PA2918, PA1880, PA3972, PA1327, PA4986, PA3429, PA3430, PA3535, PA4978, PA0799, PA1914, PA3860, PA0366, PA1046, PA1991, PA2024, PA3961, PA5181, PA5546,</i>	<i>PA2069, PA2735, PA3330, PA4615, phuS, PA5150, PA0730, PA2402</i>
Secreted Factors (toxins, enzymes, alginate)	<i>PA4078</i>	<i>ambC, ambD, aprE, lasB, cbpD, pchC, aprA, pvdF, hsiC2, pvdD, piv, pasP, rhlA</i>

Signal transduction	PA0175, <i>aer2</i> , <i>pprB</i> , PA4781, <i>dnaA</i> , <i>vfr</i> PA0177, PA0178, <i>bfiS</i> , <i>dctQ</i> , PA4036, <i>ercS</i> , <i>pprA</i> , PA1930, PA0179, PA5072, PA4520, PA2133, PA2920, PA2072, PA5442, <i>siaA</i> , PA2867, PA4982, PA1336, <i>kdpD</i> , PA3462, <i>cttP</i> , PA2571, <i>ladS</i> , PA0575, PA1951, <i>ercS</i> , PA4112, PA2573, PA4633, <i>muxA</i> , <i>muxB</i> , <i>muxC</i> , PA1181, <i>bdlA</i> , PA2177, <i>retS</i> , PA2652, PA4108, <i>pctA</i> , <i>pctB</i> , <i>algR</i> , PA2654, PA4915, PA1458, PA3349
Transcription	<i>gbdR</i> , PA3420, PA2121, <i>liuR</i> , <i>rpoD</i> , <i>rhlR</i> , PA0456, PA0547, <i>glcC</i> , PA3995, PA3038, PA1759, <i>pmpR</i> , <i>sigX</i> , <i>exsA</i> , <i>nusA</i> , <i>trmD</i> , <i>bkdR</i> , PA3921, <i>pcrR</i> , PA0159, <i>rimM</i> , <i>pvdS</i> , PA3599 <i>acoR</i> , <i>cspD</i> , PA3973, PA1760, PA4659, <i>cgrC</i> , <i>cca</i> , <i>mifR</i> , <i>mifS</i> , PA2572, PA3225, PA3341, <i>orn</i> , <i>rnc</i> , PA2897
Translation	PA5470, <i>selB</i> , <i>sprP</i> , <i>clpA</i> , <i>ftsH</i> , <i>surA</i> , <i>prc</i> , <i>trxA</i> , <i>infC</i> , PA0459, <i>era</i> , PA2618, <i>fusA2</i> <i>thrS</i> , <i>ppiB</i> , <i>rpmA</i> , <i>rpmL</i> , <i>rplU</i> , <i>rpsH</i> , <i>lysS</i> , <i>infB</i> , <i>aspS</i> , <i>rpsA</i> , <i>rplT</i> , <i>rplF</i> , <i>efp</i> , <i>rpsI</i> , <i>rplC</i> , <i>rpsJ</i> , <i>rplM</i> , <i>rplJ</i> , <i>rnpA</i> , <i>rpsO</i> , <i>rpsL</i> , <i>tufB</i> , <i>rpsD</i> , <i>rpsB</i> , <i>rmf</i> , <i>rplA</i> , <i>rplK</i> , <i>rpsR</i> , <i>rplB</i> , <i>rpsG</i> , <i>rplD</i> , <i>rplW</i> , PA4671, <i>fusA1</i> , <i>rplL</i> , <i>tsf</i> , <i>rplO</i> , <i>rpsE</i> , <i>rplQ</i> , <i>rplE</i> , <i>rpsK</i> , <i>tufA</i> , <i>rpsC</i> , <i>rplX</i> , <i>rpsF</i> , <i>rplP</i> , <i>rpmC</i> , <i>rplN</i> , PA3600, PA3601
Phage, transposon, or plasmid	PA2633
Drug resistance	PA4144, PA5471 <i>mexB</i> , <i>tolQ</i>
DNA replication, repair	PA5348, <i>polB</i> , <i>pslN</i> , <i>phr</i> , <i>nth</i> , <i>gyrB</i> , <i>recF</i> , <i>uvrB</i> , <i>gyrA</i> , <i>hupB</i> , PA4010, <i>recB</i> , <i>recC</i> , <i>recD</i> , <i>priA</i> , PA3272

Xenobiotics metabolism	PA1253, <i>pcaB</i> , <i>fdhA</i> , PA2475			PA3331		
Antibiotic resistance	<i>triA</i> , <i>triC</i> , <i>clpS</i>			-		
Hypothetical, unclassified, unknown	PA3923, PA0787, PA1761, PA3080, PA0174, PA0859, PA3089, PA1416, PA1888, PA4362, PA0333, PA3073, PA1874, PA5101, PA5208, PA3668, PA2874, PA3456, PA2448, PA1762, PA0344, PA2366, PA1218, PA1221, PA3123, PA4326, PA4658, PA0052, PA0309, PA1545, PA1414, PA5507, PA1463, PA3493, PA2937, PA1679, PA0484, PA0586, PA4510, PA3796, PA5180,	PA0741, PA0543, PA0826, PA0585, PA1975, PA5543, PA1488, PA2779, PA4925, PA4702, PA3071, PA3074, PA4682, PA2368, PA0348, PA2365, PA2875, PA2778, PA3986, PA5412, PA0345, PA2363, PA1219, PA0902, PA4926, PA3613, PA4877, PA0959, PA0812, PA1784, PA4027, PA2075, PA3091, PA3494, PA2481, PA4955, PA4611, PA0587, PA4511, PA4703, PA2684,	PA5145, PA3922, PA3638, PA4780, PA0049, PA5545, PA1887, PA1208, PA0891, PA3072, PA3075, PA0788, PA2370, PA5047, PA2872, PA3688, PA2002, PA1259, PA0383, PA5271, PA1216, PA1220, PA1274, PA4927, PA5290, PA3354, PA0391, PA2544, PA1943, PA1728, PA2632, PA3492, PA4368, PA5307, PA0855, PA5527, PA4509, PA2504, PA2360, PA2927,	PA5061, <i>opgG</i> , PA1324, PA0563, PA0805, PA2427, PA1766, PA2377, PA0938, PA5339, PA2034, PA1560, PA4090, PA4469, PA0050, PA0284,	PA0315, PA1939, PA1830, PA3332, PA5540, PA1134, PA0974, PA2412, PA4515, PA0431, PA0781, PA4063, PA4570,	PA2462, PA4578, PA2581, PA0572, PA4638, PA3729, PA4141, PA4390, PA2384, PA2033, PA1559, PA4220, PA4471,
	(continued on next page)					

PA5019,	PA2542,	PA2543,
PA2562,	PA3848,	PA2174,
PA0388,	PA2727,	PA3833,
PA4607,	PA4608,	PA4311,
PA4312,	PA4313,	PA3945,
PA5359,	PA4017,	PA0769,
PA4879,	PA4523,	PA3250,
PA4656,	PA2372,	PA1789,
PA2433,	PA4735,	PA3919,
PA0168,	PA0522,	PA0614,
PA1558, <i>pslO</i> ,	PA2829,	PA2898,
PA4298,	PA4577,	PA4605,
PA4826,	PA5341	

*Log 2 fold change of before starvation vs. after starvation

3.3.6 qPCR validation

The results from the transcriptomic-based analysis were validated by qPCR. A Total of 10 genes, including two housekeeping genes, were selected for validation. The aldehyde dehydrogenase gene *hdhA*, showed a 5.05 fold increase in expression during starvation by qPCR, while the transcriptomic data indicated a 6.9 fold increase. The exopolysaccharide related gene *pelD* showed a 7.3 fold increase compared to transcriptomic data, which indicated a 3.08 fold change. The pyoverdine biosynthetic gene *pvdA* was down regulated -4.81 vs -12.23 as determined by qRT-PCR and transcriptomic analyses respectively. A similar trend was observed for the fumarate hydratase gene, *fumC1*, which was down regulated by -5.91 fold (transcriptomic analysis) compared to -1.25 (qRT-PCR). The global virulence regulator, *vfr* was down regulated -2.77 and -8.6 as shown by transcriptomic and qRT-PCR analysis, respectively. The 7.2 fold repression of *rhlA* was also confirmed by qRT-PCR, -3.2 fold (Figure 3.7). The house keeping genes *rpoS* and *proC* did not showed any significant change in accordance with the transcriptomic data.

Figure 3.7: qRT-PCR validation of gene expression for glucose starved *P. aeruginosa* cells in the planktonic phase. Each data point represents the average of two independent starvation experiments, where the qRT-PCR was performed in triplicate for each experimental replicate. Error bars represent the standard deviation (n=6).

3.3.7 Pathway analysis

A more detailed pathway analysis was carried out based on KEGG pathway analysis, with a specific focus on metabolic pathways (Figure 3.8) and it is evident that even though most of the Krebs's cycle genes were down regulated, one, *aceA*, was up regulated creating a shunt between isocitrate and succinate via isocitrate lyase. Similarly many other shunt pathways were induced, including the glyoxylate shunt which is involved in the conversion of glyoxylate to acetyl-CoA without the krebb's cycle intermediate steps.

Figure 3.8: KEGG pathway analysis of genes differentially expressed during planktonic starvation. The red colour represents genes that were down regulated, the purple coloured lines represent genes that were induced. The green coloured lines represent genes and pathways that have been identified in *P. aeruginosa* PAO1 experimentally or where homologues are present in the genome. The black colour represents reference pathways with no experimental evidence for their presence in *P. aeruginosa*.

3.3.8 Biofilm starvation is associated with controlled dispersal

The results from planktonic cells and biofilms, based on transcriptomic and the c-di-GMP reporter indicate that during starvation, intracellular c-di-GMP levels first

increase by 2.1 fold and then decrease by 2.3 fold. Despite the decrease in c-di-GMP, there was no observable dispersal for the duration of the time-lapse imaging, approximately 3 h. As this observation was not in line with previous studies (Huynh *et al.*, 2012), this response was investigated further. To more closely monitor biofilm dispersal during starvation the effluent tube of the flow cell was connected to an online biofilm monitor, which continuously detects and records the turbidity of the effluent (Huynh *et al.*, 2012). In this experiment, at the end of the 4th day of biofilm growth, the dispersed cells in the effluent reached an OD of 0.1. At this time point starvation was induced by supplying medium without carbon source to the flow cell. Immediately after the start of the starvation, the biofilm underwent a massive dispersal event (Figure 3.9). Within 1 h, the dispersal reached a maximum effluent optical density of 0.16 and then started to decrease. Within 2 h, the amount of dispersal achieved a steady rate of 0.12, based on optical density, and this level of dispersal continued for almost 24 h. Around 24 h after the start of starvation, the rate of dispersal was no different from a non-starved biofilm, OD 0.1, and thus may represent erosion dispersal. Thus, the results indicate that dispersal due to starvation was not only immediate but also continued for up to 24 h.

Figure 3.9: Quantification of cells in the biofilm effluent using online biofilm monitor. Biofilm dispersal was quantified as the change in optical density of the effluent. ○, non-starved; ▲, starved. Time zero is the start of starvation. Each data point is the average optical density of the effluent from three flowcells. The error bars were removed for clarity. Data was recorded automatically every 5 min using a data logger and the DAQ factory express software.

3.3.9 Incomplete biofilm dispersal is associated with dispersal resistant colony variants

Since the results above show that biofilm dispersal in response to starvation was slow and continuous for the first 24 h (4 d old biofilm), the biofilm was monitored for 3 d further under continuous starvation. Even though amount of biomass decreased substantially, not all of the biofilm dispersed after 72 h, the duration of the experiment (Figure 3.10) and some of the colonies remained attached in the flow cell (Figure 3.11). Based on the fluorescence response from the *pcdrA::gfp* (ASV) reporter, the biofilm colonies remaining after dispersal were suggested to have very high intracellular c-di-GMP levels.

Figure 3.10: Characterization of *P. aeruginosa* biofilms during starvation. The biofilm was stained for viability with the BacLight™ Live-Dead® reagents, and the biovolume was calculated from 3 independent experiment from CLSM images using the Imaris software package. Error bars represent standard deviations (n=15).

Figure 3.11: Orthogonal view of *P. aeruginosa* microcolonies before and after starvation. A) biofilm before starvation B) colonies after 72 h of starvation. Biofilms were stained with Syto9 and imaged by CLSM. Orthogonal view showing top panel x-z plane, right y-z and middle corresponding x-y plane. Magnification 20x. Scalebar: 50 μm.

Biofilm effluent was serially diluted and plated onto LB agar plates at 24 h intervals and viable cells were enumerated. On day 4, just before the start of starvation, the effluent was found to have high numbers of wild type *P. aeruginosa* (2.8×10^6 cfu/ml) and a small number (4×10^5 cfu/ml) of small colony variants

(SCVs). After 1 h of starvation, the effluent contained 1.65×10^5 cfu/ml wild type and 1.13×10^5 cfu/ml SCV. A similar trend was observed after 24 h, 48 h and 72 h of starvation, where the effluent always contained a higher number of wild type *P. aeruginosa* and relatively fewer SCVs. In contrast, on day 7 (3 d of starvation) when the flow cell was finally disconnected and flushed with medium to extract biomass, the biofilm biomass was found to contain rugose small colony variants (RSCVs). At this time point the numbers of bacteria in the extracted biomass included 8×10^4 cfu/ml RSCVs, 1.6×10^5 cfu/ml SCVs and 3.16×10^6 cfu/ml wild type bacteria (Figure 3.12).

Figure 3.12: Colony variants of *P. aeruginosa* during biofilm starvation. Effluent from biofilms during starvation was serially diluted and plated onto LB agar for enumeration. On the 7th day, the biomass was harvested from the flow cells, serially diluted and plated onto LB agar to quantify morphotypic variants.

Five colonies of each type (WT, wild type phenotype; SCV, small colony variant; RSCV, rugose small colony variant) of colony variant (Figure 3.13) were isolated and glycerol stocks were prepared for future use.

Figure 3.13: Morphotypic variants isolated from starved biofilms. Representative images of colonies isolated from biofilms after 3 d of starvation. A-E, colonies classified as having the wild type phenotype, PWT1 to 5; F-J, SCV phenotypic variants SCV1 to 5 and K-O, RSCV phenotypic variants RCV1 to 5. Scale bar: 1 mm.

3.3.10 Characterization of morphotypic variants isolated from starved biofilms

To further characterize the morphotypic variants isolated from starved biofilms, the isolates were tested for c-di-GMP production, biofilm formation and dispersal in response to starvation. All colonies with a wild type phenotype had *pcdrA::gfp* expression levels (559 ± 11 , 553 ± 80 , 525 ± 25 RFU), that were similar to the parental strain (555 ± 42 RFU), indicating they have similar concentrations of c-di-GMP (Figure 3.14). SCVs were found to have slightly higher c-di-GMP levels (734 ± 37 , 682 ± 28 , 567 ± 58 RFU) compared to wild type. All of the PRSCVs (Starvation in sensitive rugose small colony variant) had very high c-di-GMP levels compared to wild type, although PRSCV2 (1289 ± 134 RFU) had only half of the c-di-GMP levels of PRSCV1 (2367 ± 152 RFU) and PRSCV3 (1871 ± 324 RFU). The PRSCVs were

also found to form pellicles at the air-liquid interface of planktonic cultures (data not shown), a phenotype characteristic of the high c-di-GMP mutant *wspF*.

Figure 3.14: C-di-GMP levels of colonies that fail to disperse upon starvation measured using the *cdrA::gfp* reporter. Individual isolates were grown in M9 glucose medium overnight and 2 ml of culture was transferred to a 24 well plate in triplicate. GFP fluorescence and OD were measured using a Tecan plate reader. RFUs were calculated as GFP/OD₆₀₀. Each data point is the average of three technical replicates. Error bars represent standard deviations (n=3). Asterisks indicate that the values are significantly different between strains compared (one-way ANOVA, **** <P 0.0001).

To understand the biofilm specific characteristics of these variants, representatives from the 15 starvation resistant isolates were grown in flow cells for 4 d fed with M9-glucose, then starved for 3 d and the biomass was quantified by image analysis. From the image analysis (Figure 3.15) it was found that the wild type isolates responded to starvation in a fashion similar to wild type cells and dispersed when starved. The wild type biofilm had a total biovolume of $5.66 \pm 1.54 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ on day 4, before the start of starvation. Within 24 h of starvation, the biomass reduced to $2.44 \pm 6.6 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$, a 56.8 % reduction, indicating that dispersal had occurred. Similarly, the wild type isolate PWT1 also showed a reduction in biomass from $4.6 \pm 2.46 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ to $1.57 \pm 4.96 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ (65.8% reduction in biomass within 24 h). In contrast, the biovolume of the small colony isolate PSCV1 was $3.77 \pm 6.97 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ before the start of starvation, and did not change for 72 h after the onset of starvation, $3.65 \pm 1.74 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ and $3.65 \pm 5.38 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$, respectively. The biovolume of the PSCV2 isolate showed a small decrease from $7.29 \pm 3.32 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ before the start of starvation to $6.02 \pm 1.54 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ at 24 h, $5.02 \pm 6.84 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$, 48 h, $4.59 \pm 1.75 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ after 72 h of starvation (17%, 16% and 8% reduction in biovolume, respectively). In contrast, the PRSCV1 biomass did not show any significant reduction during the 3 d of starvation.

3.3.11 Genetic analysis of starvation resistant colony variant

The data above suggest that the RSCVs differed from SCVs and the wild type isolates with respect to their starvation dispersal response and because the isolates were stable upon serial passage. Therefore, individual isolates were genome sequenced to determine genetic changes that might explain why the RSCVs did not disperse upon glucose starvation. Sequence analysis revealed that the wild type isolates harbored one or more mutations (

Table 3.4). For example, the PWT1 had a deletion in *filH* and a substitution in *mexT*. The *mexT* mutation has been shown to elevate activity of the multi-drug efflux

system (MexE–MexF–OprN multidrug efflux system) resulting in increased resistance to a range of antibiotics. MexT also regulates type III secretion, pyocyanin production and early surface attachment, and hence the starvation response is likely to impact these phenotypes as well (Tian *et al.*, 2009). PWT2 has an insertion mutation in PA4321, which is a hypothetical, unclassified gene. PWT3, and PWT5 had several mutations in *pilT*, known to be important for twitching motility. PWT4 had a mutation in *fha1*, codes for a component of the type VI secretion system, mutation of which causes alteration in regulation of the Hcp (hemolysin co-regulated protein) secretion system. The system exports three proteins, Tse1-3. The Tse2 protein was found to be the toxin component of a toxin- immunity system and to arrest the growth of prokaryotic and eukaryotic cells. It provided a major growth advantage for *P. aeruginosa* in evading host immune response (Hood *et al.*, 2010, Mougous *et al.*, 2007). The small colony variants had mutations in *wspF*, coding for a protein of the chemosensory signal-transduction system. In addition to the *wspF* mutation, PSCV1 and PSCV3 had a mutation in *fha1*. PSCV3-5 had mutations in on *flhA*, an important gene in the flagellar biosynthesis pathway. Both PRSCV1 and PRSCV5 had a single point mutation in the *wspF* gene. Mutation in *wspF* has been shown to cause changes in the expression of 560 genes in *P. aeruginosa*, and is known to increase the biofilm forming capacity and is associated with the wrinkly colony morphotype. These RSCVs had morphologies that were consistent with the previously described *wspF* mutant. The Wsp chemosensory system regulates biofilm formation in *P. aeruginosa* through modulation of intracellular c-di-GMP levels. The WspF protein is a methylesterase involved in adaptation to chemotactic stimuli. The chemotaxis signal transduction system also includes the response regulator CheY. In *P. aeruginosa*, the response regulator of the Wsp chemosensory system, WspR, is a CheY homolog that

contains the conserved GGDEF domain and hence, is capable of synthesizing c-di-GMP. Loss of *wspF* results in increased cellular c-di-GMP, possibly by stimulating WspR catalyzed c-di-GMP synthesis (Hickman et al., 2005). PRSCV2 had a mutation in *aguR* in addition to *wspF*. PRSCV3 had multiple mutations in *wspF* and a mutation in the hypothetical protein PA4321. Similarly PRSCV4 had mutation in *wspF* and the hypothetical protein PA4543.

Figure 3.15: Biofilm starvation dispersal response of isolated collected after 7 d of biofilm starvation. Representative morphotypic isolates were grown in flow cells and starvation was induced at day 4. Biomass biovolumes were calculated from 3 independent experiments by CLSM images using IMARIS software. Error bars represent standard deviations (n=15).

Table 3.4: Identification of genetic differences between the *P. aeruginosa* wild type and non-dispersing isolates.

Strain	Location of mutation	Types of mutation	Deleted sequence	Inserted sequence	Gene
PWT1	1195638..1195642	Deletion	GGTCA		<i>fliH</i>
	2807698..2807701	Substitution	CAGC		<i>mexT</i>
PWT2	4850499^4850500	Insertion			PA4321
PWT3	437529..437543	Deletion	CAAG GGCCT GGTCG C		<i>pilT</i>
	437539..437541	Substitution	GTC		<i>pilT</i>
PWT4	98901^98902	Insertion	-	TGGC TG	<i>fhaI</i>
PWT5	437539..437541	Substitution	GTC		<i>pilT</i>
PSCV1	98901^98902	Insertion	-		<i>fhaI</i>
	4145465	SNV*	C		<i>wspF</i>
PSCV2	331470	SNV	G	T	<i>aguR</i>
	4145465	SNV	C	T	<i>wspF</i>
PSCV3	98901^98902	Insertion	-	TGGC TGTG GCTG	<i>fhaI</i>
PSCV4	1580512	SNV	C		<i>flhA</i>
(Continued on next page)					
	1587908	Substitution	G		<i>flhA</i>

PSCV5	98901^98902	Insertion	-		<i>FhaI</i>
PRSCV1	4145465	SNV	C		<i>wspF</i>
PRSCV2	331470	SNV	G	T	<i>aguR</i>
	4145465	SNV	C	T	<i>wspF</i>
PRSCV3	4145465	SNV	C	T	<i>wspF</i>
	4145465	SNV	C	T	<i>wspF</i>
	4850499^4850500	Insertion	-		PA4321
PRSCV4	4145465	SNV	C	T	<i>wspF</i>
	5090567	SNV	G	A	PA4543
PRSCV5	4145465	SNV	C	T	<i>wspF</i>

*SNV, Single nucleotide variants

3.3.12 Starvation resistant morphotypes evolve over multiple starvation cycles

Since the biofilm after starvation was rich in mutants, those variants were tested for their response to carbon starvation in a continuous starvation-replenishment-starvation experiment. The results showed that there was significant increase in biomass during starvation and re-growth cycle (Figure 3.16). During the first 4 d of biofilm growth, the biofilms achieved a biovolume of approximately $4 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$. Even though there was some dispersal during the subsequent 3 d of starvation, the biofilm retained $1.8 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ at the end of the 7th d of starvation. On the 7th day, the flow cells were switched back to medium containing glucose and allowed to grow

for a further 3 d. Interestingly, the biofilm achieved an even higher biovolume of $6 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ at the end of the 10th day. The same flow cells were re-starved for 3 d and were found to retain $4 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$, which was approximately equal to the non-starved biofilm on day 4. Subsequent replenishment with glucose containing medium resulted in an increase in biomass while further restarvation resulted in dispersal leaving a final biomass of $2.1 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$.

Figure 3.16: The effect of repeated cycles of biofilm formation and starvation on total biofilm formation. Representative data from one of two experiments. Days 4, 11, 14 and 18 are after biofilm growth with M9 glucose. Days 7, 14, 18 after three days of subsequent starvation. Biovolumes were calculated from three independent experiments by CLSM images using Imaris software. Error bars represent standard deviations (n=15). Asterisks indicate that the values are significantly different between strains compared (one-way ANOVA, **** <P 0.0001; ** <P 0.005, ns Not significant).

The c-di-GMP distribution of starvation insensitive colonies left in the flow cell after each starvation cycle was visualized using ratiometric imaging and quantified

(Figure 3.17). The average pixel frequency were increased from 28675 to 165965 at the end of second starvation cycle, followed by a reduction to 90069 after the 3rd starvation cycle (Figure 3.17 D). The c-di-GMP distribution map also showed an increase in localised regions of high c-di-GMP after every starvation cycle. It was also observed that the of micrcolonies with high c-di-GMP concentrations were smaller but greater in number, than observed for the first and second starvation periods. Interestingly, the reduction in c-di-GMP, as determined by a reduction in pixel frequency correlated with a reduction of total biomass at the end of the third starvation cycle (Figure 3.16).

Figure 3.17: C-di-GMP distribution map of starvation insensitive colonies after cycles of starvation. Representative images of A) day 7, B) day 14 and C) day 21. D) Average pixel frequency of starvation insensitive colonies after each starvation cycle. n=3. The scale bar represents 50 μ m.

3.4 Discussion

3.4.1 C-di-GMP displayed a transient increase during induction of dispersal

It has been shown in many studies that the intracellular levels of c-di-GMP play a key role in regulating the switch between bacterial life styles, either as free floating planktonic bacteria or as sessile biofilms. Elevated levels of c-di-GMP is commonly associated with biofilm growth and reductions in this second messenger can result in the dispersal of biofilms (Chua *et al.*, 2014). Dispersal cues responsible for altering the intracellular concentrations of c-di-GMP include oxygen limitation, certain chemical cues such as nitric oxide and nutrient starvation (Stewart & Franklin, 2008, Barraud *et al.*, 2009, Huynh *et al.*, 2012). While starvation is typically associated with reductions in c-di-GMP, data presented here, based on the temporal monitoring of c-di-GMP using the *cdrA* reporter, showed that c-di-GMP increased immediately upon starvation, followed by a reduction in the intracellular concentration of c-di-GMP (Figure 3.4). To rule out that the increase in fluorescence was an artifact during imaging, or an inherent property of GFP, the results were verified through several control experiments. Time lapse images taken of an unstarved microcolony showed that there was no change of GFP when the biofilm was is fed with M9 medium containing glucose (Appendix A, Figure A.2). An increase in the ratio of CFP and GFP can be due to a reduction in the CFP signal or increase in the GFP signal. In order to verify that the increase in ratio observed here was not due to a decrease in CFP signal, fluorescence intensity of the raw images were calculated and no decrease in CFP was observed over 3 h, the duration of time-lapse imaging performed in this experiment (Appendix A, Figure A.2)with a stable GFP expressing plasmid-under the control of a constitutive promoter indicated that the GFP signal did not change due to

starvation treatment. In addition, chemical measurements of c-di-GMP were performed under the same conditions which indicated that the c-di-GMP was increased in response to starvation (Figure 3.5). Hence, we can conclude that the increase in ratio CFP-GFP is indicative of an actual increase in c-di-GMP. The transient increase in the *wspF* mutant was not observed in Figure 3.1, even though the experiment was performed in 10 min intervals, which is most likely due to the low sensitivity of the Tecan plate reader. In contrast, the data presented Figure 3.2, were generated from confocal imaging, which is more sensitive than the plate reader. Further work could include more frequent sampling points for both the chemical quantification as well as the fluorescence imaging to fully analyse the temporal differences in c-di-GMP of *wspF* mutants. C-di-GMP has been shown to exert both transcriptional and translational control of *cdrA* gene expression (Cooley *et al.* 2015). A promoter fusion only reports transcriptional changes and such regulation occurs much more slowly than post-translational regulation. Thus, the response as observed from the GFP reporter may either under estimate the degree of c-di-GMP activity or may represent a delayed response compared to the actual pool of c-di-GMP.

Transcriptomic data also showed that c-di-GMP concentration is higher after 2 h of the start of starvation compared to non starved cells. Almblad *et al.* (2015) showed that there was an inverse relationship between intracellular levels of c-di-GMP, and virulence regulator *vfr*. The overproduction of c-di-GMP caused a decrease in transcription of *vfr* (Almblad *et al.*, 2015). The transcription of *vfr* was downregulated 2.8 fold during planktonic starvation, and this was verified by qRT-PCR, which also showed down regulation of 8.6 fold, further supporting the transient increase in c-di-GMP upon initiation of starvation.

While the data presented here clearly show an increase in c-di-GMP upon induction of dispersal, this contradicts previously published data showing that c-di-GMP concentrations decrease during active dispersal (Cotter & Stibitz, 2007, Waters *et al.*, 2008, Barraud *et al.*, 2009, Schleheck *et al.*, 2009). However, Roy & Sauer (2014), showed that the sensory protein BdlA forms a multi protein complex with the phosphodiesterase DipA and a membrane bound diguanylate cyclase, NicD, resulting in the phosphorylation of NicD, during planktonic growth, where NicD is inactive. NicD, upon sensing an environmental cue, is dephosphorylated and increases its c-di-GMP cyclase activity, contributing to activation of BdlA via phosphorylation and temporarily elevated c-di-GMP levels. BdlA, in turn, activates DipA, with the increased phosphodiesterase activity resulting in the reduction of c-di-GMP levels (Roy *et al.*, 2012). This provides a potential explanation for the transient increase in c-di-GMP in response to starvation. Additionally, since the outer edges of the microcolonies contain cells in direct contact with media, the starvation will be first sensed by the bacteria at the periphery of the microcolony giving rise to a high c-di-GMP at the boundary of microcolony.

3.4.2 Bacteria prepare for famine in response to starvation

The results presented here showed that 64% of the genes that were significantly altered in expression during planktonic starvation were in fact upregulated. Of the many down regulated genes, many were associated with energy expensive, secreted products such as toxins, pyoverdine etc. When the genes and their associated pathways were examined in detail, it was clear that metabolic bypass reactions were induced rather than shutting down the entire pathway (Figure 3.8). For example, all of the genes in the citric acid cycle were repressed during starvation, except for those

involved in the glyoxylate shunt. The glyoxylate pathway is a bypass reaction in the citric acid cycle to convert acetyl-CoA into succinate for the synthesis of carbohydrates (Berg *et al.*, 2002). This allows the organism to utilize simple carbon compounds such as acetate in the absence of glucose (Ensign, 2006). The glyoxylate pathway additionally plays an important role in gluconeogenesis (Berg *et al.*, 2002). Previous studies on bacterial starvation have shown that post transcriptional modifications are more common than transcriptional changes in gene expression in response to starvation. For example, the putative sigma factor KatF of *E. coli* showed only a 2 fold increase in transcription during nutrient starvation whereas the protein fusion containing the longest fragment of KatF was induced eight fold under the same conditions (McCann *et al.*, 1993). Using 2-D gel electrophoresis, several starvation related proteins were identified, including the increased synthesis of 55 proteins during carbon starvation, 35 during phosphorus starvation and 47 during nitrogen starvation (Matin, 1991). These numbers are likely to also underestimate the total post-transcriptional changes since only the more obvious spots were taken into account in manual analysis of 2-D gel electrophoresis. Approximately 100 proteins were differentially regulated during starvation of flowcell grown *P. aeruginosa* PAO1 biofilms identified using a peptide-fingerprint mass-spectrometry (iTRAQ) analysis (Huynh *et al.*, 2012). In addition Cooley *et al.*, (2015) showed that the CdrA protein was post-translationally controlled via c-di-GMP, in addition to the already established transcriptional control. Thus, changes in mRNA may underestimate the global response of cells during carbon starvation.

It has been shown that bacteria under nutrient limitation are highly tolerant to certain antibiotics. This tolerance may not simply be due to bacteria entering an inactive state, but rather is likely to be the result of a specific regulatory response mediated by

the stringent response, which is controlled by (p)ppGPP, which controls a variety of genes and pathways associated with increased tolerance (Nguyen *et al.*, 2011). Hunt *et al.* (2004), using a combination of experimental and computer modeling approaches, showed that biofilm detachment in response to the stop of flow, was not due to the accumulation of waste products but instead was due to nutrient starvation (Hunt *et al.*, 2004). Similarly, glucose starvation induced dispersal has shown to be energy dependent and is mediated by cAMP (Huynh *et al.*, 2012). This suggests that bacteria during starvation do not simply shut off metabolism and activity but rather, actively prepare for starvation conditions.

There were only 78 genes differentially expressed during biofilm starvation, compared to 902 genes in planktonic starvation. Out of the 78 differentially expressed genes during biofilm starvation, 41 (52%) were common between planktonic and biofilm starvation. Likewise, 46% of planktonic starvation altered genes were common to the differences between biofilm and planktonic growth. This suggests that some cells within the biofilm might already be experiencing starvation, so that the gene expression in biofilm is mostly similar to that of starved planktonic cells. Nutrient and oxygen gradients have been demonstrated for biofilms supporting this hypothesis (De Beer *et al.*, 1994, Khl *et al.*, 2007). So it likely that the localized starvation in the biofilm might cause cells to express stress resistance genes even when the entire biofilm is not under such stress. Therefore, when the biofilm experiences an actual stress condition, it may already be genetically prepared to respond to that stress. This might be a key mechanisms by which biofilm are more tolerant to stressors than planktonic cells.

3.4.3 Dispersal in response to starvation is sustained over time

Looking more in depth at the dispersal response over time revealed that even though the c-di-GMP concentrations increased initially in response to starvation for up to 90 min, dispersal started immediately and is supported by data presented by Huynh *et al.* (2012). Dispersal, as measured by an increase in the optical density of the biofilm effluent, reached its peak within the first 30 min of starvation. The rate of dispersal subsequently decreased, but was maintained over a period of 2 h. It is possible that the physiological heterogeneity of the biofilm cells results in some cells being prepared for immediate dispersal and others, already starved for nutrients that are less able to respond to complete starvation and hence are less likely to disperse. This heterogeneity is clearly reflected in the differences in c-di-GMP distributions across different parts of the biofilm.

3.4.4 Starvation insensitive bacteria are mutants expressing high concentrations of c-di-GMP

The presence of morphological variants in the biofilm has been reported many times (Boles *et al.*, 2004, Koh *et al.*, 2007), where the appearance of morphotypic variants such as small colony variants (SCVs) and the rugose small colony variants (RSCVs) have been speculated to be a primary factor in the persistence of *P. aeruginosa* during chronic infection of cystic fibrosis patients (Starkey *et al.*, 2009). When the biofilms were starved for 3 d, not all of the biofilm is dispersed and indeed some microcolonies remain. Surprisingly, some of the colonies have high c-di-GMP as shown by the *pcdrA::gfp* monitor strain. Isolation of these variants revealed three distinct morphotypes and sequence analysis revealed that all of the isolates, which were effectively resistant to starvation induced dispersal, were mutants, including

those isolates with the wild type morphology. Despite those mutations, the wild type isolates behaved phenotypically as the parent strain in response to starvation and their intracellular c-di-GMP concentrations. The wild type looking, starvation-insensitive isolate PWT1 had mutations in the *filH* and *mexT* genes. *filH* encodes a protein important for flagellar biosynthesis (Minamino & Macnab, 2000) and a mutation may cause the *P. aeruginosa* to be defective in motility. This might be a reason why the bacteria did not disperse from the flow cell even after an extended period of starvation. Interestingly, some isolates contained a mutation in *mexT*, a LysR-family regulator, which modulates multi-drug efflux systems, resulting in increased resistance to diverse antibiotics (Tian *et al.*, 2009). Some of the SCVs had mutations in *wspF*, a methylesterase whose mutation results in elevated levels of c-di-GMP as well as mutations in flagella associated genes. As for the wild type isolates, none of the SCVs showed elevated levels of c-di-GMP (Figure 3.14). In contrast, all of the RSCVs contained *wspF* mutations and showed elevated levels of c-di-GMP although the degree of elevated c-di-GMP differed between RSCV isolates (Table 3.4), which might be due to secondary mutations (e.g. *aguR*, PA4321, PA4543) that may antagonize c-di-GMP production via phosphodiesterase activity.

The ratiometric imaging and quantification method developed here for quantification of the intracellular messenger c-di-GMP has helped to improve our understanding of several key phenomena associated with biofilm development. The expression of c-di-GMP is clearly heterogenous across the biofilm and changes in response to exposure to dispersal cues. Unexpectedly, one change in response to starvation was the transient increase in c-di-GMP, which was unexpected based on previous studies. While c-di-GMP associated fluorescence ultimately decreased during starvation, in accordance with previous data, some parts of the biofilm, such as

the edges of the microcolonies retained relatively high c-di-GMP levels. These data therefore suggest that global quantification of c-di-GMP is not sufficient to understand its role in biofilm development and dispersal, and that mapping of c-di-GMP across the biofilm can help to explain heterogeneity in the biofilm and incomplete dispersal responses. Future work aimed at understanding why the c-di-GMP is unevenly distributed will be of particular interest in better defining biofilm development, dispersal and differential responses to signalling cues as well as antibiotics.

CHAPTER 4. C-di-GMP DISTRIBUTION DURING MIXED SPECIES BIOFILM DEVELOPMENT

4.1 Introduction

In nature, biofilms are typically a diverse combination of species and may include not only bacteria but also eukaryotes. These multi-species biofilms involve community interactions such as cooperation, competition, mutualism, ammensilism and altruism. Many of these interactions are synergistic in nature where the combined community response is greater than the sum of the individual species contribution. For example, a co-culture of *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus* showed a four fold increase in biomass compared with that of single-species biofilms and two–five fold increase when compared with that of three-species biofilms (Ren *et al.*, 2015). Whereas elimination of *X. retroflexus* from the above consortium resulted in significantly reduced biomass to less biomass. Interestingly >97% of the cell number in the mixed species is *X. retroflexus*, suggesting that despite their relatively low abundance, the other three community members exert a strong influence in the enhancement of community biofilm formation (Ren *et al.*, 2015). These results highlight the importance of studying mixed species communities. As a consequence of this understanding, many researchers have now developed defined model mixed species communities to investigate inter-species interactions. For example, a model biofilm system formed by *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis* and *Acinetobacter lwoffii* was found to produce 167% increase in biomass compared to the amount of biofilm formed by the individual species (Burmølle *et al.*, 2006). One mechanism that allows for the increased biofilm biomass

of the community is likely to involve metabolic cooperation to enhance resource utilization. For example, a recent study on microbial fuel cells (MFC) has shown a mutualistic relationship between *Shewanella oneidensis* and *Escherichia coli*, where *S. oneidensis* is unable to metabolize glucose, while *E. coli* ferments glucose to produce metabolites including formate, which is then utilized by *S. oneidensis* as an electron donor in the MFC (Wang *et al.*, 2015). Co-metabolic processes and the sharing of other resources is typically facilitated by organisms developing close spatial organization, something that is facilitated within the structured environment of a biofilm (Vieubl Gonod *et al.*, 2006). Alternatively, cooperation can be achieved by the formation of microniches within the biofilm, where for example, aerobic respiration consumes the available oxygen, allowing microaerophiles or anaerobes to be active in the deeper layers of the biofilm (Kim & Jaff, 2008). Using multiplex fluorescence *in situ* hybridization (FISH) technique, six oral Gram-negative and Gram-positive bacteria were visualized in an *in vitro* study. The study showed the spatial localization of similar groups of organisms giving rise to a heterogeneous population and species-specific coaggregation (Thurnheer *et al.*, 2004). For example, the oral bacterium *Treponema denticola* coaggregated only with *Porphyromonas gingivalis*, whereas *Fusobacterium nucleatum* colocalized with all other species of the community (Kolenbrander, 2000). Instead of being driven by the sharing of metabolites, these interactions are believed to be mediated by inter-species communication. One such example can be found in dual species biofilm formed by *Actinomyces naeslundii* and *Streptococcus oralis* (Rickard *et al.*, 2006). When a *luxS* mutant of *S. oralis* was mixed with *A. naeslundii*, the resulting biofilm had 10 fold less biomass than the wild type mixed biofilm. When synthetic autoinducer 2, 4,5-dihydroxy-2, 3-pentanedione (DPD), a product of the LuxS enzyme, was exogenously

added to the system, the mutualism was restored, demonstrating the importance of interspecies communication in multispecies biofilm development (Rickard *et al.*, 2006).

The intracellular second messenger c-di-GMP plays a central role in the development of monospecies biofilms and in some instances, such as for *Vibrio cholerae*, has been shown to also respond to quorum sensing (QS) signaling to control biofilm development (Waters *et al.*, 2008). The major regulator of QS in *V. cholerae*, HapR, controls the transcription of c-di-GMP metabolizing proteins that lower intracellular c-di-GMP levels at higher cell densities, causing dispersal of the biofilm. At low cell density, HapR binds to the transcriptional activator VpsT resulting in an increase in biofilm formation. The activation of VpsT is regulated by VpsR, which shows a high binding affinity to c-di-GMP. This joint regulation of HapR and VpsR through a shared common regulatory pathway gives rise to cell density dependent biofilm formation in *V. cholerae* (Srivastava *et al.*, 2011). This suggests that, as for many other genes and regulatory pathways, c-di-GMP production differs in mixed species biofilms and this is most likely a reflection of species specific interactions.

We have recently established a reproducible model mixed species biofilm community composed of *Pseudomonas aeruginosa* PAO1 expressing the yellow fluorescent protein, *Pseudomonas protegens* Pf5 expressing the cyan fluorescent protein and *Klebsiella pneumoniae* KP1 expressing the red fluorescent protein (Lee *et al.*, 2013). This mixed species community shows synergy, as defined by the increased overall biomass of the mixed species biofilm compared to the biofilms formed individually by the component species. Further, the mixed species biofilm demonstrated community level stress and antibiotic tolerance, which was extended to

all of the community members, despite some of them being individually sensitive to those treatments. While the exact mechanism of these cooperative behaviours has not been elucidated, one possible explanation can be through metabolic cooperation along with communication between member species. Since c-di-GMP is one of the central second messengers controlling biofilm formation and preliminary data suggested that c-di-GMP was increased in this three species biofilm (unpublished data), the spatial and temporal localization of c-di-GMP within this mixed species model was investigated using the methodologies developed in Chapters 2 and 3. In this study, the *P. aeruginosa* strain expressing the *pcdrA::gfp* (ASV) reporter, was introduced into the mixed species biofilm along with the two other species and the c-di-GMP concentrations were monitored during biofilm development.

4.2 Materials and methods

4.2.1 Bacterial strains and culture media

P. aeruginosa PAO1, *P. protegens* Pf5 and *K. pneumoniae* KP1 tagged respectively with the cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) and *Discosoma* sp. red fluorescent protein (DsRed) (Lee et al., 2013) were used for biofilm development studies presented here. For c-di-GMP visualization studies, untagged *P. protegens* and *K. pneumoniae* were grown along with *P. aeruginosa* tagged with the stably expressing, genome integrated CFP along with the plasmid encoded *pcdrA::gfp* (ASV) as the c-di-GMP reporter. Bacteria were either cultured in Luria Bertani agar (10 g/l Tryptone, 5 g/l Yeast extract and 5 g/l NaCl, 15 h/L agar) or M9 minimal medium (48 mM Na₂HPO₄; 22 mM KH₂PO₄; 9 mM NaCl; 19 mM NH₄Cl; 2 mM MgSO₄; 0.1 mM CaCl₂) with 20 mM glucose as the sole carbon source.

4.2.2 Biofilm development in flow cells

Biofilms were cultivated in three-channel flow cells (channel dimensions, 1 x 4 x 40 mm) at room temperature. The flow cells were fed with M9 minimal medium containing 20 mM glucose as the carbon source at a flow rate of 9 ml/h. Each channel of the flow cell was inoculated with 0.5 ml of an overnight culture diluted to approximately 1×10^8 cfu/ml total bacteria with either single strain or the three bacterial strains mixed together. Mixed species biofilms were established by inoculating single species cultures of *P. aeruginosa* PAO1, *P. protegens* Pf5 and *K. pneumoniae* KP1 in the ratio of 5:5:1.

4.2.3 Microscopy and image analysis

All biofilm images were acquired using a Carl Zeiss Confocal Laser Scanning Microscope CLSM 780 (Carl Zeiss, Germany) using a 20x (Plan-Apochromat 20x/0.8) objective. Three separate fluorescent channels were used for three species imaging. For biofilm growth studies, a total of five images were taken for each flow channel, covering a total area of approximately $9 \times 10^5 \mu\text{m}^2$. For image analysis, a total of 15 image stacks (5 from each experiment) were quantified for each biofilm type using IMARIS (Bitplane AG, Switzerland). For ratiometric imaging and quantification, two separate optical channels were used to image the biofilm biomass and c-di-GMP reporter. Stably expressed CFP was used to quantify the biomass and GFP was quantified as a reporter for c-di-GMP in *P. aeruginosa*. The laser voltage was measured using a Sanwa™ CD800a digital multimeter and kept at 0.587 V throughout the experiment and pinholes of 50 μm and 58 μm were used for CFP and GFP, respectively. An emission bandwidth of 440-503 nm was used for CFP and 497-598 nm was used for GFP while the master gain was set to 700 and the digital gain of

1.00 was used for all the images. Images were processed using ImageJ Version 1.47 (rsb.info.nih.gov/ij/).

4.3 Results

4.3.1 Monospecies biofilm development

All three organisms were able to form biofilms when grown in M9-glucose medium (Figure 4.1). *P. aeruginosa* biofilms reached a maximum biomass of $6 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ by day 5 which decreased to $5.6 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ on day 7 suggesting dispersion of biofilm biomass around day 5. *P. protegens* showed similar changes in biomass, reaching a maximum biomass of $3.8 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ on day 5, which decreased to $2.9 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ at the end of day 7. In contrast, the *K. pneumoniae* biomass increased continually across the entire 7 d of the experiment, reaching a maximum biomass of $3.8 \times 10^6 \mu\text{m}^3/\mu\text{m}^2$ at the end of day 7 with no obvious dispersal event. *P. aeruginosa* made the most biofilm, while *P. protegens* and *K. pneumoniae* made much less as monospecies biofilms.

4.3.2 Mixed species biofilm development

As a first step towards understanding the c-di-GMP spatial distribution and dynamics in a mixed-species biofilm, the model biofilm consisting of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* was established and its development was studied here. All three organisms were able to co-exist in M9-glucose medium and *P. aeruginosa* constituted 4-17%, *P. protegens* 27-67% and *K. pneumoniae* 13-55% of the community (Figure 4.2). The community composition was dynamic, where, except for day 1, *P. protegens* was the dominant organism in the community. *P. protegens* showed the highest biovolume on day 5 of $8.42 \times 10^4 \mu\text{m}^3/\mu\text{m}^2$ whereas *K.*

pneumoniae on day 1 was found to be the dominant organism with a total biomass of $6.97 \times 10^4 \mu\text{m}^3/\mu\text{m}^2$. *P. aeruginosa* reached a maximum biomass of $2.15 \times 10^4 \mu\text{m}^3/\mu\text{m}^2$ by day 1 after which it decreased to $6.38 \times 10^3 \mu\text{m}^3/\mu\text{m}^2$ on day 3 and $5.84 \times 10^3 \mu\text{m}^3/\mu\text{m}^2$ on day 5, which was the lowest amount of biomass for *P. aeruginosa*.

Figure 4.1: Monospecies biofilms development. Monospecies biofilms of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* were grown in flow cells in 20 mM glucose M9-minimal medium and their development was quantified by image analysis. Biovolumes were calculated from CLSM images using IMARIS. Error bars represent standard deviations (n=15).

Tagging of individual species with fluorescent proteins allows for the spatial organization of individual organisms in the mixed species community to be observed. *P. aeruginosa* showed well defined microcolonies after 24 h of growth, whereas, *P. protegens* formed a uniform, monolayer biofilm. *K. pneumoniae* formed comparatively smaller microcolonies (10-20 μm in diameter) during the initial days of development, but on day 7, *K. pneumoniae* microcolonies were considerably larger

(>50 μm in diameter) and contained cells of *P. protegens* and *P. aeruginosa* (Figure 4.3).

Figure 4.2: The proportion of species during mixed species biofilm development. The mixed species biofilm, composed of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* were grown in flow cells using 20 mM glucose and M9 minimal medium. Biovolumes were calculated from CLSM images using IMARIS. Data points are average of 15 images from three independent experiments.

Figure 4.3: Structure and spatial localization of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* during mixed species biofilm development. Representative images of confocal micrographs of mixed species biofilm grown in M9 glucose medium for 7 d are presented. Images A - D represent days 1, 3, 5 and 7. *P. aeruginosa* was labelled with the yellow fluorescent protein, *P. protegens* was labelled with the cyan fluorescent protein, and *K. pneumoniae* was labelled with the red fluorescent protein. Magnification was 20x. Scale bars are 100 μm .

4.3.3 C-di-GMP distribution during the development of a mixed species biofilm

A mixed species biofilm community was grown by mixing the c-di-GMP reporter strain *P. aeruginosa* PAO1 mini-Tn7 CFP *pcdrA::gfp* (ASV) with untagged *P. protegens* and *K. pneumoniae* to visualize and quantify c-di-GMP of *P. aeruginosa* in the mixed species community. Images were taken at 24 h intervals and ratiometric imaging was performed. The average pixel frequency, an indication of c-di-GMP of *P. aeruginosa*, increased from 4793 on day 2 to 6616 on day 3 and reached a maximum of 116306 on day 4 (Figure 4.4). Subsequently, the fluorescence response decreased significantly to 2963 on day 5 and to below the detection limit on day 6. On day 7, the average pixel frequency was only 117.

Figure 4.4: Quantification of c-di-GMP concentrations in *P. aeruginosa* in mixed species biofilms using ratiometric imaging. Mixed species biofilm were established using the *P. aeruginosa* reporter strain and non-fluorescently tagged *P. protegens* and *K. pneumoniae*. Data points are the average of three images from different parts of same flow cell at 24 h intervals. Experiments were independently repeated 3 times and representative data from one experiment are presented here. Error bars represent the standard deviation (N=3).

When the c-di-GMP distribution of *P. aeruginosa* microcolonies of the mixed species biofilm was analyzed (Figure 4.5), the fluorescent signal was homogenously distributed across the biofilm on days 2 and 3.

Figure 4.5: C-di-GMP distribution of a developing mixed species biofilm. Ratiometric images were calculated from *P. aeruginosa* tagged with the mini-Tn7 CFP and c-di-GMP expression was visualized using the *cdrA::gfp* reporter plasmid. Representative image from A) day 2, B) day 3, C) day 4, D) day 5, E) day 6, F) Day 7. Magnification 20x. Scale bar 50 μm . The 16 colour LUT in the bottom image represents the c-di-GMP concentration, white is equivalent to approximately 75 pM c-di-GMP / μg of protein and black is equivalent to approximately 0.38 pM c-di-GMP / μg of protein.

However, on day 4, there was a higher c-di-GMP concentration at the microcolony margins compared to their interiors. This pattern was also observed for the *P. aeruginosa* single species biofilm (Chapter 2). However, unlike the distribution

in the single species biofilms, smaller colonies (diameter < 50 μm) also showed this localization pattern until day 4. After day 5, almost no GFP signal could be detected, suggesting very low concentrations of c-di-GMP despite *P. aeruginosa* being present in these biofilms at 10-17 %.

4.3.4 The starvation response of a mixed species biofilm

It has been previously shown that decreased c-di-GMP concentrations in *P. aeruginosa* are associated with biofilm dispersal and also that glucose limitation is one of the key environmental cues leading to dispersal for *P. aeruginosa*. Given that the mixed species biofilms have been shown to consume most of the glucose carbon source, especially in the latter stages of development (Lee *et al.*, 2013), it is possible that these biofilms are experiencing glucose limitation. However, no dispersal of the mixed species biofilm was observed and this may suggest the mixed species biofilm responds differently to starvation than the *P. aeruginosa* monospecies biofilm. To study the starvation response of *P. aeruginosa* in the mixed species consortia, the mature biofilm on day 4 was starved as described (Chapter 3, section 3.2.4) and time lapse imaging was performed with 15 min intervals for 3 h. The c-di-GMP concentration of *P. aeruginosa* was also calculated from ratiometric images (Figure 4.7) as pixel frequency and this was found to increase from 186626 to 279161 within 15 min of starvation, e.g. a 1.5 fold increase immediately after the initiation of dispersal (Figure 4.6). Thereafter, the concentration of c-di-GMP decreased and finally reached 163671 after 3 h of starvation, which was less than the starting pixel frequency.

Figure 4.6: C-di-GMP quantification of *P. aeruginosa* in a mixed species biofilm of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* during carbon starvation as determined by ratiometric imaging. Time lapse imaging was performed at 15 min time intervals, up to 3 h. Data were plotted for average pixel frequency against time. Experiments were independently repeated 3 times and representative data from one experiment is presented here.

Figure 4.7: C-di-GMP distribution within *P. aeruginosa* in a mixed species biofilm that consists of *P. aeruginosa*, *P. protegens* and- (continued on the next page)

K. pneumoniae during carbon starvation. Time-lapse images of mature PAO1 biofilms under flow conditions as visualized by ratiometric imaging. A) before starvation, B) 15 min of starvation C) 30 min of starvation, D) 60 min of starvation, E) 120 min of starvation and F) 180 min of starvation. Images were recorded in real-time. *P. aeruginosa* was tagged with the mini-Tn7 CFP to monitor total *P. aeruginosa* biomass and c-di-GMP expression was visualized using the *cdrA::gfp* reporter plasmid. Magnification 20x. The 16 colour LUT in the bottom image represents the c-di-GMP concentration, white is equivalent to approximately 75 pM c-di-GMP / μ g of protein and black is equivalent to approximately 0.38 pM c-di-GMP / μ g of protein.

4.3.5 Characterization of the biofilm biomass of a mixed species biofilm after 3 d of carbon starvation

Ratiometric imaging was performed on the mixed species biofilm after 3 d of starvation. The average pixel frequency of colonies remaining after 3 d of starvation was 1483803 ± 413699 , representing a 12.7 fold increase compared to the non-starved biofilm (Figure 4.8).

Figure 4.8: Amount of c-di-GMP produced by *P. aeruginosa* when grown in a mixed species biofilm of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* before and after 3 d of starvation. The amount of c-di-GMP was calculated as the average pixel frequency from ratiometric imaging from a total of 9 images from 3 independent experiments. Error bar represents standard deviation (N=9).

Some representative ratiometric images of mixed species biofilm after three days of starvation are shown below (Figure 4.9).

Figure 4.9: C-di-GMP visualization of *P. aeruginosa* colonies in a mixed species biofilm of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* after 3 days of starvation. Representative ratiometric images of *P. aeruginosa* in mixed species biofilm. A) Near the inlet of the flowcell, B) near the outlet of the flowcell. Magnification 20x. Scale bar 50 μm . The 16 colour LUT in the bottom image represents the c-di-GMP concentration, white is equivalent to approximately 75 pM c-di-GMP / μg of protein and black is equivalent to approximately 0.38 pM c-di-GMP / μg of protein.

It was not possible to calculate the biomass volume that remained after starvation since two of the three organisms were not tagged with any flurophore. However, bright field imaging of the flowcell (Figure 4.10) revealed that a considerable amount of biofilm biomass remained in the flowcell even after 3 d of starvation, especially near the inlet of the flow cell.

Figure 4.10: Representative brightfield image of mixed species biofilm of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* growing in a flow cell A) before starvation and B) after 3 d of starvation. Medium flow is from the left to right. Magnification 4x, scale bar = 1 mm.

For the single species biofilm of *P. aeruginosa*, a high c-di-GMP concentration, as indicated by the fluorescent signal, was associated with a high percentage of morphotypic variants in the biofilm biomass. To determine if such variants were similarly responsible for the elevated fluorescence observed in the mixed species biofilm, the flowcell was washed with M9 medium after 3 d of starvation and plated onto LB agar. The colonies were counted according to their morphology and it was observed that the starved biofilms had high proportions of *P. aeruginosa* and *K. pneumoniae*. Three independent experiments were performed and the data from one representative experiment is given here. Enumeration of colonies revealed 3.16×10^7 cfu/ml of *P. aeruginosa* and 2.08×10^7 cfu/ml of *K. pneumoniae* which constituted 60% and 40% of the total community, respectively. All of the isolates appeared similar to their respective wild type strains and none showed morphotype variation. This contrasts dramatically when compared to earlier observations of colony morphotype variants in starved *P. aeruginosa* PA01 monospecies biofilms (Figure 3.13).

Figure 4.11: Biofilm composition of mixed species biofilm *P. aeruginosa*, *P. protegens* and *K. pneumoniae* remaining in the flow cell after 3 d of carbon starvation. The biofilm was washed with 2 ml of starvation medium, serially diluted and plated on to LB agar plates. Colonies were counted for to determine cfu after 3 d of incubation at room temperature and to identify morphotypic variants.

4.3.6 C-di-GMP expression is higher in mixed species biofilms than in monospecies biofilms of *P. aeruginosa*

To verify the observations presented here, which were based on the c-di-GMP reporter strain, the chemical concentration of c-di-GMP was estimated in a separate experiment. Mono and mixed species biofilms of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* were harvested from flow cells on the 5th day (mature biofilm), c-di-GMP was extracted as previously described (Chapter 2) and quantified using LC-MS. The biomass of the sample was calculated using the Bicinchoninic acid protein assay (BCA) (Chapter 2, section, 2.2.7) and normalized before chromatography. Since there was no absolute quantification done, the c-di-GMP concentration is expressed as relative abundance and hence has no units. In mono species biofilms, *K. pneumoniae* showed the least abundance (0.67 ± 0.84) of c-di-GMP, *P. protegens* showed the highest abundance (5.98 ± 4.63) and *P. aeruginosa* showed intermediate levels (3.64

± 3.45). C-di-GMP levels in the mixed species biofilms showed the highest values of 9.18 ± 1.16 on day 5, which was 2.9 fold more than the c-di-GMP levels of the monospecies biofilms.

Figure 4.12: Relative amount of c-di-GMP in 5 d old mono and mixed species biofilms of *P. aeruginosa*, *P. protegens* and *K. pneumoniae*. Extracted biomass was normalized with protein concentration before LC-MS analysis. Each data set is the average of three independent biofilm samples with two technical replicates. Error bars represent standard deviations (n=6).

4.4 Discussion

Given the importance of c-di-GMP in the formation and dispersal of monospecies biofilms, it was of interest to understand its dynamics in a mixed-species model biofilm community. The experiments were designed to characterise c-di-GMP levels of one of the species of the mixed-species biofilm, *P. aeruginosa*, in the mixed-species model, with a view to compare c-di-GMP distribution and dynamics to monospecies biofilms of *P. aeruginosa* under similar experimental conditions. The results showed that *P. aeruginosa* microcolonies in monospecies and mixed species biofilms were similar in their spatial distribution of c-di-GMP. The transient increase of c-di-GMP observed in response to starvation was also observed for the mixed species biofilm, although no morphotypic variants were found in the biomass that

remained in the flow cell after extended starvation, in contrast to the high percentage of such variants observed for the monospecies biofilm of *P. aeruginosa*.

The distribution of c-di-GMP of *P. aeruginosa* in the mixed species biofilm showed the same pattern as observed for the mono species biofilm (Chapter 2). The intracellular level of c-di-GMP increased during the initial stages of development and on day 4, was 17 fold higher than on day 3. Likewise, after day 4, the c-di-GMP dropped below the detection limit of the imaging method. A similar drop in concentration was also observed in mono species biofilm development (Chapter 2, Figure 2.8). Similarly the c-di-GMP localization pattern of c-di-GMP on the boundary of large colonies was also observed during mixed species biofilm development. Unlike monospecies biofilm development, the localization pattern was also visible for smaller colonies, <50 μm in diameter. It is possible that the even the small colonies could be under nutrient or oxygen limitation (De Beer *et al.*, 1994, Khl *et al.*, 2007), so that even the small colonies show a dramatically reduced c-di-GMP. Maturation of the biofilms was dependent on carbon source used, and a delayed maturation of mixed species biofilm was observed here in comparison to monospecies biofilms that dispersed around days 4-5 (Lee *et al.*, 2013). Even though there are no studies on the spatial distribution of c-di-GMP in *P. protegens*, Miller *et al.* (2011), using a FRET based biosensor found that there was an asymmetric distribution of c-di-GMP in *P. aeruginosa*, *Caulobacter crescentus* and *K. pneumoniae*. During bacterial replication, the daughter cells express differential levels of c-di-GMP, which was common for all three organisms. Interestingly, they observed a polar localization of c-di-GMP for *P. aeruginosa* and *C. crescentus* but not for *K. pneumoniae* (Christen *et al.*, 2010). It is understood that c-di-GMP has a high binding affinity to the PilZ domain (Srivastava *et al.*, 2011) and it is possible that such proteins co-localize to the polar flagellum of

P. aeruginosa and *C. crescentus*. It is also possible that there is no polar localization of c-di-GMP in *K. pneumoniae* since it is non flagellated and hence, there may be not mechanism for localization of PilZ homologies at the cell pole. The differential distribution of c-di-GMP in these three organisms also highlights that the c-di-GMP localization will vary between species. .

The starvation of a mixed species biofilm showed a similar trend of a transient increase in c-di-GMP concentration as observed in the monospecies *P. aeruginosa* biofilm, as reported in Chapter 3. Therefore, it can be concluded that *P. aeruginosa*, even in a mixed species community, exhibits a similar starvation response irrespective of whether it is present in a population or community context. To improve our understanding of how c-di-GMP changes in the mixed species community in response to environmental cues such as starvation, it will be necessary to extend the measurements to all community members. For example, the *P. protegens* disperses in response to low inorganic phosphate concentration (Pi) and this response is mediated through the c-di-GMP specific phosphodiesterases RapA. In response to low Pi, RapA lowers c-di-GMP, which inhibits secretion of the cell surface adhesin, LapA, resulting in dispersal (Monds *et al.*, 2007). To date, the starvation response of *K. pneumoniae* biofilms has not been reported but should be investigated in the future.

One of the striking differences of mixed species starvation in comparison to the monospecies biofilm starvation is the lack of morphotypic variants in the community. Similar to the monospecies starvation conditions, starvation of the community failed to disperse all of the biomass, even after 72 h of starvation. For the monospecies *P. aeruginosa* biofilm, the incomplete dispersal was linked to the presence of morphotypic variants, many of which displayed high endogenous c-di-GMP levels.

The high c-di-GMP levels were most likely due to the mutations identified in genes such as *wspF*, *pilT* and *fhaI*. In contrast, no such variants were observed for the mixed community, even after prolonged starvation. A recent study using the same mixed-species model revealed that interspecies diversity reduced intraspecies variation (Lee *et al.*, 2015). In other words, the mixed species biofilm produces a lower frequency of colony variants than the monospecies biofilm. But the reason behind incomplete dispersal in response to starvation for a mixed species biofilm given that fact that the mutation rates are less compared to monospecies biofilm are yet to be explored.

The presence of a high proportion of *K. pneumoniae* in the flow cell after 3 days of starvation could suggest that *K. pneumoniae* do not disperse in response to starvation. Therefore, it will be important to investigate the starvation response of *K. pneumoniae* under these conditions. Chemical quantification of the total c-di-GMP levels in the mixed species biofilm revealed that the c-di-GMP was higher than that of any of the single species biofilms (Figure 4.12). Since the amount of c-di-GMP associated with *P. aeruginosa* in the mixed species biofilm, as determined here using ratiometric imaging, was lower than when it grew as a monospecies biofilm, the higher overall c-di-GMP of the mixed community represents c-di-GMP contributions from *P. protegens* and *K. pneumoniae*. It is known that *P. protegens* and *K. pneumoniae* encode 49 and 37 c-di-GMP modifying proteins, respectively and therefore, it is likely they contribute to the c-di-GMP pool measured by LC-MS (Römling *et al.*, 2013). Future experiments using similarly tagged c-di-GMP reporter genes in *K. pneumoniae* and *P. protegens* would help to better refine our understanding of the distribution of c-di-GMP during mixed species biofilm development.

In summary, the ratiometric imaging technique developed here to visualize and quantify c-di-GMP *in situ* in real-time can be applied to mixed species biofilms also. The visualization pattern revealed that *P. aeruginosa* follows similar developmental patterns as that of monospecies biofilm in a consortia of mixed species biofilm. The total c-di-GMP level of *P. aeruginosa* in the mixed species biofilm measured using ratiometric method was much lesser than that of the monospecies biofilm. At the same time the total c-di-GMP measured for the total mixed species biofilm using LC-MS was in fact higher than that of any of the mono species biofilms indicating that the c-di-GMP content in a mixed species biofilm is more either due to intracellular signaling or due to resources competition. Starvation of the mixed species biofilm leads to dispersal and a transient increase of c-di-GMP similar to the monospecies biofilm. While some of the mixed species biofilm did not disperse, no morphotypic variants were detected, which contrasted with the monospecies biofilms.

CHAPTER 5. GENERAL DISCUSSION

The objective of this study was to define the spatial and temporal distribution of c-di-GMP during mono and mixed species systems during biofilm development. While chemical quantification can be used to determine c-di-GMP concentrations, this approach is best suited to homogenous cultures, where most or all cells are in the same physiological state. However, this condition does not apply to biofilms, which are increasingly understood to display wide heterogeneity across different regions of the biofilm and even within a single microcolony (Xu *et al.*, 1998, Stewart & Franklin, 2008, Anetzberger *et al.*, 2009). For these reasons, the chemical quantification method is not well suited to studies of the spatial distribution of c-di-GMP. Therefore, this study investigated a method to adapt an existing bioreporter strain, where the expression of the green fluorescent protein (GFP), changes depending on the concentration of c-di-GMP to study the distribution of c-di-GMP during biofilm development.

The *cdrA* based c-di-GMP reporter was shown to be useful for studying the c-di-GMP concentration in planktonic cultures (Rybtke *et al.*, 2012). However, the application of this sensor to flow cell biofilms was challenging since the fluorescent signals can be due to high intracellular c-di-GMP or cells clumping together at the same spot. Even though ratiometric imaging accounts for the biomass, it does have many pitfalls, potentially leading to an incorrect interpretation of the data (North, 2006). Possible issues such as chromatic shift, spherical aberrations were experimentally analyzed and corrected mathematically (Appendix A). While every attempt was made to account for these known issues, the problem of spherical aberration due to the refractive index of the medium and the biofilm could not be

addressed since there is no available blue-green biofilm mimic. Ideally, an artificial blue-green fluorescing biofilm structure (including the water channels) should be synthesized and used to identify the spherical aberration, which would allow for mathematical correction if there is any shift. Nevertheless, ratiometric imaging can be used as a tool for semi-quantitative analysis of dynamic intracellular signaling molecules. Even though image quantification allows one to measure the c-di-GMP for a given image, the quantification has to be done with care, since the calibration of images involves the assumption that the samples used for LC-MS quantification were homogenous, although for biofilms, this is not the case. While we feel the method developed here was robust and reproducible, the ratiometric image should nonetheless not be used for absolute quantification, but rather can be used with confidence for the comparison of c-di-GMP in different images or parts of an image.

The ratio imaging of developing biofilms here showed that the distribution of c-di-GMP changes in time and has localized hotspots of high c-di-GMP across the biofilm (Figure 2.8). These areas with relatively high c-di-GMP in comparison to the neighboring areas are the first report of this kind. Similar hot spots have been observed for a variety of biological systems, such as calcium hotspots in neuroblastoma cells and copper hotspots in vineyard soils (Silver *et al.*, 1990) (Jacobson *et al.*, 2007). This localized, high level of c-di-GMP could be due to either the presence of mutants that over produce c-di-GMP or this pattern of expression could reflect the local physiology of the biofilm cells. With respect to the accumulation of mutants, Conibear *et al.* (2009) showed that there was a 100 fold increase in the mutation rate in the biofilm compared to planktonic cells. The spontaneous mutations were also observed as localized hot spots of fluorescent cells (Conibear *et al.*, 2009). Similar mutations could also account for the high c-di-GMP

concentration on the microcolony periphery where clonal propagation results in localized hot spots of high *cdrA::gfp*. To determine if the high c-di-GMP producing cells are mutants, they could be isolated from the flow cell using single cell capture techniques such as micromanipulation or optical trapping (Ashkin & Dziedzic, 1987), (Thomsen *et al.*, 2004). This would also necessitate the use of flowcells that allow for the non-destructive removal of the coverslip (Cohen *et al.*, 2016) to expose the biofilm and to allow for the capture and analysis of the highly fluorescent cells.

Currently, there are only two common variants reported for *P. aeruginosa* (SCV and RSCV), but the variant analysis described in this thesis (Chapter 3, Table 3.4) showed that there are many other mutations can be found in isolates from *P. aeruginosa* biofilms. In addition, Penterman *et al.* (2014) reported variants of *P. aeruginosa* with mutations in genes associated with lipopolysaccharide (LPS) synthesis. Such variants had an apparent fitness advantage during biofilm growth since the synthesis of LPS requires energy and adhering together in a biofilm provides the required protection without high energy expenditure.

Nutrient and oxygen concentrations are known to affect the c-di-GMP levels in bacteria (Gjermansen *et al.*, 2005). Since access to these, and other, nutrients by the biofilm will be dependent on a combination of diffusion and metabolic rates of the biofilm community, it is perhaps not surprising that the distribution of c-di-GMP is uneven across the biofilm. This is supported by studies using fluorescent tracers and oxygen sensing optode that have demonstrated their heterogeneous distribution in biofilm microcolonies. In those studies, the oxygen concentrations were observed to decrease steadily from the surface of the microcolonies to the interior (Rani *et al.*, 2005). In contrast, the c-di-GMP, as indicated by the fluorescence pattern in this study

was quite different, where there was a thin, high level around the edges of the larger colonies and thus, the diffusion and consumption of oxygen alone does not explain the c-di-GMP distribution observed here.

The distribution of c-di-GMP differed depending on microcolonies size, where smaller microcolonies showed a uniform fluorescence and larger colonies only showed fluorescence around their perimeter. Since this pattern was primarily observed on day 4 at the time of dispersal, it is speculated that, based on this pattern of c-di-GMP, that dispersal initiates from these larger microcolonies. This is in agreement with other studies that have indicated that after the microcolony reaches the maturation stage (approximately 80 μm in diameter), the interior of the colony disperses from a breakout point and leaves a nonmotile, empty colony behind (Purevdorj-Gage *et al.*, 2005, Kirov *et al.*, 2007). The low levels of c-di-GMP on the interior also supports observations that there are often ‘hollow colonies’ left behind after dispersal. It is therefore proposed that the microcolony shell is maintained by a subpopulation of cells that retain the biofilm phenotype, due to high levels of c-di-GMP, while the interior cells have returned to the planktonic phase.

Masked ratiometric imaging performed during induction of dispersal, with improved temporal resolution (15 min), showed a transient increase in c-di-GMP followed by the expected decrease and active dispersal (Chapter 3, Figure 3.4 and Figure 3.9). This transient increase implies that the dispersal response is more complex than expected. It has recently been reported that, BdlA, a protein involved in dispersal of *P. aeruginosa*, is active at high intracellular levels of c-di-GMP, even when dispersal ultimately leads to a reduction in overall c-di-GMP levels (Petrova & Sauer, 2012) and is in agreement with the observations made here.

The transcriptomic data for the starved biofilms also supports the hypothesis that the initial starvation response leads to elevated c-di-GMP. Out of the 902 genes that were differentially expressed during starvation, 64% of the genes were induced in response to starvation (Chapter 3, Table 3.2). Furthermore, many of the genes that were altered in expression were associated with metabolic pathways and TCA shunt pathways, which may reflect that the cells adapt by switching to an energy conservation state (Figure 3.8). Similar pathways such as the methylglyoxal shunt have been shown to be involved with energy conservation in *E. coli* (Russell & Cook, 1995). A similar observation has been found during amino acid starvation of *E. coli* using microarray analysis. It was found that 51% of the significantly changed genes were upregulated, where 174 were involved in metabolism and 139 were involved in the RpoS-dependent general stress response (Traxler *et al.*, 2008). Hence it is becoming clear that bacteria during nutrient starvation is metabolically active and more tolerant to stress leading to a resistant or tolerant state. Since localized nutrient starvation is a common occurrence in a well fed surface attached biofilm, it might be a potential reason for enhanced stress resistance of a biofilm

The final objective of the study was to monitor c-di-GMP in mixed species biofilms. The c-di-GMP distribution of *P. aeruginosa* in a mixed species biofilm development and transient increase during starvation was similar to the *P. aeruginosa* monospecies biofilm, indicating that *P. aeruginosa* followed a similar developmental cycle in mixed species consortia. However, there were some clear differences between the single and mixed species biofilm results, where there were fewer c-di-GMP hotspots observed and almost no colony variants were observed in the effluent in the mixed species biofilm in comparison to the monospecies biofilm. It has been previously reported that the mixed species biofilm produces fewer variants (Lee *et al.*,

2015), although the mechanism behind this reduction is currently unknown. This supports the argument that the hotspots were in fact due to the clonal propagation of high c-di-GMP mutants. Surprisingly, the overall c-di-GMP concentration was higher in the mixed species biofilm, as measured specifically for *P. aeruginosa* (bioreporter) when compared to the whole community (LC-MS) (Chapter 4, Figure 4.14). This suggests that the c-di-GMP concentration of the other two species (*P. protegens* and *K. pneumoniae*) may also be higher than in the corresponding monospecies biofilm. The higher c-di-GMP concentration found for the mixed species biofilm may partly explain the observation that some mixed species biofilms show increased biofilm biomass (Burmølle *et al.*, 2006) and elevated stress tolerance (Lee *et al.*, 2013). The mechanism by which the mixed species biofilms increase c-di-GMP relative to the situation where they grow as single species biofilms remains to be explored. However, it is possible that changes in nutrient concentration or processes such as cell signaling could induce c-di-GMP production in the rest of the community. For example, it has been shown that QS molecule 3-oxoC12-HSL binds to the LasR transcription factor and activates the expression of transmembrane protein TpbA (Ueda & Wood, 2009). TpbA further activates membrane-anchored GGDEF protein TpbB. TpbB deactivates GGDEF enzyme activity and cause a reduction in cellular c-di-GMP level in response to a high QS concentration.

In conclusion, the methodology developed in this study was validated for the monitoring of the intracellular second messenger c-di-GMP as it changes during biofilm development or in response to dispersal cues, such as starvation. Studying these molecules *in situ*, in real time allowed us to discover a previously unknown transient increase in c-di-GMP during nutrient starvation. Visualization of c-di-GMP in a biofilm *in situ* further demonstrates biofilm heterogeneity as localized hotspots of

high c-di-GMP across the biofilm. Also a direct relationship between starvation insensitive colonies and colony variant formation were observed, together with the observation that starvation leads to an increased metabolic response possibly leading to a more stress tolerant state and localized starvation in a biofilm is a common occurrence, this supports the hypothesis that the physiological heterogeneity along with the genetic diversification observed during biofilm development might be the key to enhanced biofilm stress resistance and tolerance.

The methodology developed here is readily adaptable to other second messengers, such as cAMP, which is also an important regulator during the physiological adaptation of bacteria to changing environmental conditions. One of the limitations of the methodology is the availability of fluorophores. Additional fluorophores with high quantum yield and good color separation could help to extend the study to include more than one signaling molecule in the same system.

The control of dispersal has received significant attention in recent years as a novel anti-biofilm strategy. Since results from this thesis demonstrate that starvation induced dispersal selects for mutants that do not disperse, further studies on other dispersal agents, e.g. nitric oxide, oxygen limitation etc., should also address whether they similarly result in selection for non-dispersing mutants. Given that non-dispersing mutants were identified here, it may be necessary to combine dispersal technologies with traditional antimicrobial compounds to better eradicate biofilms.

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Appendix A

Figure A.1: Chromatic aberration analysis. A 6 μm bead expressing cyan and green colour is imaged using the same imaging parameters as that of ratiometric imaging. A) Chromatic aberration, and spherical aberration in XY plane is marginal, showing perfect overlap between cyan and blue. B) Chromatic aberration in YZ is significant, showing non-overlapping cyan and green images. C) Corrected chromatic aberration in YZ, showing overlapping cyan and green images. D) Ratio image created with non-corrected bead, showing intensity changes as 16 color LUT. E) Ratio image of corrected bead, showing identical ratio across the entire bead. White colour represents ratio of 1 in 16 color LUT.

Figure A.2: Comparison of *cdrA::gfp* and *Tn7-gfp* reporter fluorescence during starvation. A-D) *c-di-GMP* reporter *cdrA::gfp* in *P. aeruginosa* wild type, 15 min before the start of starvation, 0 min start of starvation, 30 min starvation and 3 h of starvation. E-H) Stably expressing *Tn7-GFP* in *P. aeruginosa* wild type, 15 min before the start of starvation, 0 min starvation, 30 min starvation and 3 h of starvation. I) Average grey value of plotted vs time.

Appendix B

Finding chromatic aberration

Prerequisite: Dual colour fluorescent beads of size, which is ideally equal to the object to be measured. Take z-stack images with the same settings as for the biological samples being imaged.

Find out the intensity maximum slide in each channel

1. Open the image in ImageJ software.
2. Draw the region of interest (ROI) around the bead.
3. Plot z-axis profile (Image-Stacks-Plot Z-axis profile)
4. Repeat the step in both the channels.
5. Find the slide where the intensity is maximum for both the channels from the z-axis profile
eg: in CFP intensity max is 7th slide; GFP max is 11. So shift is 4)

Correcting the chromatic aberration

6. Image-colour- split channels
7. Duplicate green channel from 8th-18 (Control +Shift+D)
8. Add slide at the last till 18
9. Make a composite image by joining the two channels (image-colour-make composite)
10. Save the image as “Aberration corrected”

Ratiometric Imaging

1. Open the “Aberration corrected” image in ImageJ software
2. Set division by zero as Nan instead of infinity in imageJ (Only need to do once after installing ImageJ) (Edit-Options- Miss-Divide by zero = NaN instead of infinity)

3. Smooth the image (Control+Shift+S)
4. Split channels (Image-colour-Split channel)
5. Create ratio image by dividing GFP channel by CFP (Process-Image calculator -Divide- GFP by CFP; Select 32 bit floating result in the pop up window)
6. Create Mask (Image-Adjust threshold (Control +Shift+T). Move the slider until the background is removed)
7. Make the mask binary (Process-Math- divide by 255)
8. Apply Mask to ratio (Process-Image calculator-multiply- Ratio and binary mask)
9. Scale down by histogram (Image-adjust brightness contrast (Control +shift+C; set 0 and 1)
10. Apply 16 colour LUT for visualization (Image-Look up Table-16 colour)
11. Plot histogram (Control +Shift+H; Save the values in Excel for future use)
12. Export the masked ratio in 16 colour LUT into .jpeg for future use.

Appendix C

Table C.1: Primers and their sequences for qRT-PCR

Primer name	Sequence 5' to 3'
hdhA-F	TACTACATCCAGCCGACCCT
hdhA-R	ACAGGTGGTAGCAGTTGGTC
pelD-F	TGCCTGTATGCCTTCGAGTT
pelD-R	GAAGAGAATCCTCAGGCGCT
pvdA-F	ATCTCATCGGTGTCGGCTTC
pvdA-R	TCGTGCTTGTGCAGGTAGTT
fumC1-F	GTGCACATCGAGAGCGTACT
fumC1-R	AACCGATCAGGGCGAAGAAG
cyaA-F	GATTCGCCCTACAAGTCGCT
cyaA-R	GCGTTCGAGATGGCGATAGA
PA4781-F	CGACCGCTACGATGAACTGA
PA4781-R	GTGAGTTCGATGCTTTCCGC
vfr-F	ACCGCAAAGAGCACCATCAT
vfr-R	GTTCGCTGCCTTCCTTTTCG
RpoS-F	CATGTGGTCAAGGAGCTCAA
RpoS-R	TCTACCGAAGTCACCCGTTC
rhlA_F	AGCTGGGACGAATACACCAC
rhlA_R	GACTCCAGGTCGAGGAAATG
pvdA forward	GGGCGCCCTGGAAGTGCTGTT
pvdA reverse	GCTCTGCCCCGCCGCGATAATG
rpoSF	CCCAAGCTTGGGTCATGCAAGGGATAACGACA
rpoSR	GGAATTCCTCAGTGGGTCTAAGGTTTTC

Table C.2: Genes that were differentially expressed during planktonic starvation and their fold change

PA Numb	gene	log2	Kegg category	Description
PA4022	<i>hdhA</i>	7.0	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA2247	<i>bkdA1</i>	6.9	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2248	<i>bkdA2</i>	6.9	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA5100	<i>hutU</i>	6.8	Amino acid metabolism	Histidine metabolism
PA2862	<i>lipA</i>	6.4	Lipid metabolism	Glycerolipid

				metabolism
PA5098	<i>hutH</i>	6.3	Amino acid metabolism	Histidine metabolism
PA0865	<i>hpd</i>	6.1	Biosynthesis of cofactors	Ubiquinone and other terpenoid-quinone biosynthesis
PA3709	PA3709	6.0	Membrane transport	Carbohydrate transport
PA1984	<i>exaC</i>	6.0	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA2015	<i>liuA</i>	5.8	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA5096	PA5096	5.4	Membrane transport	ABC transporters
PA2013	<i>liuC</i>	5.3	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2012	<i>liuD</i>	5.3	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA3710	PA3710	5.2	Amino acid metabolism	Glycine and serine and threonine metabolism
PA5097	PA5097	5.0	Membrane transport	Carbohydrate transport
PA2250	<i>lpdV</i>	5.0	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3415	PA3415	4.7	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA5093	PA5093	4.7	Amino acid metabolism	Histidine metabolism
PA5094	PA5094	4.7	Membrane transport	ABC transporters
PA5095	PA5095	4.7	Membrane transport	ABC transporters
PA5091	<i>hutG</i>	4.7	Amino acid metabolism	Histidine metabolism
PA5092	<i>hutI</i>	4.7	Amino acid metabolism	Histidine metabolism
PA2014	<i>liuB</i>	4.5	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2249	<i>bkdB</i>	4.5	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA4302	<i>tadA</i>	4.5	Membrane proteins	pilus assembly/ secretion
PA4301	<i>tadB</i>	4.5	Membrane proteins	Motility & Attachment
PA4303	<i>tadZ</i>	4.5	Membrane proteins	Motility & Attachment
PA3570	<i>mmsA</i>	4.4	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2553	PA2553	4.4	Lipid metabolism	Fatty acid degradation
PA3305.1	<i>phrS</i>	4.2	Non-coding RNA	Non-coding RNA
PA0746	PA0746	4.2	Lipid metabolism	Acyl-CoA dehydrogenases
PA0175	PA0175	4.2	Signal transduction	Two-component system
PA1190	PA1190	4.1	Membrane proteins	Membrane proteins
PA0113	PA0113	4.1	Energy metabolism	Oxidative phosphorylation
PA4024	<i>eutB</i>	4.1	Lipid metabolism	Glycerophospholipid metabolism
PA3416	PA3416	4.1	Carbohydrate	Glycolysis or

PA3417	PA3417	4.1	metabolism Carbohydrate metabolism	Gluconeogenesis Glycolysis or Gluconeogenesis
PA0176	<i>aer2</i>	4.0	Signal transduction	Two-component system
PA1253	PA1253	4.0	Xenobiotics biodegradation and metabolism	Caprolactam degradation
PA4296	<i>pprB</i>	4.0	Signal transduction	CheY-like receiver domain
PA4297	<i>tadG</i>	4.0	Membrane proteins	Motility & Attachment
PA4025	PA4025	4.0	Lipid metabolism	Glycerophospholipid metabolism
PA0744	PA0744	3.9	Lipid metabolism	phenylacetate catabolism
PA3079	PA3079	3.8	Membrane proteins	Membrane proteins
PA2265	PA2265	3.8	Carbohydrate metabolism	Pentose phosphate pathway
PA1338	<i>ggt</i>	3.8	Amino acid metabolism	Taurine and hypotaurine metabolism
PA0109	PA0109	3.8	Membrane proteins	Membrane proteins
PA1174	<i>napA</i>	3.7	Energy metabolism	Nitrogen metabolism
PA1175	<i>napD</i>	3.7	Energy metabolism	nitrate reductase activity
PA1176	<i>napF</i>	3.7	Energy metabolism	oxidation-reduction process
PA4781	PA4781	3.7	Signal transduction	cyclic di-GMP phosphodiesterase
PA0112	PA0112	3.7	Energy metabolism	Oxidative phosphorylation
PA1172	<i>napC</i>	3.7	Energy metabolism	nitrate reductase activity
PA1421	<i>gbuA</i>	3.7	Amino acid metabolism	Arginine and proline metabolism
PA0747	PA0747	3.7	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA0177	PA0177	3.7	Signal transduction	Two-component system
PA0178	PA0178	3.7	Signal transduction	Two-component system
PA2011	<i>liuE</i>	3.6	Lipid metabolism	Synthesis and degradation of ketone bodies
PA5348	PA5348	3.6	DNA replication and repair	DNA replication
PA0107	PA0107	3.6	Energy metabolism	Oxidative phosphorylation
PA0108	<i>coIII</i>	3.6	Energy metabolism	Oxidative phosphorylation

PA0106	<i>coxA</i>	3.6	Energy metabolism	Oxidative phosphorylation
PA0105	<i>coxB</i>	3.6	Energy metabolism	Oxidative phosphorylation
PA2984	PA2984	3.6	Membrane proteins	Transport
PA4812	<i>fdnG</i>	3.6	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA2262	PA2262	3.5	Membrane proteins	Transport
PA4197	<i>bfiS</i>	3.5	Signal transduction	phosphorelay sensor kinase
PA3957	PA3957	3.5	Putative enzymes	dehydrogenases
PA5380	<i>gbdR</i>	3.5	Transcription	positive regulation of cellular amino acid metabolic process
PA0230	<i>pcaB</i>	3.5	Xenobiotics biodegradation and metabolism	Benzoate degradation
PA1886	<i>polB</i>	3.5	DNA replication and repair	DNA replication
PA3568	PA3568	3.5	Carbohydrate metabolism	Propanoate metabolism
PA2557	PA2557	3.4	Lipid metabolism	Fatty acid and phospholipid metabolism
PA3420	PA3420	3.4	Transcription	Transcriptional regulators
PA5168	<i>dctQ</i>	3.4	Signal transduction	Two-component system
PA1417	PA1417	3.4	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA0745	PA0745	3.3	Putative enzymes	phenylacetate catabolic process
PA4913	PA4913	3.3	Membrane transport	ABC transporters
PA4036	PA4036	3.2	Signal transduction	Two-component system
PA4821	PA4821	3.2	Membrane proteins	Transport
PA3562	<i>fruI</i>	3.2	Carbohydrate metabolism	Fructose and mannose metabolism
PA3561	<i>fruK</i>	3.2	Carbohydrate metabolism	Fructose and mannose metabolism
PA4726.2	<i>P30</i>	3.2	Non-coding RNA	Non-coding RNA
PA1976	<i>ercS</i>	3.2	Signal transduction	phosphorelay sensor kinase
PA3760	PA3760	3.2	Non-coding RNA	Non-coding RNA
PA4293	<i>pprA</i>	3.2	Signal transduction	Two-component system
PA0860	PA0860	3.2	Membrane transport	ABC transporters
PA2121	PA2121	3.2	Transcription	Transcriptional

PA5544	PA5544	3.1	Membrane proteins	regulators
PA4726.1	<i>crcZ</i>	3.1	Non-coding RNA	Transport
PA3521	PA3521	3.1	Membrane proteins	Non-coding RNA
PA3522	PA3522	3.1	Membrane proteins	Transport
PA3523	PA3523	3.1	Membrane proteins	Transport
PA4650	<i>cupE3</i>	3.1	Membrane proteins	Cell wall / LPS / capsule; Motility & Attachment
PA1375	<i>pdxB</i>	3.1	Biosynthesis of cofactors	Vitamin B6 metabolism
PA3061	<i>pelD</i>	3.1	Membrane proteins	Cell wall / LPS / capsule; Motility & Attachment
PA3060	<i>pelE</i>	3.1	Membrane proteins	Cell wall / LPS / capsule; Motility & Attachment
PA3059	<i>pelF</i>	3.1	Membrane proteins	Cell wall / LPS / capsule; Motility & Attachment
PA1930	PA1930	3.1	Signal transduction	Two-component system
PA0164	PA0164	3.1	Amino acid metabolism	Taurine and hypotaurine metabolism
PA2891	<i>atuF</i>	3.1	Lipid metabolism	acetyl-CoA carboxylase complex
PA2597	PA2597	3.0	Putative enzymes	oxidoreductase activity
PA4207	<i>mexI</i>	3.0	Membrane proteins	Transport
PA4208	<i>opmD</i>	3.0	Membrane proteins	Transport
PA2016	<i>liuR</i>	3.0	Transcription	Transcriptional regulators
PA5356	<i>glcC</i>	3.0	Transcription	Transcriptional regulators
PA4078	PA4078	3.0	Secreted Factors (toxins, enzymes, alginate)	Adaptation, Protection
PA2958.1	<i>rgsA</i>	2.9	Non-coding RNA	Non-coding RNA
PA2264	PA2264	2.9	Carbohydrate metabolism	Pentose phosphate pathway
PA3516	PA3516	2.9	Nucleotide metabolism	Purine metabolism
PA3517	PA3517	2.9	Nucleotide metabolism	Purine metabolism
PA2263	PA2263	2.9	Carbohydrate metabolism	Pentose phosphate pathway
PA4236	<i>katA</i>	2.9	Amino acid metabolism	Tryptophan metabolism
PA1400	PA1400	2.9	Energy metabolism	gluconeogenesis
PA5355	<i>glcD</i>	2.9	Carbohydrate metabolism	Glyoxylate and dicarboxylate

PA5354	<i>glcE</i>	2.9	Carbohydrate metabolism	metabolism Glyoxylate and dicarboxylate metabolism
PA3504	PA3504	2.9	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1860	PA1860	2.9	Putative enzymes	Secondary metabolites biosynthesis
PA0195	<i>pntAA</i>	2.9	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA1973	<i>pqqF</i>	2.9	Biosynthesis of cofactors, prosthetic groups and carriers	pyrroloquinoline quinone biosynthesis
PA0195.1	<i>pntAB</i>	2.8	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA0196	<i>pntB</i>	2.8	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA0179	PA0179	2.8	Signal transduction	Two-component system
PA5072	PA5072	2.8	Signal transduction	Two-component system
PA0043	PA0043	2.8	Membrane proteins	Unknown
PA3393	<i>nosD</i>	2.8	Energy metabolism	Nitrogen metabolism
PA3394	<i>nosF</i>	2.8	Energy metabolism	ATPase
PA3395	<i>nosY</i>	2.8	Membrane transport	ABC transporters
PA3392	<i>nosZ</i>	2.8	Energy metabolism	Nitrogen metabolism
PA4520	PA4520	2.8	Signal transduction	Two-component system
PA1875	PA1875	2.8	Protein secretion	porin activity
PA1876	PA1876	2.8	Membrane transport	ABC transporters
PA1877	PA1877	2.8	Protein secretion	protein secretion by the type I secretion system
PA2008	<i>fahA</i>	2.8	Amino acid metabolism	Tyrosine metabolism
PA2007	<i>maiA</i>	2.8	Amino acid metabolism	Tyrosine metabolism
PA3032	<i>snrI</i>	2.8	Energy metabolism	generation of precursor metabolites and energy
PA3995	PA3995	2.8	Transcription	Transcriptional regulators
PA3038	PA3038	2.8	Transcription	Transcriptional regulators
PA1759	PA1759	2.8	Transcription	Transcriptional regulators
PA4084	<i>cupB3</i>	2.8	Motility & Attachment	Motility & Attachment
PA4083	<i>cupB4</i>	2.8	Motility & Attachment	Motility & Attachment

PA4500	PA4500	2.8	Membrane transport	ABC transporters
PA5213	<i>gcvPI</i>	2.8	Amino acid metabolism	Glycine and serine and threonine metabolism
PA4909	PA4909	2.7	Membrane transport	ABC transporters
PA4910	PA4910	2.7	Membrane transport	ABC transporters
PA4911	PA4911	2.7	Membrane transport	ABC transporters
PA2133	PA2133	2.7	Signal transduction	Cyclic-guanylate-specific phosphodiesterase
PA2129	<i>cupA2</i>	2.7	Motility & Attachment	Motility & Attachment
PA2130	<i>cupA3</i>	2.7	Motility & Attachment	Motility & Attachment
PA2131	<i>cupA4</i>	2.7	Motility & Attachment	Motility & Attachment
PA2132	<i>cupA5</i>	2.7	Motility & Attachment	Motility & Attachment
PA0502	PA0502	2.7	Biosynthesis of cofactors	Biotin metabolism
PA0503	PA0503	2.7	Biosynthesis of cofactors	Biotin metabolism
PA0501	<i>bioF</i>	2.7	Biosynthesis of cofactors	Biotin metabolism
PA2369	PA2369	2.7	Membrane proteins	protein secretion by the type VI secretion system
PA1861	<i>modC</i>	2.7	Membrane transport	ABC transporters
PA2244	<i>pslN</i>	2.7	DNA Replication and repair	Replication and repair
PA0743	PA0743	2.7	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2858	PA2858	2.7	Membrane proteins	transport
PA3723	PA3723	2.7	Putative enzymes	
PA2920	PA2920	2.7	Signal transduction	Two-component system
PA0349	PA0349	2.7	Membrane proteins	Transport
PA0451	PA0451	2.7	Membrane proteins	protease IV activity
PA0452	PA0452	2.7	Membrane proteins	negative regulation of proteolysis
PA2246	<i>bkdR</i>	2.6	Transcription	Transcriptional regulators
PA1617	PA1617	2.6	Putative enzymes	menaquinone biosynthetic process
PA5048	PA5048	2.6	Putative enzymes	
PA1990	<i>pqqH</i>	2.6	Putative enzymes	
PA3534	PA3534	2.6	Putative enzymes	formate metabolic process
PA3667	PA3667	2.6	Putative enzymes	cysteine desulfurase activity
PA2873	<i>tgpA</i>	2.5	Membrane proteins	protein-glutamine gamma-glutamyltransferase activity

PA2009	<i>hmgA</i>	2.5	Amino acid metabolism	Tyrosine metabolism
PA2072	PA2072	2.5	Signal transduction	GGDEF, EAL,PAS
PA0007	PA0007	2.5	Membrane proteins	Transport
PA4397	panE	2.5	Biosynthesis of cofactors	Pantothenate and CoA biosynthesis
PA3758	PA3758	2.5	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA3759	PA3759	2.5	Putative enzymes	peptidoglycan biosynthetic process
PA3921	PA3921	2.5	Transcription	Transcriptional regulators
PA3560	<i>fruA</i>	2.5	Carbohydrate metabolism	Fructose and mannose metabolism
PA5442	PA5442	2.5	Signal transduction	cyclase activity
PA0328	<i>aaaA</i>	2.5	Amino acid metabolism	protein secretion by the type V secretion system
PA1108	PA1108	2.5	Membrane proteins	Transport
PA0172	<i>siaA</i>	2.5	Signal transduction	Motility & Attachment
PA1258	PA1258	2.4	Membrane proteins	ectoine transport
PA2084	PA2084	2.4	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA0809	PA0809	2.4	Membrane proteins	Transport
PA4143	PA4143	2.4	Membrane transport	ABC transporters
PA4144	PA4144	2.4	Drug resistance	beta-Lactam resistance
PA4344	PA4344	2.4	Amino acid metabolism	Phenylalanine metabolism
PA5112	<i>estA</i>	2.4	Lipid metabolism	carboxylic ester hydrolase
PA2477	PA2477	2.4	Membrane proteins	
PA2478	PA2478	2.4	Membrane proteins	oxidation-reduction process
PA2476	<i>dsbG</i>	2.4	Folding, sorting and degradation	protein folding
PA2867	PA2867	2.4	signal transduction	Chemotaxis
PA1703	<i>pcrD</i>	2.4	Membrane transport	Bacterial secretion system
PA1704	<i>pcrR</i>	2.4	Transcription	Transcriptional regulators
PA0159	PA0159	2.4	Transcription	Transcriptional regulators
PA4845	<i>dipZ</i>	2.4	Folding, sorting and degradation	protein folding
PA3372	PA3372	2.4	Amino acid metabolism	Phosphonate and phosphinate metabolism
PA3373	PA3373	2.4	Carbohydrate	Pentose phosphate

PA3374	PA3374	2.4	metabolism Amino acid metabolism	pathway Phosphonate and phosphinate metabolism
PA3375	PA3375	2.4	Amino acid metabolism	Phosphonate and phosphinate metabolism
PA3340	PA3340	2.4	Membrane proteins	
PA2912	PA2912	2.4	Membrane transport	ABC transporters
PA2913	PA2913	2.4	Membrane transport	ABC transporters
PA2914	PA2914	2.4	Membrane transport	ABC transporters
PA2371	PA2371	2.4	Membrane transport	Bacterial secretion system
PA4147	<i>acoR</i>	2.4	Transcription	Transcriptional regulators
PA0334	PA0334	2.4	Membrane proteins	Transport
PA2443	<i>sdaA</i>	2.4	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1735	PA1735	2.4	Membrane proteins	
PA1386	PA1386	2.4	Membrane transport	ABC transporters
PA2918	PA2918	2.4	Putative enzymes	fatty acid biosynthetic process
PA5153	PA5153	2.3	Membrane transport	
PA4988	<i>waaA</i>	2.3	Cell wall / LPS / capsule	Glycan biosynthesis and metabolism
PA1373	<i>fabF2</i>	2.3	Lipid metabolism	Fatty acid biosynthesis
PA4733	<i>acsB</i>	2.3	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA2622	<i>cspD</i>	2.3	Transcription	Transcriptional regulators
PA1880	PA1880	2.3	Putative enzymes	
PA3920	PA3920	2.3	Membrane proteins	Transport
PA4982	PA4982	2.3	signal transduction	Two-component regulatory systems
PA3607	<i>potA</i>	2.3	Membrane proteins	Transport
PA3924	PA3924	2.3	Fatty acid metabolism	menaquinone biosynthetic process
PA1339	PA1339	2.3	Membrane transport	ABC transporters
PA1340	PA1340	2.3	Membrane transport	ABC transporters
PA1341	PA1341	2.3	Membrane transport	ABC transporters
PA3972	PA3972	2.3	Putative enzymes	
PA3973	PA3973	2.3	Transcription	Transcriptional regulators
PA2442	<i>gcvT2</i>	2.3	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1327	PA1327	2.3	Putative enzymes	
PA2938	PA2938	2.3	Membrane proteins	Transport
PA5170	<i>arcD</i>	2.3	Membrane proteins	Transport

PA3893	PA3893	2.3	Membrane proteins	
PA3894	PA3894	2.3	Membrane proteins	
PA4986	PA4986	2.3	Putative enzymes	
PA1217	PA1217	2.3	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA5470	PA5470	2.3	Translation	translational termination
PA5471	PA5471	2.3	Drug resistance	beta-Lactam resistance
PA3429	PA3429	2.3	Putative enzymes	photosynthesis
PA3430	PA3430	2.3	Putative enzymes	L-methionine salvage from methylthioadenosine
PA3418	<i>ldh</i>	2.2	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA1280	PA1280	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA1276	<i>cobC</i>	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA1275	<i>cobD</i>	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA1278	<i>cobP</i>	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA1277	<i>cobQ</i>	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA1279	<i>cobU</i>	2.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA3535	PA3535	2.2	Putative enzymes	protein secretion by the type V secretion system
PA0704	PA0704	2.2	Amino acid metabolism	Arginine and proline metabolism
PA4930	<i>alr</i>	2.2	Amino acid metabolism	D-Alanine metabolism
PA2638	<i>nuoB</i>	2.2	Energy metabolism	Oxidative phosphorylation
PA1336	PA1336	2.2	Signal transduction	Two-component system
PA0871	<i>phhB</i>	2.2	Amino acid metabolism	tetrahydrobiopterin biosynthetic process
PA0870	<i>phhC</i>	2.2	Amino acid metabolism	Cysteine and methionine metabolism
PA1636	<i>kdpD</i>	2.2	Signal transduction	Two-component system

PA4808	<i>selA</i>	2.2	Amino acid metabolism	Selenocompound metabolism
PA4807	<i>selB</i>	2.2	Translation	translational elongation
PA4978	PA4978	2.2	Putative enzymes	acetyl-CoA catabolic process
PA3462	PA3462	2.2	Signal transduction	Two-component system
PA1342	PA1342	2.2	Membrane transport	ABC transporters
PA0180	<i>cttP</i>	2.2	Signal transduction	Two-component system
PA0799	PA0799	2.2	Putative enzymes	
PA3352	PA3352	2.2	Motility & Attachment	Flagellar assembly
PA5421	<i>fdhA</i>	2.1	Xenobiotics biodegradation and metabolism	Chloroalkane and chloroalkene degradation
PA2571	PA2571	2.1	Signal transduction	Two-component system
PA0872	<i>phhA</i>	2.1	Amino acid metabolism	Phenylalanine metabolism
PA0297	<i>spuA</i>	2.1	Amino acid metabolism	GMP biosynthetic process
PA0811	PA0811	2.1	Membrane proteins	Transport
PA3974	<i>ladS</i>	2.1	signal transduction	Two-component regulatory systems
PA1760	PA1760	2.1	Transcription	Transcriptional regulators
PA0575	PA0575	2.1	signal transduction	GGDEF, EAL,PAS
PA4505	PA4505	2.1	Membrane transport	ABC transporters
PA4506	PA4506	2.1	Membrane transport	ABC transporters
PA2475	PA2475	2.1	Xenobiotics biodegradation and metabolism	Bisphenol degradation
PA0917	<i>kup</i>	2.1	Membrane proteins	Transport
PA1997	PA1997	2.1	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2238	<i>pslH</i>	2.1	Motility & Attachment	Cell wall / LPS / capsule
PA2239	<i>pslI</i>	2.1	Motility & Attachment	Cell wall / LPS / capsule
PA3211	PA3211	2.0	Membrane transport	ABC transporters
PA3212	PA3212	2.0	Membrane transport	ABC transporters
PA1914	PA1914	2.0	Putative enzymes	
PA4659	PA4659	2.0	Transcription	Transcriptional regulators
PA4660	<i>phr</i>	2.0	DNA replication and repair	
PA3860	PA3860	2.0	Putative enzymes	
PA0397	PA0397	2.0	Membrane proteins	Transport
PA0366	PA0366	2.0	Putative enzymes	oxidoreductase

PA5508	pauA7	2.0	Amino acid metabolism	activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
PA2367	PA2367	2.0	Membrane transport	Alanine and aspartate and glutamate metabolism
PA0899	aruB	2.0	Amino acid metabolism	Bacterial secretion system
PA0898	aruD	2.0	Amino acid metabolism	Arginine and proline metabolism
PA2633	PA2633	2.0	Phage, transposon, or plasmid	Arginine and proline metabolism
PA2640	nuoE	2.0	Energy metabolism	
PA2641	nuoF	2.0	Energy metabolism	Oxidative phosphorylation
PA1951	PA1951	2.0	Signal transduction	Oxidative phosphorylation
PA1046	PA1046	2.0	Putative enzymes	Motility & Attachment
PA2126	cgrC	2.0	Transcription	
PA3495	nth	1.9	DNA Replication and repair	Transcriptional regulators
PA1991	PA1991	1.9	Putative enzymes	Base excision repair
PA1992	ercS	1.9	signal transduction	lactaldehyde reductase activity
PA2439	PA2439	1.9	Membrane proteins	Two-component regulatory systems
PA5103	puuR	1.9	Membrane transport	negative regulation of proteolysis
PA4112	PA4112	1.9	Signal transduction	ABC transporters
PA4590	pra	1.9	Membrane proteins	PAS probable sensor/response regulator hybrid
PA2482	PA2482	1.9	Energy metabolism	Transport
PA3311	PA3311	1.9	Membrane proteins	oxidation-reduction process
PA1931	PA1931	1.9	Nucleotide metabolism	Transport
PA1932	PA1932	1.9	Nucleotide metabolism	Purine metabolism
PA1933	PA1933	1.9	Nucleotide metabolism	Purine metabolism
PA0081	fha1	1.9	Membrane transport	Purine metabolism
PA0584	cca	1.9	Transcription	Bacterial secretion system
PA5511	mifR	1.9	Transcription	tRNA
				adenylyltransferase activity
				Transcriptional regulators

PA5512	<i>mifS</i>	1.9	Transcription	Transcriptional regulators
PA3909	<i>eddB</i>	1.9	Nucleotide metabolism	DNA catabolic process
PA5272	<i>cyaA</i>	1.9	Nucleotide metabolism	Purine metabolism
PA2024	PA2024	1.8	Putative enzymes	catechol 2,3-dioxygenase activity
PA1074	<i>braC</i>	1.8	Membrane transport	ABC transporters
PA4010	PA4010	1.8	DNA Replication and repair	Base excision repair
PA4011	PA4011	1.8	Membrane proteins	
PA4284	<i>recB</i>	1.8	DNA Replication and repair	Homologous recombination
PA4285	<i>recC</i>	1.8	DNA Replication and repair	Homologous recombination
PA4283	<i>recD</i>	1.8	DNA Replication and repair	Homologous recombination
PA3961	PA3961	1.8	Putative enzymes	ATP-dependent helicase
PA5050	<i>priA</i>	1.8	DNA Replication and repair	Homologous recombination
PA2570	<i>lecA</i>	1.8	Cell wall / LPS / capsule	response to stimulus
PA2573	PA2573	1.8	Signal transduction	Two-component system
PA4633	PA4633	1.8	Signal transduction	Two-component system
PA2528	<i>muxA</i>	1.8	Signal transduction	Two-component system
PA2527	<i>muxB</i>	1.8	Signal transduction	Two-component system
PA2526	<i>muxC</i>	1.8	Signal transduction	Two-component system
PA1181	PA1181	1.8	signal transduction	cyclase activity
PA1562	<i>acnA</i>	1.8	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1423	<i>bdlA</i>	1.8	Signal transduction	Two-component system
PA2494	<i>mexF</i>	1.7	Membrane proteins	transport
PA2495	<i>oprN</i>	1.7	Membrane proteins	transport
PA2572	PA2572	1.7	Transcription	regulation of nitrogen utilization
PA0476	PA0476	1.7	Membrane proteins	transport
PA2361	PA2361	1.7	Membrane transport	Bacterial secretion system
PA2362	PA2362	1.7	Membrane transport	Bacterial secretion system
PA4624	<i>cdrB</i>	1.7	Motility & Attachment	Cell wall / LPS / capsule
PA3526	PA3526	1.7	Membrane proteins	
PA0324	PA0324	1.7	Membrane transport	ABC transporters

PA0325	PA0325	1.7	Membrane transport	ABC transporters
PA0326	PA0326	1.7	Membrane transport	ABC transporters
PA1946	<i>rbsB</i>	1.7	Membrane transport	ABC transporters
PA5181	PA5181	1.7	Putative enzymes	
PA3225	PA3225	1.7	Transcription	Transcriptional regulators
PA2177	PA2177	1.7	Signal transduction	Two-component system
PA1242	<i>sprP</i>	1.7	Translation	protein secretion
PA2231	<i>pslA</i>	1.7	Cell wall / LPS / capsule	Cell wall / LPS / capsule
PA2232	<i>pslB</i>	1.7	Cell wall / LPS / capsule	Fructose and mannose metabolism
PA2233	<i>pslC</i>	1.7	Cell wall / LPS / capsule	Cell wall / LPS / capsule
PA3465	PA3465	1.7	Membrane proteins	
PA1085	<i>flgJ</i>	1.7	Motility & Attachment	Cell wall / LPS / capsule
PA4163	PA4163	1.7	Amino acid metabolism	Arginine and proline metabolism
PA0901	<i>aruE</i>	1.6	Amino acid metabolism	Arginine and proline metabolism
PA2620	<i>clpA</i>	1.6	Translation	ATP binding
PA4856	<i>retS</i>	1.6	Signal transduction	phosphorelay response regulator activity
PA5113	PA5113	1.6	Membrane proteins	
PA5114	PA5114	1.6	Motility & Attachment	hydrolase activity, acting on glycosyl bonds
PA3341	PA3341	1.6	Transcription	
PA3272	PA3272	1.6	DNA replication and repair	
PA1337	<i>ansB</i>	1.6	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA4550	<i>fimU</i>	1.6	Motility & Attachment	pilus assembly
PA4551	<i>pilV</i>	1.6	Motility & Attachment	membrane
PA4552	<i>pilW</i>	1.6	Motility & Attachment	type IV pilus biogenesis
PA4553	<i>pilX</i>	1.6	Motility & Attachment	type IV pilus-dependent motility
PA3930	<i>cioA</i>	1.6	Energy metabolism	Oxidative phosphorylation
PA0387	PA0387	1.6	Nucleotide metabolism	Purine metabolism
PA0447	<i>gcdH</i>	1.6	Lipid metabolism	Fatty acid degradation
PA4880	PA4880	1.6	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA2642	<i>nuoG</i>	1.6	Energy metabolism	Oxidative phosphorylation

PA2643	<i>nuoH</i>	1.6	Energy metabolism	Oxidative phosphorylation
PA2652	PA2652	1.5	Signal transduction	Two-component system
PA3690	PA3690	1.5	Membrane proteins	
PA0459	PA0459	1.5	Translation	
PA4108	PA4108	1.5	Signal transduction	cyclic di-GMP phosphodiesterase
PA3858	PA3858	1.5	Membrane transport	ABC transporters
PA4951	<i>orn</i>	1.5	Transcription	RNA metabolic process
PA5173	<i>arcC</i>	1.5	Nucleotide metabolism	Purine metabolism
PA3351	<i>flgM</i>	1.5	Motility & Attachment	Flagellar assembly
PA2235	<i>pslE</i>	1.5	Motility & Attachment	Cell wall / LPS / capsule
PA2236	<i>pslF</i>	1.5	Motility & Attachment	Cell wall / LPS / capsule
PA2237	<i>pslG</i>	1.5	Motility & Attachment	Cell wall / LPS / capsule
PA4309	<i>pctA</i>	1.4	Signal transduction	Two-component system
PA5265	PA5265	1.4	Membrane proteins	
PA5266	PA5266	1.4	Membrane transport	Bacterial secretion system
PA4496	PA4496	1.4	Membrane transport	ABC transporters
PA1041	PA1041	1.4	Membrane proteins	type VI protein secretion system complex
PA5546	PA5546	1.4	Putative enzymes	3-demethylubiquinone-9 3-O-methyltransferase activity
PA4310	<i>pctB</i>	1.4	Signal transduction	Two-component system
PA3857	<i>pcs</i>	1.4	Lipid metabolism	Glycerophospholipid metabolism
PA5261	<i>algR</i>	1.4	Signal transduction	Two-component system
PA0156	<i>triA</i>	1.3	Antibiotic resistance	transport
PA0157	<i>triB</i>	1.3	Membrane proteins	transport
PA0158	<i>triC</i>	1.3	Antibiotic resistance	metal ion:hydrogen antiporter activity
PA0771	<i>era</i>	1.3	Translation	ribosome binding
PA0770	<i>rnc</i>	1.3	Transcription	RNA processing
PA2618	PA2618	1.3	Translation	
PA2654	PA2654	1.3	Signal transduction	Two-component system
PA5171	<i>arcA</i>	1.3	Amino acid metabolism	Arginine and proline metabolism

PA0782	<i>putA</i>	1.3	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA4915	PA4915	1.3	Signal transduction	Two-component system
PA2897	PA2897	1.2	Transcription	cellular modified amino acid biosynthetic process
PA3115	<i>fimV</i>	1.2	Motility & Attachment	
PA2634	<i>aceA</i>	1.2	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA5172	<i>arcB</i>	1.2	Amino acid metabolism	Arginine and proline metabolism
PA1458	PA1458	1.2	Signal transduction	Two-component system
PA4655	<i>hemH</i>	1.2	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA3353	PA3353	1.1	c-di-GMP-binding protein	c-di-GMP-binding protein
PA2071	<i>fusA2</i>	1.0	Translation	translational elongation
PA2621	<i>clpS</i>	1.0	Antibiotic resistance	bacterial-type flagellar swarming motility
PA2939	PA2939	1.0		
PA3349	PA3349	0.9	Signal transduction	Two-component system
PA4751	<i>ftsH</i>	-0.9	Translation	ATP-dependent Zn proteases
PA0576	<i>rpoD</i>	-1.0	Transcription	regulation of transcription, DNA-templated
PA3459	PA3459	-1.0	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA0426	<i>mexB</i>	-1.1	Drug resistance	beta-Lactam resistance
PA0595	<i>ostA</i>	-1.1	Membrane proteins	ABC transporters
PA0593	<i>pdxA</i>	-1.1	Biosynthesis of cofactors	Vitamin B6 metabolism
PA0594	<i>surA</i>	-1.1	Translation	
PA0266	<i>gabT</i>	-1.1	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA3257	<i>prc</i>	-1.2	Translation	
PA0971	<i>tolA</i>	-1.2	Membrane proteins	intrinsic component of periplasmic side of plasma membrane
PA0972	<i>tolB</i>	-1.2	Membrane transport	

PA5077	<i>opgH</i>	-1.2	Motility & Attachment	osmoregulated periplasmic glucan biosynthetic process
PA4403	<i>secA</i>	-1.2	Folding, sorting and degradation	Protein export
PA2069	PA2069	-1.2	Putative enzymes	
PA3648	<i>opr86</i>	-1.2	Membrane transport	
PA2760	<i>oprQ</i>	-1.2	Motility & Attachment	
PA5312	<i>pauC</i>	-1.2	Amino acid metabolism	Arginine and proline metabolism
PA1580	<i>gltA</i>	-1.2	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4938	<i>purA</i>	-1.3	Nucleotide metabolism	Purine metabolism
PA2951	<i>etfA</i>	-1.3	Energy metabolism	
PA2952	<i>etfB</i>	-1.3	Energy metabolism	
PA4406	<i>lpxC</i>	-1.3	Cell wall / LPS / capsule	Glycan biosynthesis and metabolism
PA4168	<i>fpvB</i>	-1.3	Membrane transport	
PA5227.1	<i>ssrS</i>	-1.3	Non-coding RNA	
PA1787	<i>acnB</i>	-1.3	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA3477	<i>rhlR</i>	-1.3	Transcription	Transcriptional regulators
PA1770	<i>ppsA</i>	-1.3	Carbohydrate metabolism	Pyruvate metabolism
PA3014	<i>faoA</i>	-1.4	Lipid metabolism	Fatty acid degradation
PA0004	<i>gyrB</i>	-1.4	DNA replication and repair	
PA0003	<i>recF</i>	-1.4	DNA Replication and repair	Homologous recombination
PA0001	<i>dnaA</i>	-1.4	Signal transduction	Two-component system
PA5240	<i>trxA</i>	-1.4	Translation	
PA2735	PA2735	-1.4	Putative enzymes	
PA2623	<i>icd</i>	-1.4	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4919	<i>pncB1</i>	-1.4	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA4514	PA4514	-1.5	Membrane transport	
PA2743	<i>infC</i>	-1.5	Translation	
PA2744	<i>thrS</i>	-1.5	Translation	Aminoacyl-tRNA biosynthesis
PA0456	PA0456	-1.5	Transcription	
PA5541	<i>pyrQ</i>	-1.5	Nucleotide metabolism	Pyrimidine metabolism
PA3769	<i>guaA</i>	-1.5	Nucleotide metabolism	Purine metabolism
PA4747	<i>secG</i>	-1.6	Folding, sorting and degradation	Protein export

PA1793	<i>ppiB</i>	-1.6	Translation	
PA3138	<i>uvrB</i>	-1.6	DNA Replication and repair	Nucleotide excision repair
PA4749	<i>glmM</i>	-1.6	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA3482	<i>metG</i>	-1.6	Amino acid metabolism	Selenocompound metabolism
PA5119	<i>glnA</i>	-1.6	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA5322	<i>algC</i>	-1.6	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA5161	PA5161	-1.6	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5163	<i>rmlA</i>	-1.6	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5164	<i>rmlC</i>	-1.6	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5162	<i>rmlD</i>	-1.6	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5117	<i>typA</i>	-1.6	Adaptation, Protection	cell adhesion involved in single-species biofilm formation
PA2304	<i>ambC</i>	-1.6	Secreted Factors (toxins, enzymes, alginate)	
PA2303	<i>ambD</i>	-1.6	Secreted Factors (toxins, enzymes, alginate)	
PA4848	<i>accC</i>	-1.6	Lipid metabolism	Fatty acid biosynthesis
PA4922	<i>azu</i>	-1.6	Energy metabolism	oxidation-reduction process
PA5013	<i>ilvE</i>	-1.6	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA4761	<i>dnaK</i>	-1.6	Folding, sorting and degradation	RNA degradation
PA2976	<i>rne</i>	-1.6	Folding, sorting and degradation	RNA degradation
PA4567	<i>rpmA</i>	-1.6	Translation	Ribosome
PA2742	<i>rpmI</i>	-1.7	Translation	Ribosome
PA4568	<i>rplU</i>	-1.7	Translation	Ribosome
PA1583	<i>sdhA</i>	-1.7	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA0316	<i>serA</i>	-1.7	Amino acid metabolism	Glycine and serine and threonine metabolism
PA4249	<i>rpsH</i>	-1.7	Translation	Ribosome
PA3700	<i>lysS</i>	-1.7	Translation	Aminoacyl-tRNA biosynthesis
PA3168	<i>gyrA</i>	-1.7	DNA replication and repair	
PA1302	PA1302	-1.7	Membrane transport	
PA1246	<i>aprD</i>	-1.7	Membrane transport	ABC transporters
PA1247	<i>aprE</i>	-1.7	Secreted Factors (toxins, enzymes, alginate)	

PA1750	PA1750	-1.7	Amino acid metabolism	Phenylalanine and tyrosine and tryptophan biosynthesis
PA3724	<i>lasB</i>	-1.7	Secreted Factors (toxins, enzymes, alginate)	
PA5016	<i>aceF</i>	-1.7	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0973	<i>oprL</i>	-1.7	Membrane transport	
PA3333	<i>fabH2</i>	-1.7	Lipid metabolism	Fatty acid biosynthesis
PA0985	<i>pyoS5</i>	-1.7	Membrane proteins	
PA3166	<i>pheA</i>	-1.8	Amino acid metabolism	Phenylalanine and tyrosine and tryptophan biosynthesis
PA3167	<i>serC</i>	-1.8	Amino acid metabolism	Glycine and serine and threonine metabolism
PA4744	<i>infB</i>	-1.8	Translation	
PA1804	<i>hupB</i>	-1.8	DNA replication and repair	
PA5239	<i>rho</i>	-1.8	Folding, sorting and degradation	RNA degradation
PA3163	<i>cmk</i>	-1.8	Nucleotide metabolism	Pyrimidine metabolism
PA3165	<i>hisC2</i>	-1.8	Amino acid metabolism	Histidine metabolism
PA3001	PA3001	-1.8	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA1586	<i>sucB</i>	-1.8	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA0963	<i>aspS</i>	-1.8	Translation	Aminoacyl-tRNA biosynthesis
PA0852	<i>cbpD</i>	-1.8	Secreted Factors (toxins, enzymes, alginate)	
PA5128	<i>secB</i>	-1.9	Folding, sorting and degradation	Protein export
PA4450	<i>murA</i>	-1.9	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA1777	<i>oprF</i>	-1.9	Membrane transport	
PA4670	<i>prs</i>	-1.9	Carbohydrate metabolism	Pentose phosphate pathway
PA3408	<i>hasR</i>	-1.9	Membrane transport	
PA3162	<i>rpsA</i>	-1.9	Translation	Ribosome
PA1800	<i>tig</i>	-1.9	Cell division	protein folding
PA3330	PA3330	-2.0	Putative enzymes	
PA3331	PA3331	-2.0	Xenobiotics biodegradation and metabolism	Bisphenol degradation
PA2741	<i>rplT</i>	-2.0	Translation	Ribosome
PA3149	<i>wbpH</i>	-2.0	Motility & Attachment	Cell wall / LPS / capsule
PA3148	<i>wbpI</i>	-2.0	Carbohydrate	Amino sugar and

			metabolism	nucleotide sugar metabolism
PA2389	<i>pvdR</i>	-2.0	Membrane transport	
PA2390	<i>pvdT</i>	-2.0	Membrane transport	ABC transporters
PA4248	<i>rplF</i>	-2.0	Translation	Ribosome
PA5496	<i>nrdJb</i>	-2.0	Nucleotide metabolism	
PA0547	PA0547	-2.0	Transcription	
PA5505	PA5505	-2.0	Membrane transport	ABC transporters
PA5497	<i>nrdJa</i>	-2.0	Nucleotide metabolism	Purine metabolism
PA1178	<i>oprH</i>	-2.0	Adaptation, Protection	lipopolysaccharide binding
PA0291	<i>oprE</i>	-2.0	Membrane transport	
PA3790	<i>oprC</i>	-2.1	Membrane transport	
PA0792	<i>prpD</i>	-2.1	Carbohydrate metabolism	Propanoate metabolism
PA3150	<i>wbpG</i>	-2.1	Motility & Attachment	Cell wall / LPS / capsule
PA0139	<i>ahpC</i>	-2.1	Adaptation, Protection	alkyl hydroperoxide reductase activity
PA4615	PA4615	-2.1	Putative enzymes	phenylacetate catabolic process
PA3158	<i>wbpB</i>	-2.1	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA4370	<i>icmP</i>	-2.1	Membrane proteins	
PA0904	<i>lysC</i>	-2.1	Amino acid metabolism	Glycine and serine and threonine metabolism
PA5323	<i>argB</i>	-2.1	Amino acid metabolism	Arginine and proline metabolism
PA5539	PA5539	-2.1	Biosynthesis of cofactors	Folate biosynthesis
PA2851	<i>efp</i>	-2.1	Translation	translational elongation
PA4432	<i>rpsI</i>	-2.1	Translation	Ribosome
PA3792	<i>leuA</i>	-2.1	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA4263	<i>rplC</i>	-2.2	Translation	Ribosome
PA4065	PA4065	-2.2	Membrane transport	transport
PA0964	<i>pmpR</i>	-2.2	Transcription	
PA3529	PA3529	-2.2	Adaptation, Protection	peroxiredoxin
PA4442	<i>cysN</i>	-2.2	Nucleotide metabolism	Purine metabolism
PA4264	<i>rpsJ</i>	-2.2	Translation	Ribosome
PA0969	<i>tolQ</i>	-2.2	Drug resistance	Microbial metabolism in diverse environments
PA4433	<i>rplM</i>	-2.2	Translation	Ribosome
PA1493	<i>cysP</i>	-2.3	Energy metabolism	Sulfur metabolism
PA5530	PA5530	-2.3	Membrane transport	

PA4740	<i>pnp</i>	-2.3	Nucleotide metabolism	Purine metabolism
PA4222	PA4222	-2.3	Membrane transport	ABC transporters
PA4223	PA4223	-2.3	Membrane transport	ABC transporters
PA4226	<i>pchE</i>	-2.3	Biosynthesis secondary metabolites	pyochelin biosynthe
PA4225	<i>pchF</i>	-2.3	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4224	<i>pchG</i>	-2.3	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA3686	<i>adk</i>	-2.3	Nucleotide metabolism	Purine metabolism
PA3865.1	PA3865.1	-2.3	Adaptation, Protection	
PA3866	<i>lpdG</i>	-2.3	Adaptation, Protection	
PA4774	PA4774	-2.4	Amino acid metabolism	Cysteine and methionine metabolism
PA3190	PA3190	-2.4	Membrane transport	ABC transporters
PA3117	<i>asd</i>	-2.4	Amino acid metabolism	Glycine and serine and threonine metabolism
PA4773	PA4773	-2.4	Amino acid metabolism	Cysteine and methionine metabolism
PA2411	PA2411	-2.4	Adaptation, Protection	Secondary metabolites biosynthesis
PA1584	<i>sdhB</i>	-2.4	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA2532	<i>tpx</i>	-2.4	Adaptation, Protection	response to stimulus
PA4709	<i>phuS</i>	-2.4	Putative enzymes	
PA4272	<i>rplJ</i>	-2.4	Translation	Ribosome
PA5569	<i>rnpA</i>	-2.4	Translation	tRNA processing
PA4741	<i>rpsO</i>	-2.5	Translation	Ribosome
PA5554	<i>atpD</i>	-2.5	Energy metabolism	Oxidative phosphorylation
PA1432	<i>lasI</i>	-2.5	Adaptation, Protection	transferase activity, transferring acyl groups other than amino-acyl groups
PA4243	<i>secY</i>	-2.5	Folding, sorting and degradation	Protein export
PA5030	PA5030	-2.5	Membrane transport	
PA1847	<i>nfuA</i>	-2.5	Adaptation, Protection	
PA3182	<i>pgl</i>	-2.5	Carbohydrate metabolism	Pentose phosphate pathway
PA3183	<i>zwf</i>	-2.5	Carbohydrate metabolism	Pentose phosphate pathway
PA0002	<i>dnaN</i>	-2.5	Nucleotide metabolism	Purine metabolism
PA4268	<i>rpsL</i>	-2.5	Translation	Ribosome

PA3452	<i>mqaA</i>	-2.6	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA3181	PA3181	-2.6	Carbohydrate metabolism	Pentose phosphate pathway
PA1588	<i>sucC</i>	-2.6	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1589	<i>sucD</i>	-2.6	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4270	<i>rpoB</i>	-2.6	Nucleotide metabolism	Purine metabolism
PA5150	PA5150	-2.6	Putative enzymes	fatty acid biosynthetic process
PA4277	<i>tufB</i>	-2.6	Translation	
PA1776	<i>sigX</i>	-2.6	Transcription	
PA1713	<i>exsA</i>	-2.6	Transcription	
PA1585	<i>sucA</i>	-2.6	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4239	<i>rpsD</i>	-2.6	Translation	Ribosome
PA4704.1	<i>prfF1</i>	-2.6	Non-coding RNA	
PA1775	<i>cmpX</i>	-2.6	Membrane proteins	
PA4745	<i>nusA</i>	-2.6	Transcription	
PA3013	<i>foaB</i>	-2.7	Lipid metabolism	Fatty acid degradation
PA0970	<i>tolR</i>	-2.7	Membrane transport	
PA4833	PA4833	-2.7	Membrane proteins	
PA3656	<i>rpsB</i>	-2.7	Translation	Ribosome
PA3194	<i>edd</i>	-2.7	Carbohydrate metabolism	Pentose phosphate pathway
PA3049	<i>rmf</i>	-2.7	Translation	
PA4238	<i>rpoA</i>	-2.7	Nucleotide metabolism	Purine metabolism
PA2385	<i>pvdQ</i>	-2.7	Adaptation, Protection	amidase activity
PA2624	<i>idh</i>	-2.7	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4269	<i>rpoC</i>	-2.8	Nucleotide metabolism	Purine metabolism
PA5429	<i>aspA</i>	-2.8	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA0652	<i>vfr</i>	-2.8	Signal transduction	Two-component system
PA5559	<i>atpE</i>	-2.8	Energy metabolism	Oxidative phosphorylation
PA5015	<i>aceE</i>	-2.8	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA4273	<i>rplA</i>	-2.8	Translation	Ribosome
PA4274	<i>rplK</i>	-2.8	Translation	Ribosome
PA4934	<i>rpsR</i>	-2.9	Translation	Ribosome
PA4260	<i>rplB</i>	-2.9	Translation	Ribosome
PA5558	<i>atpF</i>	-2.9	Energy metabolism	Oxidative phosphorylation
PA4231	<i>pchA</i>	-2.9	Biosynthesis of	Ubiquinone and other

			cofactors	terpenoid-quinone biosynthesis
PA4230	<i>pchB</i>	-2.9	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4229	<i>pchC</i>	-2.9	Secreted Factors (toxins, enzymes, alginate)	
PA4228	<i>pchD</i>	-2.9	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA3743	<i>trmD</i>	-2.9	Transcription	
PA4267	<i>rpsG</i>	-2.9	Translation	Ribosome
PA3744	<i>rimM</i>	-2.9	Transcription	rRNA processing
PA4262	<i>rplD</i>	-2.9	Translation	Ribosome
PA4261	<i>rplW</i>	-2.9	Translation	Ribosome
PA4671	PA4671	-3.0	Translation	Ribosome
PA1249	<i>aprA</i>	-3.0	Secreted Factors (toxins, enzymes, alginate)	
PA4710	<i>phuR</i>	-3.0	Membrane transport	
PA4266	<i>fusA1</i>	-3.1	Translation	
PA3584	<i>glpD</i>	-3.1	Lipid metabolism	Glycerophospholipid metabolism
PA5557	<i>atpH</i>	-3.1	Energy metabolism	Oxidative phosphorylation
PA4271	<i>rplL</i>	-3.1	Translation	Ribosome
PA0283	<i>sbp</i>	-3.1	Energy metabolism	Sulfur metabolism
PA3655	<i>tsf</i>	-3.1	Translation	translational elongation
PA4244	<i>rplO</i>	-3.1	Translation	Ribosome
PA0084	<i>tssC1</i>	-3.1	Protein secretion	type VI protein secretion system complex
PA0432	<i>sahH</i>	-3.1	Amino acid metabolism	Cysteine and methionine metabolism
PA4933	PA4933	-3.1	Membrane proteins	
PA3931	PA3931	-3.2	Membrane transport	ABC transporters
PA4246	<i>rpsE</i>	-3.2	Translation	Ribosome
PA4237	<i>rplQ</i>	-3.2	Translation	Ribosome
PA2397	<i>pvdE</i>	-3.3	Membrane transport	ABC transporters
PA4251	<i>rplE</i>	-3.3	Translation	
PA5556	<i>atpA</i>	-3.3	Energy metabolism	Oxidative phosphorylation
PA0730	PA0730	-3.3	Putative enzymes	3-oxoadipate enol-lactonase activity
PA4467	PA4467	-3.3	Membrane proteins	
PA4696	<i>ilvI</i>	-3.3	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA4240	<i>rpsK</i>	-3.3	Translation	Ribosome
PA4385	<i>groEL</i>	-3.3	Folding, sorting and	RNA degradation

			degradation	
PA4265	<i>tufA</i>	-3.3	Translation	translational elongation
PA4257	<i>rpsC</i>	-3.4	Translation	Ribosome
PA4694	<i>ilvC</i>	-3.4	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA4252	<i>rplX</i>	-3.4	Translation	Ribosome
PA2393	PA2393	-3.5	Amino acid metabolism	
PA2424	<i>pvdL</i>	-3.5	Adaptation, Protection	siderophore biosynthetic process
PA5555	<i>atpG</i>	-3.5	Energy metabolism	Oxidative phosphorylation
PA3814	<i>iscS</i>	-3.5	Biosynthesis of cofactors	Thiamine metabolism
PA2396	<i>pvdF</i>	-3.5	Secreted Factors (toxins, enzymes, alginate)	pyoverdine biosynthetic process
PA4156	PA4156	-3.5	Membrane transport	
PA1658	<i>hsiC2</i>	-3.6	Secreted Factors (toxins, enzymes, alginate)	protein secretion by the type VI secretion system
PA1317	<i>cyoA</i>	-3.6	Energy metabolism	Oxidative phosphorylation
PA1156	<i>nrdA</i>	-3.7	Nucleotide metabolism	Purine metabolism
PA1318	<i>cyoB</i>	-3.7	Energy metabolism	Oxidative phosphorylation
PA2399	<i>pvdD</i>	-3.7	Secreted Factors (toxins, enzymes, alginate)	siderophore biosynthetic process
PA2402	PA2402	-3.7	Putative enzymes	
PA3195	<i>gapA</i>	-3.7	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA4443	<i>cysD</i>	-3.8	Nucleotide metabolism	Purine metabolism
PA4935	<i>rpsF</i>	-3.8	Translation	Ribosome
PA2426	<i>pvdS</i>	-3.8	Transcription	sigma factor activity
PA5531	<i>tonB1</i>	-3.8	Motility & Attachment	bacterial-type flagellar swarming motility
PA2425	<i>pvdG</i>	-3.8	Adaptation, Protection	response to stimulus
PA0546	<i>metK</i>	-3.8	Amino acid metabolism	Cysteine and methionine metabolism
PA3599	PA3599	-3.8	Transcription	Transcriptional regulators
PA3407	<i>hasAp</i>	-3.8	Membrane transport	
PA0085	<i>hcp1</i>	-3.9	Membrane transport	Bacterial secretion system
PA4256	<i>rplP</i>	-3.9	Translation	Ribosome
PA4255	<i>rpmC</i>	-3.9	Translation	Ribosome
PA4175	<i>piv</i>	-3.9	Secreted Factors (toxins, enzymes, alginate)	
PA4704.2	<i>prfF2</i>	-3.9	Non-coding RNA	

PA2413	<i>pvdH</i>	-4.0	Amino acid metabolism	Glycine and serine and threonine metabolism
PA3813	<i>iscU</i>	-4.1	Energy metabolism	iron-sulfur cluster assembly
PA2400	<i>pvdJ</i>	-4.1	Adaptation, Protection	response to stimulus
PA2394	<i>pvdN</i>	-4.2	Adaptation, Protection	cysteine desulfurase activity
PA3397	<i>fpr</i>	-4.2	Biosynthesis of cofactors, prosthetic groups and carriers	ferredoxin-NADP+ reductase activity
PA2395	<i>pvdO</i>	-4.2	Adaptation, Protection	pyoverdine biosynthetic process
PA2392	<i>pvdP</i>	-4.3	Adaptation, Protection	response to stimulus
PA0423	<i>pasP</i>	-4.4	Secreted Factors (toxins, enzymes, alginate)	
PA4253	<i>rplN</i>	-4.4	Translation	Ribosome
PA1155	<i>nrdB</i>	-4.5	Nucleotide metabolism	Purine metabolism
PA2204	PA2204	-4.8	Membrane transport	
PA2386	<i>pvdA</i>	-4.8	Adaptation, Protection	response to stimulus
PA4221	<i>fptA</i>	-4.9	Membrane proteins	
PA0672	<i>hemO</i>	-5.0	Biosynthesis of cofactors, prosthetic groups and carriers	
PA4837	PA4837	-5.0	Membrane transport	
PA3478	<i>rhlB</i>	-5.2	Membrane transport	
PA3450	PA3450	-5.2	Adaptation, Protection	peroxiredoxin activity
PA4468	<i>sodM</i>	-5.3	Adaptation, Protection	superoxide dismutase activity
PA2398	<i>fpvA</i>	-5.5	Membrane transport	
PA4470	<i>fumC1</i>	-5.9	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA3479	<i>rhlA</i>	-7.5	Secreted Factors (toxins, enzymes, alginate)	
PA3600	PA3600	-8.1	Translation	Ribosome
PA3601	PA3601	-8.1	Translation	Ribosome
PA5419	<i>soxG</i>	*	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1173	<i>napB</i>	*	Energy metabolism	Nitrogen metabolism
PA1497	PA1497	*	Membrane proteins	

- represents down regulation

* represents up regulation; but fold change could not be calculated since the transcript is not present in the sample before starvation treatment.

Table C.3: Changes in gene expression during biofilm starvation

PA Number	genes	log2	Kegg category	Description
PA3519	PA3519	3.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3920	PA3920	3.3	Membrane transport	metal ion transmembrane

PA3186	<i>oprB</i>	2.3	Membrane transport	transporter
PA5152	PA5152	2.1	Membrane transport	carbohydrate transport
PA2826	PA2826	2.0	Amino acid metabolism	ectoine transport
PA2825	<i>ospR</i>	2.0	Transcription	Glutathione metabolism
PA2291	PA2291	1.9	Membrane transport	regulation of cellular response to oxidative stress
PA4913	PA4913	1.9	Membrane transport	Carbohydrate-selective porin
PA2862	<i>lipA</i>	1.7	Lipid metabolism	ABC transporters
PA5348	PA5348	1.7	Replication and repair	Glycerolipid metabolism
PA3922	PA3922	1.7	Hypothetical, unclassified, unknown	Bacterial nucleoid DNA-binding protein
PA3038	PA3038	1.7	Membrane transport	Hypothetical, unclassified, unknown
PA4497	PA4497	1.6	Membrane transport	outer membrane porin
PA3923	PA3923	1.6	Hypothetical, unclassified, unknown	ABC transporters
PA2445	<i>gcvP2</i>	1.4	Amino acid metabolism	Hypothetical, unclassified, unknown
PA4496	PA4496	1.3	Membrane transport	Glycine and serine and threonine metabolism
PA3188	PA3188	1.3	Membrane transport	ABC transporters
PA3189	PA3189	1.3	Membrane transport	ABC transporters
PA1074	<i>braC</i>	1.3	Membrane transport	ABC transporters
PA5100	<i>hutU</i>	1.2	Amino acid metabolism	ABC transporters
PA0833	PA0833	1.2	Membrane proteins	Histidine metabolism
PA5112	<i>estA</i>	1.2	Secreted Factors (toxins, enzymes, alginate); Fatty acid and phospholipid metabolism; Protein secretion/export apparatus; Motility & Attachment	type VI protein secretion system complex
PA5056	<i>phaC1</i>	1.2	Carbohydrate metabolism	carboxylic ester hydrolase activity
PA0300	<i>spuD</i>	1.1	Membrane transport	Butanoate metabolism
PA5153	PA5153	1.1	Membrane transport	ABC transporters
PA3187	PA3187	1.1	Membrane transport	ABC transporters
PA0506	PA0506	1.0	Metabolism of terpenoids and polyketides	ABC transporters
PA2567	PA2567	1.0	Hypothetical, unclassified, unknown	Geraniol degradation
PA3190	PA3190	0.9	Membrane transport	Hypothetical, unclassified, unknown
				ABC transporters

PA0958	<i>oprD</i>	0.8	Drug resistance	beta-Lactam resistance
PA3309	PA3309	-0.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2976	<i>rne</i>	-0.8	Folding, sorting and degradation	RNA degradation
PA5170	<i>arcD</i>	-0.9	Membrane transport	arginine:ornithine antiporter activity
PA3162	<i>rpsA</i>	-0.9	Translation	Ribosome
PA1324	PA1324	-0.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3692	<i>lptF</i>	-0.9	Membrane proteins	peptidoglycan precursor
PA1414	PA1414	-0.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1155	<i>nrdB</i>	-0.9	Nucleotide metabolism	Purine metabolism
PA5475	PA5475	-1.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4262	<i>rplD</i>	-1.0	Translation	Ribosome
PA4261	<i>rplW</i>	-1.0	Translation	Ribosome
PA5474	PA5474	-1.0	Translation	Ribosome
PA4433	<i>rplM</i>	-1.0	Translation	Ribosome
PA4243	<i>secY</i>	-1.0	Folding, sorting and degradation	Protein export
PA3572	PA3572	-1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0084	<i>tssCI</i>	-1.1	Membrane proteins	type VI protein secretion system complex
PA4264	<i>rpsJ</i>	-1.1	Translation	Ribosome
PA4248	<i>rplF</i>	-1.1	Translation	Ribosome
PA2381	PA2381	-1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4263	<i>rplC</i>	-1.2	Translation	Ribosome
PA3397	<i>fpr</i>	-1.2	Energy metabolism	ferredoxin-NADP+ reductase activity
PA5173	<i>arcC</i>	-1.2	Nucleotide metabolism	Purine metabolism
PA3120	<i>leuD</i>	-1.2	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA5172	<i>arcB</i>	-1.3	Amino acid metabolism	Arginine and proline metabolism
PA2851	<i>efp</i>	-1.3	Translation	translational elongation
PA3744	<i>rimM</i>	-1.3	Transcription	rRNA processing
PA5171	<i>arcA</i>	-1.4	Amino acid metabolism	Arginine and proline metabolism

PA4568	<i>rplU</i>	-1.4	Translation	Ribosome
PA2171	PA2171	-1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5297	<i>poxB</i>	-1.5	Carbohydrate metabolism	Pyruvate metabolism
PA2204	PA2204	-1.5	Membrane transport	ABC transporters
PA4748	<i>tpiA</i>	-1.5	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3743	<i>trmD</i>	-1.6	Transcription	methyltransferase activity
PA4670	<i>prs</i>	-1.6	Carbohydrate metabolism	Pentose phosphate pathway
PA5227.1	<i>ssrS</i>	-1.7	Non-coding RNA	Non-coding RNA
PA2753	PA2753	-1.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5034	<i>hemE</i>	-1.7	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA5569	<i>rnpA</i>	-1.8	Translation	tRNA processing
PA2262	PA2262	-1.8	Membrane proteins	Carbohydrate transport
PA4563	<i>rpsT</i>	-1.9	Translation	Ribosome
PA2840	PA2840	-2.4	Folding, sorting and degradation	RNA degradation
PA3938	PA3938	-2.5	Energy metabolism	Sulfur metabolism
PA3790	<i>oprC</i>	-3.0	Membrane transport	Fe transport
PA3530	<i>bfd</i>	*	Membrane proteins	bacterioferritin-associated ferredoxin
PA0980	PA0980	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0014	PA0014	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1353	PA1353	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3684	PA3684	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown

- represents down regulation

* represents up regulation; but fold change could not be calculated since the transcript is not present in the sample before starvation treatment.

represents down regulation; but fold change could not be calculated since the transcript is not present in the sample after starvation treatment.

Table C.4: Differential gene expression with fold change during planktonic to biofilm lifestyle change.

PA Number	gene	log2	Kegg category	Description
PA0519	<i>nirS</i>	8.9	Energy metabolism	Nitrogen metabolism
PA3305.1	<i>phrS</i>	7.5	Non-coding RNA	
PA5475	PA5475	6.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4587	<i>ccpR</i>	6.0	Energy metabolism	cytochrome-c peroxidase
PA0524	<i>norB</i>	5.8	Energy metabolism	Nitrogen metabolism
PA0523	<i>norC</i>	5.8	Energy metabolism	Nitrogen metabolism
PA4352	PA4352	5.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0141	PA0141	5.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5027	PA5027	5.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1746	PA1746	5.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5427	<i>adhA</i>	5.2	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0512	PA0512	5.2	Energy metabolism	Biosynthesis of cofactors
PA0513	PA0513	5.2	Energy metabolism	Biosynthesis of cofactors
PA0515	PA0515	5.2	Energy metabolism	Biosynthesis of cofactors
PA0511	<i>nirJ</i>	5.2	Biosynthesis of cofactors	Heme, porphyrin
PA0514	<i>nirL</i>	5.2	Transcription	Transcriptional regulators
PA1556	<i>ccoO2</i>	5.0	Energy metabolism	Oxidative phosphorylation
PA0517	<i>nirC</i>	5.0	Energy metabolism	Biosynthesis of cofactors
PA0516	<i>nirF</i>	5.0	Energy metabolism	Biosynthesis of cofactors
PA0518	<i>nirM</i>	5.0	Energy metabolism	Sulfur metabolism
PA3309	PA3309	4.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3393	<i>nosD</i>	4.7	Energy metabolism	nitrogen cycle metabolic process
PA3394	<i>nosF</i>	4.7	Energy metabolism	daunorubicin-transporting ATPase activity
PA3395	<i>nosY</i>	4.7	Energy metabolism	
PA3392	<i>nosZ</i>	4.7	Energy metabolism	Nitrogen metabolism
PA1557	<i>ccoN2</i>	4.7	Energy metabolism	Oxidative phosphorylation
PA2119	PA2119	4.6	Putative enzymes	L-threonine 3-dehydrogenase activity

PA4348	PA4348	4.4	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3613	PA3613	4.4	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4133	PA4133	4.3	Energy metabolism	Oxidative phosphorylation
PA1546	<i>hemN</i>	4.2	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA4131	PA4131	4.1	Energy metabolism	respiratory chain complex IV assembly
PA4236	<i>katA</i>	4.1	Amino acid metabolism	Tryptophan metabolism
PA4067	<i>oprG</i>	4.1	Membrane proteins	
PA3391	<i>nosR</i>	4.0	Membrane proteins	
PA2662	PA2662	3.9	Membrane proteins	
PA2663	<i>ppyR</i>	3.9	Membrane proteins	
PA4235	<i>finA</i>	3.9	Metabolism of cofactors and vitamins	Porphyrin metabolism
PA5171	<i>arcA</i>	3.9	Amino acid metabolism	Arginine and proline metabolism
PA4328	PA4328	3.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2265	PA2265	3.8	Carbohydrate metabolism	Pentose phosphate pathway
PA3531	<i>bfrB</i>	3.8	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA3573	PA3573	3.7	Adaptation, Protection	drug transport
PA5170	<i>arcD</i>	3.7	Membrane proteins	arginine:ornithine antiporter activity
PA1414	PA1414	3.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1551	PA1551	3.6	Energy metabolism	respiratory chain complex IV assembly
PA0200	PA0200	3.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5173	<i>arcC</i>	3.5	Nucleotide metabolism	Purine metabolism
PA1172	<i>napC</i>	3.4	Energy metabolism	nitrate reductase activity
PA5172	<i>arcB</i>	3.3	Amino acid metabolism	Arginine and proline metabolism
PA0195.	<i>pntAB</i>	3.2	Metabolism of	Nicotinate and nicotinamide

1			cofactors and vitamins	metabolism
PA0196	<i>pntB</i>	3.2	Metabolism of cofactors and vitamins	Nicotinate and nicotinamide metabolism
PA4129	PA4129	3.2	Adaptation, Protection	
PA4130	PA4130	3.2	Energy metabolism	Sulfur metabolism
PA5100	<i>hutU</i>	3.2	Amino acid metabolism	Histidine metabolism
PA3415	PA3415	3.2	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0879	PA0879	3.2	Lipid metabolism	Fatty acid degradation
PA3572	PA3572	3.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4429	PA4429	3.1	Energy metabolism	Oxidative phosphorylation
PA4430	PA4430	3.1	Energy metabolism	Oxidative phosphorylation
PA4431	PA4431	3.1	Energy metabolism	Oxidative phosphorylation
PA1415	PA1415	3.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3337	<i>rfaD</i>	3.1	Energy metabolism	Lipopolysaccharide biosynthesis
PA2264	PA2264	3.1	Carbohydrate metabolism	Pentose phosphate pathway
PA5208	PA5208	3.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1789	PA1789	3.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0836	<i>ackA</i>	3.0	Amino acid metabolism	Taurine and hypotaurine metabolism
PA5051	<i>argS</i>	3.0	Translation	Aminoacyl-tRNA biosynthesis
PA4880	PA4880	3.0	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA4812	<i>fdnG</i>	2.9	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA2646	<i>nuoK</i>	2.9	Energy metabolism	Oxidative phosphorylation
PA2647	<i>nuoL</i>	2.9	Energy metabolism	Oxidative phosphorylation
PA1896	PA1896	2.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3416	PA3416	2.9	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3417	PA3417	2.9	Carbohydrate	Glycolysis or

			metabolism	Gluconeogenesis
PA1429	PA1429	2.9	Membrane proteins	
PA1897	PA1897	2.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5267	<i>hcpB</i>	2.8	Membrane transport	Bacterial secretion system
PA4542	<i>clpB</i>	2.8	Translation	denatured protein binding
PA3188	PA3188	2.7	Membrane transport	ABC transporters
PA3189	PA3189	2.7	Membrane transport	ABC transporters
PA2639	<i>nuoD</i>	2.7	Energy metabolism	Oxidative phosphorylation
PA0263	<i>hcpC</i>	2.7	Membrane transport	Bacterial secretion system
PA2247	<i>bkdA1</i>	2.7	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2248	<i>bkdA2</i>	2.7	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA3187	PA3187	2.7	Membrane transport	ABC transporters
PA1512	<i>hcpA</i>	2.7	Membrane transport	Bacterial secretion system
PA0887	<i>acsA</i>	2.7	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0865	<i>hpd</i>	2.7	Metabolism of cofactors and vitamins	Ubiquinone and other terpenoid-quinone biosynthesis
PA0409	<i>pilH</i>	2.6	Signal transduction	Two-component system
PA5474	PA5474	2.6	Translation	pyrroloquinoline quinone biosynthetic process
PA2323	PA2323	2.6	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0451	PA0451	2.6	Membrane proteins	protease IV activity
PA0452	PA0452	2.6	Membrane proteins	negative regulation of proteolysis
PA1174	<i>napA</i>	2.6	Energy metabolism	Nitrogen metabolism
PA1175	<i>napD</i>	2.6	Energy metabolism	nitrate reductase activity
PA1176	<i>napF</i>	2.6	Energy metabolism	oxidation-reduction process
PA4333	PA4333	2.5	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4525	<i>pilA</i>	2.5	Signal transduction	Two-component system
PA3614	PA3614	2.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4611	PA4611	2.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4571	PA4571	2.5	Energy metabolism	oxidation-reduction process

PA2638	<i>nuoB</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA1927	<i>metE</i>	2.4	Amino acid metabolism	Cysteine and methionine metabolism
PA1748	PA1748	2.4	Lipid metabolism	Fatty acid degradation
PA2637	<i>nuoA</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA3879	<i>narL</i>	2.4	Signal transduction	Two-component system
PA3878	<i>narX</i>	2.4	Signal transduction	Two-component system
PA2642	<i>nuoG</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA2643	<i>nuoH</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA2640	<i>nuoE</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA2641	<i>nuoF</i>	2.4	Energy metabolism	Oxidative phosphorylation
PA5231	PA5231	2.3	Membrane proteins	daunorubicin-transporting ATPase activity
PA5232	PA5232	2.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0391	PA0391	2.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5355	<i>glcD</i>	2.3	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA5354	<i>glcE</i>	2.3	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA1562	<i>acnA</i>	2.3	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA0527	<i>dnr</i>	2.3	Transcription	Transcriptional regulators
PA5479	<i>gltP</i>	2.3	Membrane transport	
PA2262	PA2262	2.3	Membrane proteins	
PA1596	<i>htpG</i>	2.3	Chaperones	heat shock protein
PA3120	<i>leuD</i>	2.3	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA0408	<i>pilG</i>	2.2	Signal transduction	Two-component system
PA3038	PA3038	2.2	Membrane transport	
PA2648	<i>nuoM</i>	2.2	Energy metabolism	Oxidative phosphorylation
PA2195	<i>hcnC</i>	2.2	Amino acid metabolism	Cyanoamino acid metabolism
PA4550	<i>fimU</i>	2.2	Motility & Attachment	pilus assembly
PA4551	<i>pilV</i>	2.2	Motility & Attachment	membrane
PA4552	<i>pilW</i>	2.2	Motility & Attachment	type IV pilus biogenesis
PA4553	<i>pilX</i>	2.2	Motility & Attachment	type IV pilus-dependent motility
PA1552	<i>ccoPI</i>	2.2	Energy metabolism	Oxidative phosphorylation
PA1552.1	<i>ccoQI</i>	2.2	Energy metabolism	Oxidative phosphorylation

PA0411	<i>pilJ</i>	2.1	Signal transduction	Two-component system
PA5528	PA5528	2.1	Membrane proteins	
PA2322	PA2322	2.1	Membrane transport	
PA4132	PA4132	2.1	Adaptation, Protection	cellular modified amino acid biosynthetic process
PA4527	<i>pilC</i>	2.1	Motility & Attachment	
PA0996	<i>pqsA</i>	2.0	Lipid metabolism	
PA0997	<i>pqsB</i>	2.0	Fatty acid metabolism	
PA0998	<i>pqsC</i>	2.0	Fatty acid metabolism	
PA3418	<i>ldh</i>	2.0	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA5490	<i>cc4</i>	2.0	Energy metabolism	electron carrier
PA3121	<i>leuC</i>	1.9	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA2649	<i>nuoN</i>	1.9	Energy metabolism	Oxidative phosphorylation
PA0999	<i>pqsD</i>	1.9	Lipid metabolism	Biosynthesis of cofactors
PA1000	<i>pqsE</i>	1.9	Energy metabolism	Biosynthesis of cofactors
PA4296	<i>pprB</i>	1.9	Transcription	phosphate-containing compound metabolic process
PA0387	PA0387	1.9	Nucleotide metabolism	Purine metabolism
PA0388	PA0388	1.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4715	PA4715	1.9	Putative enzymes	
PA0604	<i>agtB</i>	1.9	Membrane transport	ABC transporters
PA5035	<i>gltD</i>	1.9	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA4746	PA4746	1.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5044	<i>pilM</i>	1.9	Motility & Attachment	type IV pilus biogenesis
PA5043	<i>pilN</i>	1.9	Motility & Attachment	type IV pilus biogenesis
PA5042	<i>pilO</i>	1.9	Motility & Attachment	type IV pilus-dependent motility
PA5041	<i>pilP</i>	1.9	Motility & Attachment	type IV pilus-dependent motility
PA5494	PA5494	1.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1544	<i>anr</i>	1.8	Transcription	
PA0459	PA0459	1.8	Translation	

PA3186	<i>oprB</i>	1.8	Membrane transport	
PA4022	<i>hdhA</i>	1.8	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA0579	<i>rpsU</i>	1.8	Translation	Ribosome
PA3310	PA3310	1.8	Membrane proteins	
PA4758	<i>carA</i>	1.8	Nucleotide metabolism	Pyrimidine metabolism
PA4723	<i>dksA</i>	1.8	Transcription	
PA2381	PA2381	1.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2171	PA2171	1.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4563	<i>rpsT</i>	1.7	Translation	Ribosome
PA0366	PA0366	1.7	Putative enzymes	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
PA3821	<i>secD</i>	1.7	Folding, sorting and degradation	Protein export
PA2618	PA2618	1.7	Translation	
PA0035	<i>trpA</i>	1.7	Amino acid metabolism	Glycine and serine and threonine metabolism
PA0036	<i>trpB</i>	1.7	Amino acid metabolism	Glycine and serine and threonine metabolism
PA4670	<i>prs</i>	1.7	Carbohydrate metabolism	Pentose phosphate pathway
PA4481	<i>mreB</i>	1.7	Cell wall / LPS / capsule	
PA4262	<i>rplD</i>	1.7	Translation	Ribosome
PA4261	<i>rplW</i>	1.7	Translation	Ribosome
PA4922	<i>azu</i>	1.7	Energy metabolism	oxidation-reduction process
PA0788	PA0788	1.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3688	PA3688	1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4761	<i>dnaK</i>	1.6	Folding, sorting and degradation	RNA degradation
PA2263	PA2263	1.6	Carbohydrate metabolism	Pentose phosphate pathway
PA1894	PA1894	1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1895	PA1895	1.6	Membrane proteins	
PA4684	PA4684	1.6	Hypothetical,	Hypothetical, unclassified,

			unclassified, unknown	unknown
PA4685	PA4685	1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4686	PA4686	1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3723	PA3723	1.6	Putative enzymes	
PA3225	PA3225	1.6	Transcription	
PA4433	<i>rplM</i>	1.6	Translation	Ribosome
PA2629	<i>purB</i>	1.6	Nucleotide metabolism	Purine metabolism
PA3115	<i>fimV</i>	1.6	Membrane proteins	
PA4526	<i>pilB</i>	1.6	Motility & Attachment	
PA1658	<i>hsiC2</i>	1.6	Protein secretion	protein secretion by the type VI secretion system
PA4241	<i>rpsM</i>	1.6	Translation	Ribosome
PA2754	PA2754	1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4272	<i>rplJ</i>	1.6	Translation	Ribosome
PA1656	<i>hsiA2</i>	1.6	Protein secretion	
PA5046	PA5046	1.6	Carbohydrate metabolism	Pyruvate metabolism
PA0020	PA0020	1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3615	PA3615	1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1805	<i>ppiD</i>	1.5	Translation	
PA4602	<i>glyA3</i>	1.5	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1076	PA1076	1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4495	PA4495	1.4	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2760	<i>oprQ</i>	1.4	Membrane transport	
PA0835	<i>pta</i>	1.4	Amino acid metabolism	Taurine and hypotaurine metabolism
PA1787	<i>acnB</i>	1.4	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA5264	PA5264	1.4	Hypothetical, unclassified,	Hypothetical, unclassified, unknown

			unknown	
PA4254	<i>rpsQ</i>	1.4	Translation	Ribosome
PA3525	<i>argG</i>	1.4	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA4432	<i>rpsI</i>	1.4	Translation	Ribosome
PA5036	<i>gltB</i>	1.4	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA4568	<i>rplU</i>	1.4	Translation	Ribosome
PA1583	<i>sdhA</i>	1.4	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA0779	<i>asrA</i>	1.4	Adaptation, Protection	#N/A
PA4329	<i>pykA</i>	1.4	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA4554	<i>pilY1</i>	1.4	Motility & Attachment	
PA2851	<i>efp</i>	1.4	Translation	#N/A
PA5568	PA5568	1.3	Folding, sorting and degradation	Protein export
PA5210	PA5210	1.3	Membrane transport	Bacterial secretion system
PA1561	<i>aer</i>	1.3	Signal transduction	Two-component system
PA2620	<i>clpA</i>	1.3	Translation	#N/A
PA5436	PA5436	1.3	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1288	PA1288	1.3	Membrane proteins	
PA5555	<i>atpG</i>	1.3	Energy metabolism	Oxidative phosphorylation
PA4932	<i>rplI</i>	1.3	Translation	Ribosome
PA4740	<i>pnp</i>	1.3	Nucleotide metabolism	Purine metabolism
PA4578	PA4578	1.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4249	<i>rpsH</i>	1.3	Translation	Ribosome
PA5557	<i>atpH</i>	1.3	Energy metabolism	Oxidative phosphorylation
PA4523	PA4523	1.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0353	<i>ilvD</i>	1.2	Amino acid metabolism	Valine and leucine and isoleucine biosynthesis
PA1554	<i>ccoN1</i>	1.2	Energy metabolism	Oxidative phosphorylation
PA0401	PA0401	1.2	Nucleotide metabolism	Pyrimidine metabolism
PA0402	<i>pyrB</i>	1.2	Nucleotide metabolism	Pyrimidine metabolism
PA4266	<i>fusA1</i>	1.2	Translation	translational elongation
PA0958	<i>oprD</i>	1.2	Lipid metabolism	Carboxylesterase type B
PA5040	<i>pilQ</i>	1.2	Motility & Attachment	type IV pilus biogenesis

PA4744	<i>infB</i>	1.2	Translation	cellular protein metabolic process
PA5192	<i>pckA</i>	1.2	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA4263	<i>rplC</i>	1.2	Translation	Ribosome
PA4366	<i>sodB</i>	1.2	Adaptation, Protection	phosphotransferase activity, nitrogenous group as acceptor
PA5560	<i>atpB</i>	1.1	Energy metabolism	Oxidative phosphorylation
PA5242	<i>ppk</i>	1.1	Energy metabolism	Oxidative phosphorylation
PA4311	PA4311	1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4312	PA4312	1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4313	PA4313	1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4934	<i>rpsR</i>	1.1	Translation	Ribosome
PA4656	PA4656	1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4655	<i>hemH</i>	1.1	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA4919	<i>pncB1</i>	1.1	Metabolism of cofactors and vitamins	Nicotinate and nicotinamide metabolism
PA4671	PA4671	1.1	Translation	Ribosome
PA5554	<i>atpD</i>	1.0	Energy metabolism	Oxidative phosphorylation
PA4240	<i>rpsK</i>	1.0	Translation	Ribosome
PA0588	PA0588	1.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4264	<i>rpsJ</i>	1.0	Translation	Ribosome
PA4606	PA4606	1.0	Adaptation, Protection	
PA0316	<i>serA</i>	1.0	Amino acid metabolism	Glycine and serine and threonine metabolism
PA1609	<i>fabB</i>	0.9	Lipid metabolism	Fatty acid biosynthesis
PA5556	<i>atpA</i>	0.9	Energy metabolism	Oxidative phosphorylation
PA4251	<i>rplE</i>	0.9	Translation	Ribosome
PA0291	<i>oprE</i>	0.9	Membrane proteins	Porin
PA4238	<i>rpoA</i>	0.9	Nucleotide metabolism	Purine metabolism
PA4277	<i>tufB</i>	0.9	Translation	translational elongation
PA3149	<i>wbpH</i>	0.9	Cell wall / LPS / capsule	glycogen (starch) synthase activity
PA3148	<i>wbpI</i>	0.9	Carbohydrate	Amino sugar and nucleotide

PA4385	<i>groEL</i>	0.9	metabolism Folding, sorting and degradation	sugar metabolism RNA degradation
PA4253	<i>rplN</i>	0.9	Translation	Ribosome
PA3190	PA3190	0.9	Membrane transport	ABC transporters
PA1800	<i>tig</i>	0.9	Cell wall / LPS / capsule	protein folding
PA4246	<i>rpsE</i>	0.9	Translation	Ribosome
PA4463	PA4463	0.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3656	<i>rpsB</i>	0.8	Translation	Ribosome
PA3622	<i>rpoS</i>	-0.8	Transcription	sigma factor activity
PA0506	PA0506	-1.0	Metabolism of terpenoids and polyketides	Geraniol degradation
PA5227.1	<i>ssrS</i>	-1.0	Non-coding RNA	Non-coding RNA
PA3397	<i>fpr</i>	-1.0	Biosynthesis of cofactors	ferredoxin-NADP+ reductase activity
PA5178	PA5178	-1.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2160	PA2160	-1.0	Carbohydrate metabolism	Starch and sucrose metabolism
PA5312	<i>pauC</i>	-1.0	Amino acid metabolism	Arginine and proline metabolism
PA0139	<i>ahpC</i>	-1.0	Adaptation, Protection	alkyl hydroperoxide reductase activity
PA0059	<i>osmC</i>	-1.1	Adaptation, Protection	response to oxidative stress
PA2794	PA2794	-1.1	Cell wall / LPS / capsule	
PA3729	PA3729	-1.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0546	<i>metK</i>	-1.1	Amino acid metabolism	Cysteine and methionine metabolism
PA0084	<i>tssC1</i>	-1.1	Protein secretion	type VI protein secretion system complex
PA0426	<i>mexB</i>	-1.2	Drug resistance	beta-Lactam resistance
PA2445	<i>gcvP2</i>	-1.2	Amino acid metabolism	Glycine and serine and threonine metabolism
PA3013	<i>foaB</i>	-1.2	Lipid metabolism	Fatty acid degradation
PA4614	<i>mscL</i>	-1.2	Membrane proteins	
PA3476	<i>rhII</i>	-1.2	Adaptation, Protection	transferase activity, transferring acyl groups other than amino-acyl groups

PA0266	<i>gabT</i>	-1.2	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA5161	PA5161	-1.3	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis
PA5163	<i>rmlA</i>	-1.3	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis
PA5164	<i>rmlC</i>	-1.3	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis
PA5162	<i>rmlD</i>	-1.3	Biosynthesis of other secondary metabolites	Streptomycin biosynthesis
PA4726.11	<i>crcZ</i>	-1.3	Non-coding RNA	Non-coding RNA
PA0563	PA0563	-1.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2423	PA2423	-1.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0547	PA0547	-1.4	Transcription	biotin biosynthetic process
PA4370	<i>icmP</i>	-1.4	Membrane proteins	
PA3814	<i>iscS</i>	-1.4	Metabolism of cofactors and vitamins	Thiamine metabolism
PA2062	PA2062	-1.4	Metabolism of cofactors and vitamins	Thiamine metabolism
PA3795	PA3795	-1.4	Putative enzymes	
PA4843	PA4843	-1.4	Signal transduction	cyclase
PA4973	<i>thiC</i>	-1.4	Metabolism of cofactors and vitamins	Thiamine metabolism
PA3623	PA3623	-1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1939	PA1939	-1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1093	PA1093	-1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1156	<i>nrdA</i>	-1.5	Nucleotide metabolism	Purine metabolism
PA4687	<i>hitA</i>	-1.5	Membrane transport	ABC transporters
PA0085	<i>hcp1</i>	-1.5	Membrane	Bacterial secretion system

			transport	
PA3460	PA3460	-1.5	Putative enzymes	
PA3461	PA3461	-1.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3014	<i>faoA</i>	-1.6	Lipid metabolism	Fatty acid degradation
PA0660	PA0660	-1.6	Energy metabolism	Nitrogen metabolism
PA2372	PA2372	-1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4115	PA4115	-1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5441	PA5441	-1.6	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1151	<i>imm2</i>	-1.7	Adaptation, Protection	
PA1505	<i>moaA2</i>	-1.7	Metabolism of cofactors and vitamins	Folate biosynthesis
PA2946	PA2946	-1.7	Membrane proteins	
PA5061	PA5061	-1.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2592	PA2592	-1.7	Membrane transport	ABC transporters
PA2540	PA2540	-1.7	Lipid metabolism	3-oxoadipate enol-lactonase activity
PA0432	<i>sahH</i>	-1.8	Amino acid metabolism	Cysteine and methionine metabolism
PA3477	<i>rhIR</i>	-1.8	Transcription	
PA2304	<i>ambC</i>	-1.8	Secreted Factors (toxins, enzymes, alginate)	
PA2303	<i>ambD</i>	-1.8	Secreted Factors (toxins, enzymes, alginate)	
PA1244	PA1244	-1.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3485	PA3485	-1.9	Adaptation, Protection	
PA2367	PA2367	-1.9	Membrane transport	Bacterial secretion system
PA3450	PA3450	-1.9	Adaptation, Protection	peroxiredoxin activity
PA2483	PA2483	-1.9	Putative enzymes	oxidoreductase activity
PA4176	<i>ppiC2</i>	-1.9	Translation	protein folding
PA2302	<i>ambE</i>	-1.9	Secreted Factors (toxins, enzymes, alginate)	NotAvailable
PA5057	<i>phaD</i>	-1.9	Energy metabolism	carbon utilization

PA5023	PA5023	-2.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3311	PA3311	-2.0	Signal transduction	cyclase
PA0792	<i>prpD</i>	-2.0	Carbohydrate metabolism	Propanoate metabolism
PA2366	PA2366	-2.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3385	<i>amrZ</i>	-2.0	Transcription	DNA binding
PA2365	PA2365	-2.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4324	PA4324	-2.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0805	PA0805	-2.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4515	PA4515	-2.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4615	PA4615	-2.2	Putative enzymes	phenylacetate catabolic process
PA3815	<i>iscR</i>	-2.2	Adaptation, Protection	iron-sulfur cluster binding
PA0315	PA0315	-2.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1190	PA1190	-2.2	Membrane proteins	
PA4833	PA4833	-2.2	Membrane proteins	cytolysis
PA5030	PA5030	-2.3	Membrane proteins	xenobiotic transporter activity
PA2204	PA2204	-2.3	Membrane transport	
PA0423	<i>pasP</i>	-2.3	Secreted Factors (toxins, enzymes, alginate)	
PA1287	PA1287	-2.3	Amino acid metabolism	Glutathione metabolism
PA1432	<i>lasI</i>	-2.3	Adaptation, Protection	transferase activity, transferring acyl groups other than amino-acyl groups
PA3584	<i>glpD</i>	-2.3	Lipid metabolism	Glycerophospholipid metabolism
PA2407	PA2407	-2.4	Membrane transport	ABC transporters
PA2408	PA2408	-2.4	Membrane transport	ABC transporters
PA2409	PA2409	-2.4	Membrane transport	ABC transporters

PA2410	PA2410	-2.4	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1901	<i>phzC2</i>	-2.4	Amino acid metabolism	Phenylalanine and tyrosine and tryptophan biosynthesis
PA1902	<i>phzD2</i>	-2.4	Secreted Factors (toxins, enzymes, alginate)	phenazine biosynthetic process
PA1903	<i>phzE2</i>	-2.4	Secreted Factors (toxins, enzymes, alginate)	phenazine biosynthetic process
PA4212	<i>phzC1</i>	-2.4	Amino acid metabolism	Phenylalanine and tyrosine and tryptophan biosynthesis
PA4213	<i>phzD1</i>	-2.4	Secreted Factors (toxins, enzymes, alginate)	phenazine biosynthetic process
PA4214	<i>phzE1</i>	-2.4	Secreted Factors (toxins, enzymes, alginate)	phenazine biosynthetic process
PA3049	<i>rmf</i>	-2.5	Translation	
PA2560	PA2560	-2.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4514	PA4514	-2.5	Membrane transport	
PA1131	PA1131	-2.5	Membrane proteins	
PA1130	<i>rhIC</i>	-2.5	Cell wall / LPS / capsule	transferase activity
PA4217	<i>phzS</i>	-2.6	Putative enzymes	phenazine biosynthetic process
PA0039	PA0039	-2.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2786	PA2786	-2.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3813	<i>iscU</i>	-2.8	Biosynthesis of cofactors	iron-sulfur cluster assembly
PA4638	PA4638	-2.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1433	PA1433	-2.8	Signal transduction	cyclase
PA3093	PA3093	-2.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3094	PA3094	-2.8	Transcription	
PA2434	PA2434	-2.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown

PA2435	PA2435	-2.8	Membrane proteins	metal ion transmembrane transporter activity
PA0041	PA0041	-2.8	Secreted Factors (toxins, enzymes, alginate)	
PA2532	<i>tpx</i>	-2.8	Adaptation, Protection	response to stimulus
PA2414	PA2414	-2.9	Energy metabolism	
PA2415	PA2415	-2.9	Membrane proteins	
PA1148	<i>toxA</i>	-2.9	Secreted Factors (toxins, enzymes, alginate)	
PA2764	PA2764	-2.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3724	<i>lasB</i>	-2.9	Secreted Factors (toxins, enzymes, alginate)	metalloendopeptidase activity
PA0938	PA0938	-2.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2290	<i>gcd</i>	-2.9	Carbohydrate metabolism	Pentose phosphate pathway
PA0123	PA0123	-2.9	Transcription	
PA0929	PA0929	-3.0	Membrane transport	phosphate-containing compound metabolic process
PA0930	PA0930	-3.0	Membrane transport	phosphorelay signal transduction system
PA3452	<i>mqaA</i>	-3.0	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA2531	PA2531	-3.0	Amino acid metabolism	Histidine metabolism
PA0527.1	<i>rsmY</i>	-3.0	Non-coding RNA	
PA2403	PA2403	-3.0	Membrane proteins	
PA2404	PA2404	-3.0	Membrane proteins	
PA2405	PA2405	-3.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2406	PA2406	-3.0	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0707	<i>toxR</i>	-3.0	Transcription	
PA2462	PA2462	-3.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5531	<i>tonB1</i>	-3.2	Membrane transport	bacterial-type flagellar swarming motility
PA4710	<i>phuR</i>	-3.3	Membrane transport	transmembrane transporter activity
PA2394	<i>pvdN</i>	-3.3	Adaptation, Protection	cysteine desulfurase activity
PA4065	PA4065	-3.3	Membrane	transport

			transport	
PA3361	<i>lecB</i>	-3.4	Motility & Attachment	cellular component movement
PA2069	PA2069	-3.5	Putative enzymes	
PA5539	PA5539	-3.5	Metabolism of cofactors and vitamins	Folate biosynthesis
PA5540	PA5540	-3.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2389	<i>pvdR</i>	-3.7	Membrane transport	transport
PA2390	<i>pvdT</i>	-3.7	Membrane transport	ABC transporters
PA2451	PA2451	-3.8	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5541	<i>pyrQ</i>	-3.9	Nucleotide metabolism	Pyrimidine metabolism
PA4063	PA4063	-3.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2392	<i>pvdP</i>	-3.9	Adaptation, Protection	response to stimulus
PA1302	PA1302	-3.9	Membrane proteins	transmembrane transporter activity
PA3559	PA3559	-3.9	Carbohydrate metabolism	Pentose and glucuronate interconversions
PA3554	<i>arnA</i>	-3.9	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA3552	<i>arnB</i>	-3.9	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA3553	<i>arnC</i>	-3.9	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA3555	<i>arnD</i>	-3.9	Adaptation, Protection	beta-L-Ara4N-lipid A biosynthetic process
PA3557	<i>arnE</i>	-3.9	Adaptation, Protection	beta-L-Ara4N-lipid A biosynthetic process
PA3558	<i>arnF</i>	-3.9	Adaptation, Protection	beta-L-Ara4N-lipid A biosynthetic process
PA3556	<i>arnT</i>	-3.9	Adaptation, Protection	beta-L-Ara4N-lipid A biosynthetic process
PA4175	<i>piv</i>	-3.9	Secreted Factors (toxins, enzymes, alginate)	catalytic activity
PA1249	<i>aprA</i>	-3.9	Secreted Factors (toxins, enzymes, alginate)	zinc ion binding
PA4168	<i>fpvB</i>	-4.0	Membrane transport	outer membrane

PA5150	PA5150	-4.0	Fatty acid metabolism	fatty acid biosynthetic process
PA1559	PA1559	-4.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1560	PA1560	-4.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1246	<i>aprD</i>	-4.1	Secreted Factors (toxins, enzymes, alginate)	alkaline protease secretion protein AprD
PA1247	<i>aprE</i>	-4.1	Secreted Factors (toxins, enzymes, alginate)	
PA1318	<i>cyoB</i>	-4.2	Energy metabolism	Oxidative phosphorylation
PA4090	PA4090	-4.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4141	PA4141	-4.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2396	<i>pvdF</i>	-4.3	Secreted Factors (toxins, enzymes, alginate)	pyoverdine biosynthetic process
PA0672	<i>hemO</i>	-4.3	Biosynthesis of cofactors	
PA1248	<i>aprF</i>	-4.5	Secreted Factors (toxins, enzymes, alginate)	porin activity
PA1922	PA1922	-4.5	Membrane transport	cobalamin binding
PA1923	PA1923	-4.5	Metabolism of cofactors and vitamins	Porphyrin and chlorophyll metabolism
PA1924	PA1924	-4.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1925	PA1925	-4.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2386	<i>pvdA</i>	-4.5	Adaptation, Protection	response to stimulus
PA2385	<i>pvdQ</i>	-4.5	Adaptation, Protection	amidase activity
PA2397	<i>pvdE</i>	-4.6	Membrane transport	ABC transporters
PA2393	PA2393	-4.7	Amino acid metabolism	dipeptidase precursor
PA0781	PA0781	-4.7	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2400	<i>pvdJ</i>	-4.7	Adaptation,	response to stimulus

			Protection	
PA2398	<i>fpvA</i>	-4.8	Membrane proteins	transport
PA4227	<i>pchR</i>	-4.8	Transcription	
PA2411	PA2411	-4.8	Adaptation, Protection	
PA4709	<i>phuS</i>	-4.9	Putative enzymes	
PA2399	<i>pvdD</i>	-5.0	Secreted Factors (toxins, enzymes, alginate)	siderophore biosynthesis
PA3599	PA3599	-5.0	Transcription	
PA2377	PA2377	-5.1	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2402	PA2402	-5.2	Putative enzymes	
PA2424	<i>pvdL</i>	-5.2	Adaptation, Protection	
PA2426	<i>pvdS</i>	-5.2	Transcription	
PA4469	PA4469	-5.3	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4156	PA4156	-5.3	Membrane transport	
PA4468	<i>sodM</i>	-5.3	Adaptation, Protection	
PA4837	PA4837	-5.4	Membrane proteins	
PA3478	<i>rhlB</i>	-5.5	Secreted Factors (toxins, enzymes, alginate)	
PA3479	<i>rhlA</i>	-5.5	Secreted Factors (toxins, enzymes, alginate)	
PA3600	PA3600	-5.8	Translation	Ribosome
PA3601	PA3601	-5.8	Translation	Ribosome
PA4222	PA4222	-5.8	Membrane transport	ABC transporters
PA4223	PA4223	-5.8	Membrane transport	ABC transporters
PA4226	<i>pchE</i>	-5.8	Metabolism of terpenoids and polyketides	Biosynthesis of siderophore group nonribosomal peptides
PA4225	<i>pchF</i>	-5.8	Metabolism of terpenoids and polyketides	Biosynthesis of siderophore group nonribosomal peptides
PA4224	<i>pchG</i>	-5.8	Metabolism of terpenoids and polyketides	Biosynthesis of siderophore group nonribosomal peptides
PA4471	PA4471	-5.9	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4470	<i>fumC1</i>	-5.9	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA2413	<i>pvdH</i>	-5.9	Amino acid metabolism	Glycine and serine and threonine metabolism

PA4218	<i>ampP</i>	-6.5	Membrane proteins	siderophore transporter activity
PA4570	PA4570	-6.5	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4220	PA4220	-7.2	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4221	<i>fptA</i>	-7.2	Membrane proteins	transport
PA4231	<i>pchA</i>	-7.8	Secreted Factors (toxins, enzymes, alginate)	pyochelin biosynthetic process
PA4230	<i>pchB</i>	-7.8	Secreted Factors (toxins, enzymes, alginate)	metabolic process
PA4229	<i>pchC</i>	-7.8	Secreted Factors (toxins, enzymes, alginate)	pyochelin biosynthetic process
PA4228	<i>pchD</i>	-7.8	Secreted Factors (toxins, enzymes, alginate)	siderophore biosynthetic process
PA0149	PA0149	#	Transcription	
PA0150	PA0150	#	Membrane proteins	
PA0780	<i>pruR</i>	#	Transcription	
PA0980	PA0980	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1029	PA1029	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1711	<i>exsE</i>	#	Transcription	
PA1899	<i>phzA2</i>	#	Secreted Factors (toxins, enzymes, alginate)	phenazine biosynthetic process
PA2036	PA2036	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3371	PA3371	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4867	<i>ureB</i>	#	Nucleotide metabolism	Purine metabolism
PA5157	PA5157	#	Transcription	
PA1353	PA1353	#	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4708	<i>phuT</i>	#	Membrane transport	ABC transporters
PA4782	PA4782	#	Hypothetical,	Hypothetical, unclassified,

			unclassified, unknown	unknown
PA2453	PA2453	*	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0526	PA0526	*	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1173	<i>napB</i>	*	Energy metabolism	Nitrogen metabolism
PA3278	PA3278	*	Membrane proteins	
PA4577	PA4577	*	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4605	PA4605	*	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown

- represents down regulation

* represents up regulation; but fold change could not be calculated since the transcript is not present in the sample before starvation treatment.

represents down regulation; but fold change could not be calculated since the transcript is not present in the sample after starvation treatment.

Table C.5: Genes that show similar transcriptional changes for both planktonic and biofilm cells during starvation

PA Numb	gene	Kegg category	Description
PA0139	<i>ahpC</i>	Adaptation, Protection	alkyl hydroperoxide reductase activity
PA2411	PA2411	Adaptation, Protection	Secondary metabolites biosynthesis
PA2532	<i>tpx</i>	Adaptation, Protection	response to stimulus
PA1432	<i>lasI</i>	Adaptation, Protection	transferase activity, transferring acyl groups other than amino-acyl groups
PA2385	<i>pvdQ</i>	Adaptation, Protection	amidase activity
PA2424	<i>pvdL</i>	Adaptation, Protection	siderophore biosynthetic process
PA2400	<i>pvdJ</i>	Adaptation, Protection	response to stimulus
PA2394	<i>pvdN</i>	Adaptation, Protection	cysteine desulfurase activity
PA2392	<i>pvdP</i>	Adaptation, Protection	response to stimulus
PA2386	<i>pvdA</i>	Adaptation, Protection	response to stimulus
PA3450	PA3450	Adaptation,	peroxiredoxin activity

PA4468	<i>sodM</i>	Protection Adaptation, Protection	superoxide dismutase activity
PA2247	<i>bkdA1</i>	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA2248	<i>bkdA2</i>	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA5100	<i>hutU</i>	Amino acid metabolism	Histidine metabolism
PA4236	<i>katA</i>	Amino acid metabolism	Tryptophan metabolism
PA3418	<i>ldh</i>	Amino acid metabolism	Valine and leucine and isoleucine degradation
PA5171	<i>arcA</i>	Amino acid metabolism	Arginine and proline metabolism
PA5172	<i>arcB</i>	Amino acid metabolism	Arginine and proline metabolism
PA0266	<i>gabT</i>	Amino acid metabolism	Alanine and aspartate and glutamate metabolism
PA5312	<i>pauC</i>	Amino acid metabolism	Arginine and proline metabolism
PA0316	<i>serA</i>	Amino acid metabolism	Glycine and serine and threonine metabolism
PA0432	<i>sahH</i>	Amino acid metabolism	Cysteine and methionine metabolism
PA2393	PA2393	Amino acid metabolism	
PA0546	<i>metK</i>	Amino acid metabolism	Cysteine and methionine metabolism
PA2413	<i>pvdH</i>	Amino acid metabolism	Glycine and serine and threonine metabolism
PA0865	<i>hpd</i>	Biosynthesis of cofactors	Ubiquinone and other terpenoid-quinone biosynthesis
PA0195.1	<i>pntAB</i>	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA0196	<i>pntB</i>	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA4880	PA4880	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA4655	<i>hemH</i>	Biosynthesis of cofactors	Porphyrin and chlorophyll metabolism
PA4919	<i>pncBI</i>	Biosynthesis of cofactors	Nicotinate and nicotinamide metabolism
PA5539	PA5539	Biosynthesis of cofactors	Folate biosynthesis
PA4231	<i>pchA</i>	Biosynthesis of cofactors	Ubiquinone and other terpenoid-quinone biosynthesis
PA3814	<i>iscS</i>	Biosynthesis of	Thiamine metabolism

PA3397	<i>fpr</i>	cofactors Biosynthesis of cofactors, prosthetic groups and carriers	ferredoxin-NADP+ reductase activity
PA0672	<i>hemO</i>	Biosynthesis of cofactors, prosthetic groups and carriers	
PA5161	PA5161	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5163	<i>rmlA</i>	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5164	<i>rmlC</i>	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA5162	<i>rmlD</i>	Biosynthesis secondary metabolites	Streptomycin biosynthesis
PA4226	<i>pchE</i>	Biosynthesis secondary metabolites	pyochelin biosynthe
PA4225	<i>pchF</i>	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4224	<i>pchG</i>	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4230	<i>pchB</i>	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4228	<i>pchD</i>	Biosynthesis secondary metabolites	Biosynthesis of siderophore group nonribosomal peptides
PA4022	<i>hdhA</i>	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3415	PA3415	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3416	PA3416	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA3417	PA3417	Carbohydrate metabolism	Glycolysis or Gluconeogenesis
PA2265	PA2265	Carbohydrate metabolism	Pentose phosphate pathway
PA4812	<i>fdnG</i>	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA2264	PA2264	Carbohydrate metabolism	Pentose phosphate pathway
PA2263	PA2263	Carbohydrate metabolism	Pentose phosphate pathway

PA5355	<i>glcD</i>	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA5354	<i>glcE</i>	Carbohydrate metabolism	Glyoxylate and dicarboxylate metabolism
PA1562	<i>acnA</i>	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1787	<i>acnB</i>	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1583	<i>sdhA</i>	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4670	<i>prs</i>	Carbohydrate metabolism	Pentose phosphate pathway
PA3148	<i>wbpI</i>	Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism
PA0792	<i>prpD</i>	Carbohydrate metabolism	Propanoate metabolism
PA3452	<i>mqaA</i>	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA4470	<i>fumC1</i>	Carbohydrate metabolism	Citrate cycle (TCA cycle)
PA1800	<i>tig</i>	Cell division	protein folding
PA0426	<i>mexB</i>	Drug resistance	beta-Lactam resistance
PA1174	<i>napA</i>	Energy metabolism	Nitrogen metabolism
PA1175	<i>napD</i>	Energy metabolism	nitrate reductase activity
PA1176	<i>napF</i>	Energy metabolism	oxidation-reduction process
PA1172	<i>napC</i>	Energy metabolism	nitrate reductase activity
PA3393	<i>nosD</i>	Energy metabolism	Nitrogen metabolism
PA3394	<i>nosF</i>	Energy metabolism	ATPase
PA3392	<i>nosZ</i>	Energy metabolism	Nitrogen metabolism
PA2638	<i>nuoB</i>	Energy metabolism	Oxidative phosphorylation
PA2640	<i>nuoE</i>	Energy metabolism	Oxidative phosphorylation
PA2641	<i>nuoF</i>	Energy metabolism	Oxidative phosphorylation
PA2642	<i>nuoG</i>	Energy metabolism	Oxidative phosphorylation
PA2643	<i>nuoH</i>	Energy metabolism	Oxidative phosphorylation
PA4922	<i>azu</i>	Energy metabolism	oxidation-reduction process
PA5554	<i>atpD</i>	Energy metabolism	Oxidative phosphorylation
PA5557	<i>atpH</i>	Energy metabolism	Oxidative phosphorylation
PA5556	<i>atpA</i>	Energy metabolism	Oxidative phosphorylation
PA5555	<i>atpG</i>	Energy metabolism	Oxidative phosphorylation
PA1318	<i>cyoB</i>	Energy metabolism	Oxidative phosphorylation
PA3813	<i>iscU</i>	Energy metabolism	iron-sulfur cluster assembly
PA1173	<i>napB</i>	Energy metabolism	Nitrogen metabolism
PA4761	<i>dnaK</i>	Folding, sorting and degradation	RNA degradation
PA4385	<i>groEL</i>	Folding, sorting and degradation	RNA degradation
PA0788	PA0788	Hypothetical, unclassified,	Hypothetical, unclassified, unknown

		unknown	
PA5208	PA5208	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2365	PA2365	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3688	PA3688	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2366	PA2366	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3613	PA3613	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0391	PA0391	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1414	PA1414	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4611	PA4611	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0388	PA0388	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4311	PA4311	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4312	PA4312	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4313	PA4313	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4523	PA4523	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4656	PA4656	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2372	PA2372	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1789	PA1789	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5061	PA5061	Hypothetical,	Hypothetical, unclassified,

		unclassified, unknown	unknown
PA0315	PA0315	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2462	PA2462	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1939	PA1939	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4578	PA4578	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0563	PA0563	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0805	PA0805	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA5540	PA5540	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4638	PA4638	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3729	PA3729	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4141	PA4141	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA2377	PA2377	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0938	PA0938	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4515	PA4515	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA0781	PA0781	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1559	PA1559	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA1560	PA1560	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown

PA4063	PA4063	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4220	PA4220	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4090	PA4090	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4570	PA4570	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4471	PA4471	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4469	PA4469	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4577	PA4577	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA4605	PA4605	Hypothetical, unclassified, unknown	Hypothetical, unclassified, unknown
PA3014	<i>faoA</i>	Lipid metabolism	Fatty acid degradation
PA3013	<i>foaB</i>	Lipid metabolism	Fatty acid degradation
PA3584	<i>glpD</i>	Lipid metabolism	Glycerophospholipid metabolism
PA1190	PA1190	Membrane proteins	Membrane proteins
PA2262	PA2262	Membrane proteins	Transport
PA0451	PA0451	Membrane proteins	protease IV activity
PA0452	PA0452	Membrane proteins	negative regulation of proteolysis
PA5170	<i>arcD</i>	Membrane proteins	Transport
PA3311	PA3311	Membrane proteins	Transport
PA4370	<i>icmP</i>	Membrane proteins	
PA4833	PA4833	Membrane proteins	
PA4221	<i>fptA</i>	Membrane proteins	
PA3395	<i>nosY</i>	Membrane transport	ABC transporters
PA2367	PA2367	Membrane transport	Bacterial secretion system
PA4168	<i>fpvB</i>	Membrane transport	
PA4514	PA4514	Membrane transport	
PA1302	PA1302	Membrane transport	
PA1246	<i>aprD</i>	Membrane	ABC transporters

PA2389	<i>pvdR</i>	transport Membrane transport	
PA2390	<i>pvdT</i>	Membrane transport	ABC transporters
PA0291	<i>oprE</i>	Membrane transport	
PA4065	PA4065	Membrane transport	transport
PA4222	PA4222	Membrane transport	ABC transporters
PA4223	PA4223	Membrane transport	ABC transporters
PA3190	PA3190	Membrane transport	ABC transporters
PA5030	PA5030	Membrane transport	
PA4710	<i>phuR</i>	Membrane transport	
PA2397	<i>pvdE</i>	Membrane transport	ABC transporters
PA4156	PA4156	Membrane transport	
PA0085	<i>hcpI</i>	Membrane transport	Bacterial secretion system
PA2204	PA2204	Membrane transport	
PA4837	PA4837	Membrane transport	
PA3478	<i>rhlB</i>	Membrane transport	
PA2398	<i>fpvA</i>	Membrane transport	
PA4550	<i>fimU</i>	Motility & Attachment	pilus assembly
PA4551	<i>pilV</i>	Motility & Attachment	membrane
PA4552	<i>pilW</i>	Motility & Attachment	type IV pilus biogenesis
PA4553	<i>pilX</i>	Motility & Attachment	type IV pilus-dependent motility
PA3115	<i>fimV</i>	Motility & Attachment	
PA2760	<i>oprQ</i>	Motility & Attachment	
PA3149	<i>wbpH</i>	Motility & Attachment	Cell wall / LPS / capsule
PA5531	<i>tonB1</i>	Motility & Attachment	bacterial-type flagellar swarming motility
PA5227.1	<i>ssrS</i>	Non-coding RNA	

PA3305.1	<i>phrS</i>	Non-coding RNA	Non-coding RNA
PA4726.11	<i>crcZ</i>	Non-coding RNA	Non-coding RNA
PA0387	PA0387	Nucleotide metabolism	Purine metabolism
PA5173	<i>arcC</i>	Nucleotide metabolism	Purine metabolism
PA5541	<i>pyrQ</i>	Nucleotide metabolism	Pyrimidine metabolism
PA4740	<i>pnp</i>	Nucleotide metabolism	Purine metabolism
PA4238	<i>rpoA</i>	Nucleotide metabolism	Purine metabolism
PA1156	<i>nrda</i>	Nucleotide metabolism	Purine metabolism
PA0084	<i>tssC1</i>	Protein secretion	type VI protein secretion system complex
PA3723	PA3723	Putative enzymes	
PA0366	PA0366	Putative enzymes	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
PA2069	PA2069	Putative enzymes	
PA4615	PA4615	Putative enzymes	phenylacetate catabolic process
PA4709	<i>phuS</i>	Putative enzymes	
PA5150	PA5150	Putative enzymes	fatty acid biosynthetic process
PA2402	PA2402	Putative enzymes	
PA2304	<i>ambC</i>	Secreted Factors (toxins, enzymes, alginate)	
PA2303	<i>ambD</i>	Secreted Factors (toxins, enzymes, alginate)	
PA1247	<i>aprE</i>	Secreted Factors (toxins, enzymes, alginate)	
PA3724	<i>lasB</i>	Secreted Factors (toxins, enzymes, alginate)	
PA4229	<i>pchC</i>	Secreted Factors (toxins, enzymes, alginate)	
PA1249	<i>aprA</i>	Secreted Factors (toxins, enzymes, alginate)	
PA2396	<i>pvdF</i>	Secreted Factors (toxins, enzymes, alginate)	pyoverdine biosynthetic process
PA1658	<i>hsiC2</i>	Secreted Factors (toxins, enzymes, alginate)	protein secretion by the type VI secretion system
PA2399	<i>pvdD</i>	Secreted Factors (toxins, enzymes, alginate)	siderophore biosynthetic process
PA4175	<i>piv</i>	Secreted Factors (toxins, enzymes, alginate)	
PA0423	<i>pasP</i>	Secreted Factors (toxins, enzymes, alginate)	
PA3479	<i>rhlA</i>	Secreted Factors (toxins, enzymes, alginate)	
PA4296	<i>pprB</i>	Signal transduction	CheY-like receiver domain
PA3038	PA3038	Transcription	Transcriptional regulators
PA3225	PA3225	Transcription	Transcriptional regulators

PA3477	<i>rhlR</i>	Transcription	Transcriptional regulators
PA0547	PA0547	Transcription	
PA2426	<i>pvdS</i>	Transcription	sigma factor activity
PA3599	PA3599	Transcription	Transcriptional regulators
PA2620	<i>clpA</i>	Translation	ATP binding
PA0459	PA0459	Translation	
PA2618	PA2618	Translation	
PA4568	<i>rplU</i>	Translation	Ribosome
PA4249	<i>rpsH</i>	Translation	Ribosome
PA4744	<i>infB</i>	Translation	
PA2851	<i>efp</i>	Translation	translational elongation
PA4432	<i>rpsI</i>	Translation	Ribosome
PA4263	<i>rplC</i>	Translation	Ribosome
PA4264	<i>rpsJ</i>	Translation	Ribosome
PA4433	<i>rplM</i>	Translation	Ribosome
PA4272	<i>rplJ</i>	Translation	Ribosome
PA4277	<i>tufB</i>	Translation	
PA3656	<i>rpsB</i>	Translation	Ribosome
PA3049	<i>rmf</i>	Translation	
PA4934	<i>rpsR</i>	Translation	Ribosome
PA4262	<i>rplD</i>	Translation	Ribosome
PA4261	<i>rplW</i>	Translation	Ribosome
PA4671	PA4671	Translation	Ribosome
PA4266	<i>fusA1</i>	Translation	
PA4246	<i>rpsE</i>	Translation	Ribosome
PA4251	<i>rplE</i>	Translation	
PA4240	<i>rpsK</i>	Translation	Ribosome
PA4253	<i>rplN</i>	Translation	Ribosome
PA3600	PA3600	Translation	Ribosome
PA3601	PA3601	Translation	Ribosome