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ROLE OF CELL-CELL SIGNALING, DIFFERENTIATION AND FITNESS IN MULTI-SPECIES BIOFILMS

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AUTHOR'S PUBLICATIONS

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ABBREVIATIONS

$3OC_{12}$-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
AHL	Acyl homoserine lactone
AI-2	Autoinducer-2
ATCC	American type culture collection
c-di-GMP	Cyclic diguanylate
CRP	cAMP receptor protein
Dam	Deoxyadenosine methylase
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSF	Diffusible signal factor
eDNA	Extracellular deoxyribonuclei acid
EF-Tu	Elongation factor Tu
EPS	Extracellular polymeric substances
FISH	Fluorescence *in situ* hybridization
glmS	Glucosamine-fructose-6-phosphate
H-NS	Histone-like protein
IHF	Integration host factor
INDEL	Insertion and deletion
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria bertani
Lrp	Leucine response protein

NCBI	National Center for Biotechnology Information
NO	Nitric oxide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
q-PCR	Quantitative polymerase chain reaction
QS	Quorum-sensing
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SOB	Super optimal broth
SOC	Super optimal broth with catabolite repression
SSR	Short sequence repeat
Tn5	Transposon 5
Tn7	Transposon 7
Tn10	Transposon 10

ABSTRACT

Bacteria in the environment exist mostly as sessile communities known as biofilms. Biofilms are widely found on various biological surfaces such as on the leaves and roots of plants as well as in the lungs of humans, where they are associated with diseases. Biofilms can also grow on abiotic surfaces of rocks, water pipelines, contact lenses and surgical implants. Some biofilms can form independent of a surface, called suspended biofilms, and are represented by either flocs or granules that are important for the proper functioning of wastewater treatment plants. Biofilms, which are difficult to eradicate, have many negative impacts. For example, they are reservoirs for many pathogens and their activities also lead to biocorrosion of different surfaces. Biofilms are also essential, contributing to the biogeochemical cycling in the environment and are key players in processes such as sewage treatment and bioremediation. As a result, intensive research has been carried out with the aim to manipulate biofilms to our advantage. However, a main limitation of such research to date is the focus on mono species biofilms. In reality, biofilms in the environment consist of multiple interacting species, where interspecies interactions play an important role in determining the development, structure and function of the biofilms. Hence, a mixed species biofilm model that can be explored experimentally for research and development projects, with relevance to specific environmental, industrial and medical settings, is much needed. Here, a reproducible mixed species biofilm consisting of *Pseudomonas aeruginosa* PAO1, *Pseudomonas protegens* Pf-5 and *Klebsiella pneumoniae* KP-1 was studied and compared to the respective mono species biofilms.

The three bacterial species were stably labeled with three different fluorescent proteins using a Tn7 transposon expression system. The development of the mixed species biofilms was delayed relative to all mono species biofilms. While mushroom and tower-like microcolonies were observed for mature PAO1 and Pf-5 mono species biofilms respectively, these structures were not seen for the mixed species biofilms. In contrast, KP-1 in the mixed species biofilms formed mound-like microcolonies, which were not observed when it was grown alone. The proportions and structures of the three bacterial species within the mixed species biofilms grown at the inlet end and outlet end of the

continuous-culture flow cell also differed, most likely as a result of decreasing glucose concentration and associated changes in the specific growth rate for KP-1 along the channel. Most importantly, the mixed species biofilms were more resilient to stresses such as tobramycin and sodium dodecyl sulfate than the mono species biofilms. Intriguingly, such community level resilience was found to be contributed by the resistant species to the whole community rather than selection for the resistant species. In addition, community level resilience was not observed for mixed species biofilms with loosely associated members and for mixed species planktonic cultures, suggesting that community level resistance was unique to a structured biofilm community with closely associated members.

The formation of morphotypic variants, which is typical of almost all biofilm forming bacteria to date, was also observed and quantified in this study. One morphotypic variant each was identified for PAO1 and KP-1 while four morphotypic variants were observed for Pf-5. The formation of these morphotypic variants was specific to biofilms as planktonic cultures produced either no variants or a significantly reduced number of variants. The morphotypic variants were also stable after daily passaging, while sequencing of their genomes showed that they possessed insertions/deletions and single nucleotide polymorphisms that might lead to changes in their phenotypes and morphotypes. In fact, each morphotypic variant was shown to differ from its parent strain in various phenotypes such as swimming and swarming motilities, attachment, biofilm formation as well as pyoverdine and pyocyanin productions. In addition, the morphotypic variants also formed biofilms with distinct structures and outcompeted their parent strains in co-cultured biofilms. The morphotypic variants also outperformed their respective parent strains when grown with the other two species in a mixed species biofilm. Despite their fitness, the frequency and diversity of these morphotypic variants decreased in the mixed species biofilms. Since each strain sacrificed the production of individual variants in the presence of other species, the results suggest that interspecies diversity may be more important than intraspecies diversity in microbial biofilms. Notably, the addition of PAO1 and KP-1 cell-free biofilm effluents to Pf-5 biofilms also decreased the frequency and diversity of morphotypic variants formed by Pf-5. Hence, molecules present in the

biofilm effluents of PAO1 and KP-1 may be responsible for an interspecies cell-cell signaling that regulates self-generated genetic diversity.

Subsequently, metatranscriptomic analyses were performed to determine the genes that might be involved in biofilm response to sodium dodecyl sulfate. The results showed that extracellular polymeric substances production and export as well as stress response proteins were found to be involved in the resistance of PAO1 and KP-1 biofilms to SDS. Particularly, the up regulation of *siaA* has been associated with the autoaggregation of PAO1 cells during SDS treatment, thereby protecting them from lysis by SDS. Similarly, the induction of Cpx envelope stress response proteins in SDS treated KP-1 biofilms may be responsible for protecting KP-1 cells from lysis by SDS through ensuring proper folding of the envelope proteins and maintaining the integrity of the cells. The induction of extracellular polymeric substances and relevant stress response proteins was not observed for SDS sensitive Pf-5 biofilms.

In conclusion, results from this study have shown that mixed species biofilms differ from their mono species counterparts in terms of development, structure, resilience and intraspecies diversity. As a result, studies on mono species biofilms cannot predict the behaviours of mixed species biofilm communities in nature. The mixed species biofilm model used in this study is reproducible and is one of the few emperical studies that provided evidence for the proposition that interspecies diversity could substitute intraspecies diversity. Furthermore, all three bacterial species are genetically tractable and amenable to molecular techniques such as mutagenesis and 'omics' based approaches, thus making this mixed species model an excellent system to investigate interspecies interactions using both the top down and bottom up approaches.

CHAPTER 1 . INTRODUCTION

1.1 Background

Bacteria are the most abundant organisms on Earth (Whitman *et al.*, 1998) as well as vital agents that mediate all of the major biogeochemical cycles (Falkowski *et al.*, 2008). Bacteria can be beneficial to mankind, where commensal bacteria colonize and out compete opportunistic pathogens in the intestines, thus preventing harm that these pathogens can bring (Fujimura *et al.*, 2010; Stecher and Hardt, 2008), as well as in the break down and removal of organic contaminants in sewage treatment and bioremediation plants (Wagner and Loy, 2002). Bacteria can also have detrimental effects, for example, health problems ranging from dental caries caused by acids produced by cariogenic bacteria (Marsh, 2010) to life threatening lung infections caused by *Pseudomonas aeruginosa* in the alveoli of cystic fibrosis patients (Hoiby *et al.*, 2010; Sternberg *et al.*, 1999). As a result, the desire to control bacterial activities has been a key driver in the course of microbiology.

Bacteria have been the subjects of intensive study since the formulation of the germ theory of disease by Louis Pasteur in the 19th century. Ever since, microbiologists have been studying bacteria using the pure culture approach, where planktonic bacteria in liquid cultures were studied (Madigan *et al.*, 2009). These studies have indeed provided a deeper understanding of these microorganisms and have led to many improvements in infection control. However, the realization that most bacteria in the environment exist as complex, surface associated communities called biofilms (Costerton *et al.*, 1999; Fux *et al.*, 2005; Mah and O'Toole, 2001; Potera, 1996; Stewart and William Costerton, 2001) has opened up a new chapter in microbiology. Studies of planktonic bacteria do not adequately describe their natural mode of life outside the laboratory. Biofilms are found in various environments, including water distribution systems, river rocks, contact lenses, and all indwelling biomedical devices (Batté *et al.*, 2003; Bonadonna *et al.*, 2009; Costerton and Stewart, 2001). In addition, biofilms are the leading causes of biocorrosion

of steel and concrete (Flemming, 1998; Videla, 2002) and act as reservoirs for pathogens that are potential public health hazards (Mackay *et al.*, 1998).

Currently, biofilms are described as microbial derived sessile communities characterized by cells that are irreversibly attached to either a substratum, an interface (e.g. the air-liquid boundary) or to each other, that are embedded in a matrix of extracellular polymeric substances (EPS) that are self-produced, and exhibit altered phenotypes with respect to growth rate and gene transcription compared to planktonic cells (Donlan and Costerton, 2002). The formation of a biofilm follows a cyclical process and has been suggested to represent a rudimentary developmental process analogous to that seen in eukaryotes. This process consists of multiple stages (Figure 1-1) beginning with the reversible attachment of bacteria to a surface, the irreversible attachment of bacteria associated with the production of EPS, followed by the formation of microcolonies and their subsequent growth into a mature biofilm and finally dispersal of bacteria from the mature biofilm back into the planktonic phase (Sauer *et al.*, 2002; Stoodley *et al.*, 2002).

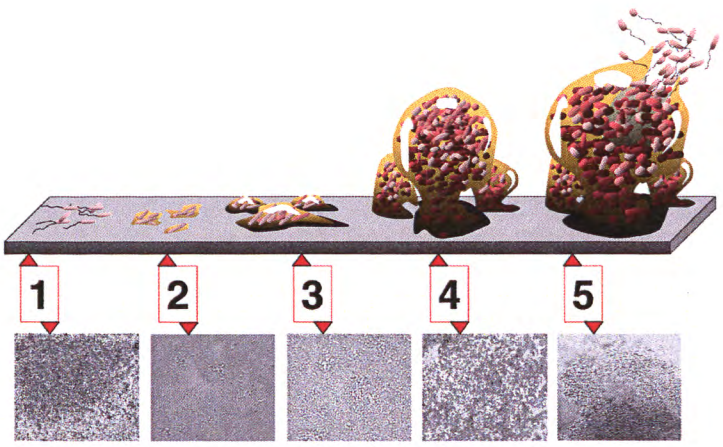


Figure 1-1. Diagram depicting five stages of biofilm development. Stage 1: reversible attachment of bacteria to the surface. Stage 2: irreversible attachment with production of EPS. Stage 3: initial development of microcolonies. Stage 4: maturation of microcolonies. Stage 5: dispersal. The bottom panels show the five stages of development, each represented by a photomicrograph of a *P. aeruginosa* biofilm when grown under continuous flow condition on a glass substratum (reproduced from Stoodley *et al.*, 2002).

CHAPTER 1. INTRODUCTION

In recent decades, studies on biofilms have grown exponentially and these have largely adopted the traditional reductionist approach by focusing mainly on mono species biofilms with the goal of dissecting the molecular and physiological details that underpin biofilm development (Sauer and Camper, 2001; Sauer *et al.*, 2002). Nonetheless, biofilms in the environment are comprised of multiple species. For example, about 100 to 200 bacterial species were found to colonize and form biofilms on hard and soft tissues of the oral cavity (Paster *et al.*, 2006). In addition, members of such multi species biofilms were reported to interact with each other instead of functioning in isolation (Elias and Banin, 2012; Rendueles and Ghigo, 2012). For example, *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13 (FR1) were reported to interact metabolically, where *Pseudomonas* sp. B13 (FR1) metabolized chlorobenzoate produced by *Burkholderia* sp. LB400 when the dual species biofilms were grown on chlorobiphenyl (Nielsen *et al.*, 2000).

It is evident that a wide range of interactions are possible within these mixed species biofilm communities. Members can compete for resources, leading to either suppression of growth or exclusion of the less competitive species (Banks and Bryers, 1991). Alternatively, co-metabolism and resource partitioning can occur (Moons *et al.*, 2009; Nielsen *et al.*, 2000), resulting in microniches that are favourable for sustaining a mixed species biofilm community. Most importantly, a diverse biofilm community also widens the range of conditions within which the community can thrive as a whole (McCann, 2000; Yachi and Loreau, 1999). For example, diverse biofilm communities were shown to be more resilient to stresses such as exposure to toxic substances, where the sensitive species were protected by the insensitive species within these communities through various mechanisms (Lindsay *et al.*, 2002; Skillman *et al.*, 1998; Whiteley *et al.*, 2001a). Within the mono species biofilms, diversity was also created through the generation of variants that differed from their parent strains in various phenotypes and this process was thought to increase the overall resilience of the biofilms by providing strains with altered stress tolerances (Boles *et al.*, 2004; Drenkard and Ausubel, 2002). These interspecies and intraspecies interactions between members of a biofilm community were facilitated by cell-cell communication systems.

The role of microbial cell-cell communication in the development of a biofilm was first described by Davies *et al.* (1998). Quorum-sensing (QS) signaling, mediated by N-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL), was found to be required for the formation and differentiation of biofilms formed by *P. aeruginosa* PAO1. Since then, research on cell-cell communication within biofilms progressed rapidly and soon, communication between Gram-positive and Gram-negative bacteria in a dual species biofilm was reported. Autoinducer-2 (AI-2), was discovered to be essential for the formation of dual species biofilms between *Streptococcus gordonii* and *Porphyromonas gingivalis* (McNab *et al.*, 2003). Other molecules such as modified peptides, fatty acid derivatives, indole and antibiotics at sub-inhibitory concentrations have also been reported to be involved in intraspecies and interspecies signaling (Ryan and Dow, 2008).

The fact that biofilms in nature are diverse, where interspecies interactions can influence their development, structures and functions, suggests that knowledge gained from studying mono species biofilms is limited. Hence, it is necessary to study biofilm communities and the interspecies interactions involved to better understand how such communities organize themselves, the impacts that diversity have on the fitness of the communities and to ultimately use this information to control and manipulate biofilm communities in the medical, industrial and environmental settings.

1.2 Objectives and scope

The detailed understanding of multi species biofilms and the interspecies interactions involved is a crucial knowledge gap necessary for the informed engineering of biofilms for a wide range of applications. Industrially, a biofilm community can be engineered to break down xenobiotic contaminants that cannot be accomplished by a biofilm population (Wolfaardt *et al.*, 1994a; Wolfaardt *et al.*, 1994b). Medically, the biofilm community in the human gut can potentially be engineered to restore health and ameliorate conditions associated with obesity (Ley, 2010). In addition, the use of probiotic strains to improve or maintain intestinal health requires continuous consumption of the probiotic strains as their persistence in the gastrointestinal tract are transient due to strong competition from endogenous lactic acid bacteria (Alander *et al.*, 1999). Therefore, understanding the

interactions between probiotic strains and the endogenous lactic acid bacteria can lead to improved probiotic strains that can become long term members of the gastrointestinal tract natural flora.

While there has been an exponential growth in biofilm research, many areas such as the effects of interspecies interactions on the development, structure and function of a multi species biofilm are largely unexplored. Therefore, this study has developed a mixed species biofilm model comprised of *P. aeruginosa* PAO1, *Pseudomonas protegens* Pf-5 and *Klebsiella pneumoniae* KP-1, accompanied with recent advances in molecular biology, next-generation sequencing and confocal laser scanning microscopy to investigate:

a) The impacts that interspecies interactions have on the development and resilience of a mixed species biofilm;
b) The effects that interspecies diversity and interactions have on intraspecies diversity; and
c) The genes involved in driving biofilm fitness.

CHAPTER 2 . LITERATURE REVIEW

2.1 Biofilm development

The development of a biofilm has been compared to the development of higher organisms (O'Toole, 2003; Webb *et al.*, 2003a). However, this has not been widely accepted and indeed a major difference distinguishes these two development processes. In a polymicrobial biofilm, different bacterial species (different genome content) coordinate their activities to achieve a specific function, which cannot be performed by the individual species alone. For example, a mixed species biofilm comprised of *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis* and *Acinetobacter Iwoffii* was more resistant to hydrogen peroxide, tetracycline and invasion by *Pseudoalteromonas tunicata* than biofilms formed by the individuals (Burmolle *et al.*, 2006). In contrast, a multi-cellular organism achieves a specific function by differentiating cells of identical genome content (Stoodley *et al.*, 2002). Nevertheless, both biofilms and multi-cellular organisms develop in stages.

The development of a biofilm is typically described as a five stages process. In each of these five stages, bacterial cells display distinct gene and protein expression profiles. For instance, it was observed that 27.3% and 4.9% of *Escherichia coli* K-12 genes were induced and repressed respectively when mature and early biofilms were compared (Domka *et al.*, 2007). Protein expression profiles of the five developmental stages of *P. aeruginosa* PAO1 biofilms also differed on average by 35% (approximately 525 proteins) (Sauer *et al.*, 2002). In addition to the changes in gene and protein expression profiles, the formation of unique structures during biofilm development has also been used to argue that it represents a developmental cycle. For example, mature *P. aeruginosa* biofilms possessed mushroom-like microcolonies (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; Lawrence *et al.*, 1991) that were made up of two subpopulations of cells. Cells that were not motile (i.e. not expressing type IV pili) formed the stalks whereas motile cells would form the caps of the mushroom-like microcolonies. In addition to *P. aeruginosa* biofilms, biofilms of *Myxococcus xanthus* were also observed to develop fruiting bodies, which

were formed by two subpopulations of cells, the sporulating cells and the stalk cells (O'Toole, 2003). Putting this evidence (i.e. complex expression profiles and the presence of subpopulations of cells) together, it is suggested that a sophisticated sensing and signaling system is present to integrate environmental cues and coordinate the responses of individual bacterial cells (Davies *et al.*, 1998).

In subsequent sections, the five stages of biofilm development 1) reversible attachment; 2) irreversible attachment; 3) early development of microcolonies; 4) maturation of microcolonies; and 5) dispersal will be reviewed. Some of the stage-specific physical and physiological properties as well as biofilm control methodologies will be discussed.

2.1.1 Reversible and irreversible attachment

Reversible attachment is the first step of biofilm development and requires bacterial cells to cross the hydrodynamic boundary layer and enter the near surface bulk region. Flagella mediated motility has been shown to help the cells cross the hydrodynamic boundary layer and enter the near surface bulk region, where they will swim parallel to the surface (O'Toole and Kolter, 1998a; Pratt and Kolter, 1998; Vigeant *et al.*, 2002). The individual cells are typically surrounded by a minute amount of EPS and are still capable of independent movement. Most of these cells will eventually leave the near surface bulk region and resume their planktonic forms, while a small number of these cells will proceed to attach irreversibly to the surface.

The transition time from reversible attachment to irreversible attachment ranges from seconds to minutes depending on the bacterial species. During irreversible attachment, bacterial cells must overcome the Gibbs energy barrier (repulsive van der Waals and electrostatic forces) before they can be in contact with the surface for irreversible attachment to take place (Palmer *et al.*, 2007). The Gibbs energy barrier is crossed when bacterial cells swimming in the near surface bulk region deviate from the equilibrium angle (due to flagella wobble) (Palmer *et al.*, 2007). Once the cells are in contact with the surface, cell surface structures such as pili and fimbriae as well as cell surface proteins and polysaccharides keep the cells firmly attached to the surface (Flint *et al.*, 1997;

Vesper, 1987; Vesper and Bauer, 1986). Other chemical forces such as van der Waals forces, electrostatic forces and hydrophobic interactions are also involved in attaching the cells to the surface (Carpentier and Cerf, 1993; Gilbert *et al.*, 1991; van Loosdrecht *et al.*, 1987).

Attached bacterial cells have been shown to switch from flagella mediated motility to type IV pili mediated twitching. Studies have shown that the flagellar filament protein FilC was induced in the planktonic cultures but not in the biofilms of *Pseudomonas putida* while type IV pili prepilin PilA was induced in the biofilms but not in the planktonic cultures (Sauer and Camper, 2001). In addition, putative surface and membrane proteins such as calcium-binding proteins, hemolysin, peptide transporters and multidrug efflux pumps were also involved in the adhesion of *P. putida* (Espinosa-Urgel *et al.*, 2000). In *Pseudomonas fluorescens*, ATP-binding cassette transporter mutants deficient in the secretion of a large adhesion protein were also unable to execute irreversible attachment (Hinsa *et al.*, 2003). *P. aeruginosa* cells that were deficient in the production of a cytoplasmic protein, called surface attachment deficiency B, were also found to be arrested at reversible attachment (Caiazza and O'Toole, 2004). Beside proteins, extracellular polysaccharides such as alginate were also reported to play a role in irreversible attachment. Studies on *P. aeruginosa* have shown that reversible attachment was independent of *algC* promoter activity, but transcription of *algC* was up regulated within 15 minutes after irreversible attachment, suggesting a role for alginate during irreversible attachment. In addition, cells that did not express alginate were washed off easily, further suggesting that alginate was involved in irreversible attachment of *P. aeruginosa* cells (Davies and Geesey, 1995).

In order to prevent biofilm development on surfaces, many approaches have been explored and involved the coating of surfaces with antimicrobial substances such as silver derivatives, quaternary ammonium and antibiotics (Isquith *et al.*, 1972; Kohnen *et al.*, 2003; Lee *et al.*, 2005). However, these approaches exhibit limited efficiency and high toxicity. Other approaches have investigated the use of natural molecules such as antimicrobial peptides, essential oils and bacteriolytic enzymes as coating materials

(Glinel *et al.*, 2012). Although these natural molecules appear to work through non-toxic mechanisms, their efficacies appear limited as immobilizing them to the surfaces in a functional conformation remains a challenge. The latest development in antimicrobial surfaces relates to changes in surface microstructures. Such surface modifications, mimicking those found on shark's skin and pilot whale's skin, have been designed and shown to prevent bacterial attachment (Baum *et al.*, 2002; Peng *et al.*, 2009). Nanostructures mimicking those found on the wing of cicada have even been shown to kill bacteria in contact (Pogodin *et al.*, 2013). Given the fact that bacteria can adapt to the environment in a short period of time, combining multiple approaches, such as those mentioned above, may be necessary to completely inhibit attachment of bacteria to surfaces. Improving the durability and stability of these coatings and micro/nanostructures in various environmental conditions have also been proven to be challenging. Therefore, understanding the other stages of biofilm development and the derivation of biofilm control measures targeted at these stages will serve as a safety net if inhibition of bacterial attachment fails.

2.1.2 Biofilm maturation

Biofilm maturation begins after irreversible attachment with the appearance of small cell aggregates termed microcolonies. Microcolonies can result from the binary division of attached cells (Heydorn *et al.*, 2000b; Tolker-Nielsen *et al.*, 2000) as well as the recruitment of single cells (Tolker-Nielsen *et al.*, 2000) or flocs (Stoodley *et al.*, 2001a) from the bulk fluid, although such recruitment is believed to play a smaller role in biofilm maturation.

Microcolonies grow in size and develop into complex structures, such as mushroom-like and mound-like colonies (Klausen *et al.*, 2003a; Klausen *et al.*, 2003b; Lawrence *et al.*, 1991) that are interspersed with water channels that are believed to facilitate the transport of liquid, thus nutrients and waste products through the biofilms (Wilking *et al.*, 2013). Such complex architecture is reported to be a result of cells being held together by the EPS. Studies have shown that both alginate and Psl (a galactose and mannose-rich exopolysaccharide) were required in the formation of mushroom-like colonies in *P.*

aeruginosa biofilms (Ghafoor *et al.*, 2011; Hentzer *et al.*, 2001). Another polysaccharide, Pel, has been linked to bioflm compactness (Ghafoor *et al.*, 2011) and structure, but this effect was suggested to be strain specific (Colvin *et al.*, 2011). Other polysaccharides are also reported to have similar structural functions in biofilms formed by other species. For example, a glucose and galactose-rich polysaccharide has been shown to be essential for the complex architecture observed for biofilms of *Vibrio cholerae* (Yildiz *et al.*, 2001). Similarly, colonic acid production was required for the formation of complex architecture in *E. coli* biofilms (Danese *et al.*, 2000). In addition to polysaccharides, extracellular deoxyribonucleic acid (eDNA) was also shown to play an important role in the early establishment of *P. aeruginosa* biofilms (Whitchurch *et al.*, 2002). Further, eDNA was found to be involved in the formation of mushroom-like colonies in later development of *P. aeruginosa* biofilms (Barken *et al.*, 2008).

In addition to EPS, the formation of mushroom-like colonies in *P. aeruginosa* biofilms is also dependent on chemotaxis, flagella and type IV pili. Non-flagellated and flagellum-linked chemotaxis deficient mutants were unable to form the caps of mushroom-like colonies in *P. aeruginosa* biofilms, suggesting that flagella and their chemosensory systems were involved (Barken *et al.*, 2008). Similarly, a non-piliated mutant (Δ*PilA*) has been shown to form the stalks but not the caps of the mushroom-like colonies in *P. aeruginosa* biofilms (Klausen *et al.*, 2003a).

Aside from structural changes, cell-cell signaling molecules such as acyl homoserine lactones (AHLs) were also detected in naturally occurring biofilms, suggesting that cell-cell signaling plays an important role in biofilm development (McLean *et al.*, 1997). Further evidence demonstrated that the differentiation and maturation of *P. aeruginosa* biofilms were controlled by $3OC_{12}$-HSL, an AHL enzymatically produced by LasI (Davies *et al.*, 1998). The *lasI* gene is part of the *lasI/rhlI* QS system, which regulates many other genes involved in biofilm maturation. For instance, QS controlled DNA release was linked to the formation of the cap portion of the mushroom-like colonies in *P. aeruginosa* biofilms (Barken *et al.*, 2008). Protein analyses and microarray studies on mature biofilms have also demonstrated that over 800 proteins and 70 genes were

differentially expressed in mature biofilms relative to planktonic cells. These include proteins and genes involved in metabolism (e.g. arginine deiminase and carbamate kinase), membrane transport and secretion (e.g. porin E1 and ATP-binding cassette transporter), as well as adaptation and protective mechanisms (e.g. superoxide dismutase and alkyl hydroxyperoxide reductase) (Sauer *et al.*, 2002; Whiteley *et al.*, 2001b).

Biofilm control measures targeted at this stage of biofilm development generally employ strategies that disrupt the EPS. For example, a mixture of polysaccharide hydrolyzing enzymes was used to remove biofilms of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *P. fluorescens* and *P. aeruginosa* from steel and polypropylene surfaces (Johansen *et al.*, 1997). Dispersin B was also used to degrade *N*-acetylglucosamine found in the EPS of *S. epidermidis* biofilms formed on plastic surfaces (Kaplan *et al.*, 2004). In *P. aeruginosa* biofilms, alginate lyase was also demonstrated to degrade alginate and lead to increased cell detachment (Boyd and Chakrabarty, 1994). In addition to EPS, cell-cell signaling is another potential target. A synthetic halogenated furanone compound derived from the secondary metabolites produced by the Australian macroalga *Delisea pulchra* was shown to interfere with AHL-mediated QS and production of virulence factors in *P. aeruginosa* biofilms (Hentzer *et al.*, 2002). Other QS inhibitors such as baicalin hydrate and cinnamaldehyde have also been observed to substantially increase the susceptibility of *P. aeruginosa* and *Burkholderia cepacia* biofilms to tobramycin. Similarly, *S. aureus* biofilms treated with hamamelitannin (a QS inhibitor) were also more susceptible to vancomycin. *In vivo* studies using *Galleria mellonella* larvae, *Caenorhabditis elegans* and mice also indicated that combined treatments with antibiotics and QS inhibitors increased the survival of infected individuals (Brackman *et al.*, 2011).

2.1.3 Dispersal

Biofilms disperse when the conditions become unfavourable, due to the accumulation of waste products and reduction in nutrients within a dense colony. Dispersal also allows the dispersed bacterial cells to seek out and colonize new environments. A particularly interesting feature of dispersal that has been proposed to be important for the rapid, successful colonization of new habitats is the formation of *morphotypic variant*s (Kirisits

et al., 2005; Kirov *et al.*, 2007; Koh *et al.*, 2007; Mai-Prochnow *et al.*, 2006; Webb *et al.*, 2004). Colonization of new habitats is vital for the survival of the bacterial cells. It also plays an important role in the transmission of disease as the disseminated bacteria colonize new hosts (Kaplan, 2010). Biofilm dispersal is categorized as either passive or active. Passive dispersal is mediated by external factors such as erosion, sloughing by shear forces (Choi and Morgenroth, 2003) and grazing by predators (Weitere *et al.*, 2005), while active dispersal involves the regulated conversion from the sessile state to the mobile state (Kaplan, 2010). As the last stage of biofilm development, dispersal has been intensively studied and has revealed multiple signals and mechanisms that regulate this response.

In many biofilms, dispersal is initiated from within the center of mature colonies, resulting in the formation of hollow colonies (Webb *et al.*, 2003b) and this process has been termed "seeding dispersal". Seeding dispersal has been shown to be QS controlled (Purevdorj-Gage *et al.*, 2005) and involves EPS degrading enzymes (Ott *et al.*, 2001) as well as extracellular surfactants (Boles *et al.*, 2005). However, these enzymes and surfactants are not known to be transported across the bacterial cell membrane and are thus believed to be released during cell lysis. In fact, the notion of cell lysis is supported by the presence of dead cells in the center of dispersed colonies (Sutherland, 1999; Webb *et al.*, 2003b).

Cell lysis during dispersal has been well studied in *P. aeruginosa* and *P. tunicata.* Expression of a Pf4 filamentous prophage as well as its receptors (flagella and type IV pili) were shown to cause lysis of *P. aeruginosa* cells (Rice *et al.*, 2009; Webb *et al.*, 2004; Webb *et al.*, 2003b) while an autolytic protein AlpP was involved in cell lysis during dispersal of *P. tunicata* biofilms (Mai-Prochnow *et al.*, 2006; Mai-Prochnow *et al.*, 2004). AlpP is a lysine oxidase and mediates cell lysis through the production of hydrogen peroxide (Mai-Prochnow *et al.*, 2008).

Upon cell lysis, enzymes such as glycosidases, proteases and deoxyribonucleases (DNase) are released and begin breaking down the EPS. As EPS maintains the integrity of a biofilm, its degradation results in the dispersal of cells from the center of mature colonies,

giving rise to hollow colonies. It was evident that Dispersin B, a glycoside hydrolase, was involved in dispersal as a Dispersin B mutant of *Actinobacillus actinomycetemcomitans* formed biofilms that did not disperse (Kaplan *et al.*, 2003). In addition, a *S. aureus* mutant deficient in the production of an extracellular protease aureolysin formed biofilms that had a significant increase in biomass and decrease in planktonic cells in the effluent (Boles and Horswill, 2008). Similarly, biofilm formation was significantly increased for a thermonuclease-deficient mutant of *S. aureus* (Mann *et al.*, 2009). Further, cell lysis in the center of a mature colony was also suggested to play an important role in supporting surrounding cells metabolically, where lysis materials were taken up by these cells as nutrients (Mai-Prochnow *et al.*, 2006).

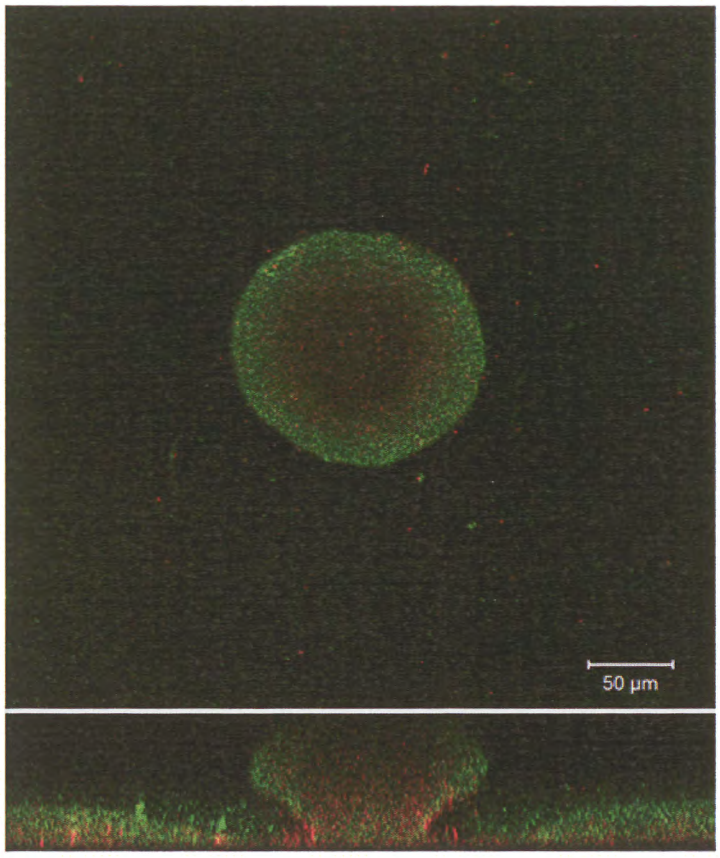


Figure 2-1. Confocal microscopy image of *P. aeruginosa* hollow colony after staining with LIVE/DEAD *Bac*Light bacterial viability probe. Upper panel shows the *x-y* plane while the lower panel shows the *y-z* plane. Green fluorescent cells are viable whereas red fluorescent cells are dead. Magnification: 20 x. Scale bar: 50 µm.

In addition to QS signaling, reactive oxygen and nitrogen intermediates have also been shown to play a role in biofilm dispersal, where low concentrations of nitric oxide (NO) produced endogenously or added exogenously induced dispersal of *P. aeruginosa* biofilms (Barraud *et al.*, 2006). Although NO is a common signal found in higher organisms and is known to be involved in numerous biological processes ranging from vasodilation to neurotransmission (Hopper and Garthwaite, 2006; Taqatqeh *et al.*, 2009; Yoon *et al.*, 2000), its mechanistic pathway during biofilm dispersal is still largely unknown. However, NO sensing proteins often have GGDEF and EAL domains (Barraud *et al.*, 2009; Iyer *et al.*, 2003), thus suggesting a link between NO sensing and the regulation of secondary messenger cyclic diguanylate (c-di-GMP). In fact, exposure of *P. aeruginosa* biofilms to NO increased the activity of phosphodiesterase and decreased the intracellular c-di-GMP concentration (Barraud *et al.*, 2009). The c-di-GMP pathway is conserved in many bacteria such as *Shewanella oneidensis*, *Salmonella enterica* serovar Typhimurium, *P. aeruginosa* and *E. coli* (Simm *et al.*, 2004; Thormann *et al.*, 2006). Typically, the biofilms disperse at low intracellular c-di-GMP concentration with the induction of genes involved in motility and the repression of genes involved in adhesins and virulence factors. In contrast, biofilm formation is favoured at high intracellular c-di-GMP concentration (Barraud *et al.*, 2009).

In addition to NO, the unsaturated fatty acid, *cis*-2-decenoic acid, was also shown to induce biofilm dispersal of *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *S. aureus*, and the yeast *Candida albicans* (Davies and Marques, 2009). *cis*-2-decenoic acid is structurally similar to diffusible signal factors (DSF) that can be found in other bacterial species such as *Xanthomonas campestris* and *Stenotrophomonas maltophilia*, where DSF is responsible for the regulation of swimming motility and the production of extracellular protease (Fouhy *et al.*, 2007). As a result, unsaturated fatty acids, which have activity across a wide range of bacteria, were suggested to be the inducer of dispersion in a mixed species biofilm by coordinating the release of enzymes that break down the EPS (Davies and Marques, 2009).

EPS degrading enzymes and molecules have attracted much attention as potential therapeutics for the treatment of biofilm related infections. In addition to the use of Dispersin B and alginate lyase mentioned above, D-amino acids and the polyamine norspermidine have been shown to break down the EPS of *B. subtilis* and *S. aureus* biofilms by interacting directly and specifically with proteins and exopolysaccharides respectively (Kolodkin-Gal *et al.*, 2010; Kolodkin-Gal *et al.*, 2012). Jamming cell-cell signaling using QS inhibitors such as halogenated furanone can prevent biofilm formation (Hentzer *et al.*, 2002), but small molecules such as NO and unsaturated fatty acids have been shown to induce biofilm dispersal *in vitro* (Barraud *et al.*, 2006; Davies and Marques, 2009), although their effectiveness *in vivo* has limited investigation. Biofilm control that combines a few strategies developed for targeting various stages of biofilm development can prove to be more comprehensive and effective. For example, Dispersin B and NO could be used together to remove biofilms. The induction of biofilm dispersal with NO would be more effective if the EPS is weakened through the degradation of *N*-acetylglucosamine by Dispersin B.

2.2 Interspecies bacterial interactions

Research on biofilms thus far has mostly been conducted on mono species biofilms. These studies have described the development of mono species biofilms and the various mechanisms associated with the different developmental stages. Nonetheless, biofilms in nature are complex communities consisting of numerous bacterial species (Dowd *et al.*, 2008; Urakawa *et al.*, 1999). In such complex biofilm communities, the relevance of data obtained from the studies of mono species biofilms is unclear since interspecies interactions are expected to play a significant role in determining the development, structure, and resilience of the biofilm communities (James *et al.*, 1995). Therefore, it is essential to study mixed species biofilms and how the various interspecies interactions affect the development, structure, and resilience of mixed species biofilms.

Numerous bacterial interactions have been discovered and can be classified according to the effect that an interaction has on each species in a binary system (Table 2-1). For example, *neutralism* refers to an interaction that does not affect any population in a binary system. *Competition* occurs when both populations suffer from poorer growth as they compete for the same growth limited nutrients. On the other hand, when one population benefits without affecting the other population, *commensalism* has taken place. When both populations benefit in the presence of each other, such an interaction will be termed as *mutualism*. Obligatory interaction (*symbiosis*), facultative interaction (*protocooperation*), interaction enhancing either the production or consumption of a product and substrate respectively (*synergism*), and finally interaction involving the exchange of a growth factor or energy source (*syntrophy*) are various forms of *mutualism* that occur in the environment. Next, when one population is indirectly affected negatively by the presence of another without cell-cell contact, this interaction is termed *ammensalism*. In contrast, *predation*, where one population is consumed by the other, and *parasitism*, where one population is invaded intracellularly by another are direct negative interactions involving cell-cell contact (James *et al.*, 1995). Last, *altruism* happens when the fitness of one member is decreased to render the whole community a competitive advantage over other communities (Sober and Wilson, 1998).

In the following sections, the most common interactions observed in biofilms are discussed. Although only one type of interaction was described in most of the literature presented, one must note that many types of interaction can occur simultaneously in a complex mixed species biofilm found in the environment.

2.2.1 Neutralism

Members in most mixed species biofilms are closely associated spatially (Cowan *et al.*, 2000; Hansen *et al.*, 2007; Stewart *et al.*, 1997). Hence, it would be rare for individuals within a microbial community to function without some form of interaction with their neighbors (Straight and Kolter, 2009). Surprisingly, no interaction was detected in a dual species biofilm comprised of *P. aeruginosa* and *K. pneumoniae* grown in an annular reactor. The specific product formation rate and glucose-oxygen stoichiometric ratio of

either *P. aeruginosa* or *K. pneumoniae* in the dual species biofilms were unaffected by the presence of each other, although both organisms utilized the same source of nutrients (Siebel and Characklis, 1991). Nevertheless, competition was observed when the rates of nutrient provision and waste removal were decreased, suggesting that competition between the two species can occur when resources are limited (Sturman *et al.*, 1994). In another study, *Pseudomonas* sp. B13 (FR1) and *Burkholderia* sp. LB400 formed separate microcolonies with no interaction when the mixed species biofilms were fed with citrate, a nutrient that could be metabolized by both species. Therefore, neutralism is indeed rare in a biofilm unless members within the biofilm are provided with an unlimited amount of nutrients that each can utilize and where waste products are not allowed to accumulate to concentrations that may be otherwise inhibitory to one or both species.

Table 2-1. List of interspecies bacterial interactions

Interspecies bacterial interactions	Definitions
Neutralism	A relationship where the members involved do not affect each other
Competition	An interaction where two species compete for nutrients and space
Ammensalism	An interaction where one species inhibits the growth of another species
Predation	An interaction where one species feeds on another species
Commensalism	An interaction where one species benefits from the presence, but does not affect another species
Mutualism	An interaction where both species benefit from the presence of one another
Altruism	A selfless act that increases the fitness of a group, but decreases the fitness of the individual

2.2.2 Competition

Competition for substrate and space in mixed species biofilms has been investigated (Zhang *et al.*, 1994, 1995). In most cases, a population's capacity to scavenge nutrients and its specific growth rate determine its fate in a mixed species biofilm. Usually, the population with a higher growth rate will outgrow the slower growing populations, leading to its dominance in the community. Nonetheless, the slow growing populations

may still persist in the biofilms (Lewis and Gattie, 1991). Specific studies including the invasion of *Hyphomicrobium* species biofilms by *Pseudomonas putida* (Banks and Bryers, 1991) and the invasion of *P. aeruginosa* biofilms by *K. pneumoniae* (Sturman *et al.*, 1994) have further demonstrated the effect that growth rate has in determining the composition of a mixed species biofilm. In these two studies, both *P. putida* and *K. pneumoniae* have a higher growth rate and thus outgrew their competitors, but failed to completely eliminate them. High growth rate is probably the key to success when nutrients are in excess, but the ability to utilize high affinity uptake systems can explain the persistent of a slower growing population at low nutrient concentrations.

Rapid occupancy of available surface is another strategy employed by one bacterial population to outcompete others in a mixed species biofilm. For example, *P. aeruginosa* was observed to rapidly colonize the available surface via swarming and twitching motilities, thus preventing the adhesion of *Agrobacterium tumefaciens* (An *et al.*, 2006).

2.2.3 Ammensalism

Competition and ammensalism are often not clearly defined and are used interchangeably in the literature. However, ammensalism is viewed here as a unique interspecies interaction that shall not be confused with competition. Unlike competition, where the survival of a population is determined by its fitness in terms of nutrient scavenging and growth rate, ammensalism is an interaction where survival of a population depends on its ability to inhibit its competitors. Such inhibition commonly does not involve cell-cell contact, but incorporates the production of either an inhibitor that can stop the growth of another population (direct) or the production of a compound that affects the formation of biofilm by another species (indirect).

Direct ammensalism involves the use of toxins that kill sensitive species in the immediate surroundings of the producer. For instance, a pre-established biofilm comprised of bacteriocin producing enteric bacteria was shown to prevent colonization by a potential competitor (Tait and Sutherland, 2002). A marine bacterium, *P. tunicata* produced a range of extracellular inhibitory compounds, including an antibacterial protein AlpP, that gave it

a competitive advantage over other bacterial species in competition for nutrient and space (Rao *et al.*, 2005). Antibiotics have also been found to be produced by *P. fluorescens* in a mixed species biofilm with *Pedobacter* sp. V48. The antibiotics produced were broad-spectrum and inhibited the growth of *Bacillus* sp. BrV52, *Brevundimonas* sp. V52, *Pedobacter* sp. V48 and many other fungus species (Garbeva and de Boer, 2009; Garbeva *et al.*, 2011).

Indirect ammensalism involves various mechanisms that target the different developmental stages of a biofilm. Compounds used in indirect ammensalism do not affect the growth of the bacteria, but affect their capacities to form and maintain biofilms. For example, group II capsules produced by *E. coli* reduced the initial attachment of a wide range of Gram-positive and Gram-negative bacteria by increasing surface hydrophilicity (Valle *et al.*, 2006). An exopolysaccharide A101 isolated from a marine *Vibrio* and a mannose-rich polysaccharide isolated from *E. coli* were also reported to prevent adhesion of *S. aureus* (Jiang *et al.*, 2011; Rendueles *et al.*, 2011). In marine biofilms, a genus specific glycoprotein also impeded the attachment and gliding motility of competing bacteria in the same genus (Burchard and Sorongon, 1998).

As discussed in the previous section, maturation of some biofilms involves QS signaling and the production of EPS. Therefore, sabotaging the cell-cell signaling network and EPS degradation are some of the indirect ammensal approaches that bacteria utilize. For example, the formation of *V. cholerae* biofilms was inhibited by *Bacillus cereus* through the production of an AHL lactonase that degraded the QS signals of *V. cholerae* (Augustine *et al.*, 2010). *P. aeruginosa* biofilms were also inhibited by bacterial extracts containing phenolic groups and aliphatic amines that interfered with *P. aeruginosa* QS signaling (Nithya *et al.*, 2010). Similarly, the abilities of other bacteria to form biofilms have also been shown to be compromised through the degradation of polysaccharides, proteins and eDNA present in the EPS. For example, Dispersin B produced by *A. actinomycetemcomitans* has been shown to disperse biofilms of *S. epidermidis* (Kaplan *et al.*, 2004) while exo-beta-D-fructosidase produced by *Streptococcus salivarius* was found to inhibit biofilm development of *Streptococcus mutans* (Ogawa *et al.*, 2011). Both

Dispersin B and exo-beta-D-fructosidase exerted their effects by degrading polysaccharides, which are major components of the biofilm EPS. EPS-associated proteins were also broken down by serine protease produced by *S. epidermidis*, affecting the formation of *S. aureus* biofilms (Iwase *et al.*, 2010). Lastly, eDNA present in the EPS of biofilms formed by Gram-positive and Gram-negative bacteria was also shown to be degraded by DNase produced by *Bacillus licheniformis* (Nijland *et al.*, 2010).

2.2.4 Predation

Predation usually involves cell-cell contact between a prey and a predator. One of the most notorious bacterial predators is *Bdellovibrios bacteriovorus*, a small Gram-negative bacterium that attaches to and invades the periplasm of other Gram-negative bacteria, where it replicates and ultimately lyses the host to be released in search of a new host. Numerous studies have shown that *B. bacteriovorus* could destroy established biofilms of *E. coli* and *P. fluorescens* (Kadouri and O'Toole, 2005; Nunez *et al.*, 2005). Similarly, *Micavibrio aeruginosavorus*, another predatory bacterium can also prey on Gram-negative bacteria by attaching on their surfaces. However, these predators differ from *B. bacteriovorus* in that they do not invade the periplasm, but rather draw off nutrients from the host and eventually killing it. *M. aeruginosavorus* was also shown to reduce existing biofilms of *P. aeruginosa*, *B. cepacia* and *K. pneumoniae* (Kadouri *et al.*, 2007). Interestingly, no bacterial predators that prey on Gram-positive bacteria have been described and this may be due to the thick peptidoglycan layer of these bacteria. In addition to *Bdellovibrio* and *Micavibrio*, bacteria are also preyed upon by protozoa. Some bacteria have developed different defensive strategies to protect themselves from predation, including alteration of their cell surface properties, the production of bioactive metabolites, increased swimming speed and the formation of biofilms (Matz and Kjelleberg, 2005). The formation of microcolonies and the production of toxins during *P. aeruginosa* biofilm development have both been shown to protect the bacteria from grazing by the flagellate *Rhynchomonas nasuta* (Matz *et al.*, 2004). Further studies have indicated that the formation of microcolonies could only protect *P. aeruginosa* biofilms in the initial stages of biofilm development from early colonizers such as *Bodo saltans* and *R. nasuta*. The production of toxins was the main strategy used in the late stages of biofilm

development against late colonizers such as *Acanthamoeba polyphaga* (Weitere *et al.*, 2005).

2.2.5 Commensalism

Commensalism is one of the most common and best studied interactions in mixed species biofilms. In many cases, members of the mixed species biofilms are spatially organized in a specific manner to facilitate the transfer of metabolites during metabolic commensalism or to create microniches, which allow the growth of members with strict growth requirements. Further, the mixed species biofilms can also be more resistant to host defenses and environmental stresses due to either the protection by insensitive members, the sharing of public goods or both.

In a consortium consisting of *Pseudomonas* sp. B13 (FR1) and *Burkholderia* sp. LB400, *Pseudomonas* sp. B13 (FR1) grew on chlorobenzoate, a metabolite produced by *Burkholderia* sp. LB400 when it fed on chlorophenyl (sole carbon source). This metabolic commensal relationship supported the growth of *Pseudomonas* sp. B13 (FR1), which was not possible in the absence of *Burkholderia* sp. LB400 (Nielsen *et al.*, 2000). In another experiment, *P. putida* strain R1 was found to survive on excess benzoate that was released from an *Acinetobacter* strain C6 when the mixed species biofilms were fed with benzyl alcohol (Christensen *et al.*, 2002). These studies have demonstrated that mixed species biofilms are fully capable of undergoing metabolic commensal relationships.

Commensalism can also involve the formation of biofilms by species that do not form biofilms on their own. For example, anaerobic bacteria could grow in the interior of a mixed species biofilm where the aerobic bacteria at the surface consumed oxygen and created a suitable anaerobic niche (Bradshaw *et al.*, 1997; Ramsing *et al.*, 1993). In addition, *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, two bacterial species that form minimal amount of biofilms individually, were observed to coaggregate and form more biofilms when co-cultured with *Actinomyces naeslundii* and *Actinomyces gerencseriae* (Filoche *et al.*, 2004). Similarly, precolonization of plastic stents with *E. coli* was shown to facilitate the subsequent attachment of other *Enterococcus* species (Leung *et al.*, 1998).

Finally, in a protective commensal relationship, betatine was degraded by *Vogesella indigofera*, thus enhancing the survival of betatine sensitive *P. putida* in a mixed species biofilm (Whiteley *et al.*, 2001a). In another study, a laboratory scale sewage treatment plant inoculated with *Pseudomonas* sp. B13 SN45RE, which was genetically engineered to enhance the degradation of chloro- and methylaromatics, was demonstrated to increase the survival of other bacteria, protozoa and metazoa within the community when challenged with the chemicals (Erb *et al.*, 1997). These studies have illustrated the detoxification of toxic substances and reduction in their concentrations, thus allowing sensitive bacteria to grow. However, protection can also be achieved through physical shielding. Physical shielding can create microniches within a mixed species biofilm where the sensitive species can survive. For instance, *p*-cresol-degrading *P. putida* surrounded the sensitive *Pseudomonas* sp. strain GJ1 when the mixed biofilms were exposed to *p*-cresol (Cowan *et al.*, 2000). In a similar manner, sensitive *P. fluorescens* was protected against chlorine dioxide by more resistant *B. cereus* (Lindsay *et al.*, 2002).

2.2.6 Mutualism

Mutualism generally refers to an interaction where each species benefits from the presence of each other and can be further subdivided into symbiosis, protocooperation, synergism and syntrophism. Bacteria-bacteria symbiosis is not common but there are a few well-studied bacteria-animal and bacteria-plant symbiosis. One well known bacteria-animal symbiosis is the association between *Vibrio fischeri* (bacteria) and *Euprymna scolopes* (a species of squid). *V. fischeri* was localized in a light organ at the ventral of *E. scolopes* and emitted light that was used by *E. scolopes* as counter-illumination, a type of camouflage that it used to eliminate its shadow. At the same time, *E. scolopes* provided nutrients and maintained a pure culture of *V. fischeri* within the light organ by protecting it from invasion by other bacterial species (Ruby and McFall-Ngai, 1992). Interaction between *Bacillus subtilis* and *Arabidopsis thaliana* demonstrated a bacteria-plant symbiosis where *B. subtilis* protected *A. thaliana* from plant pathogens such as *Pseudomonas syringae*. In return, the roots of *A. thaliana* provided a food source and

secreted L-malic acid that specifically attracted and promoted the binding and biofilm formation of *B. subtilis* (Rudrappa *et al.*, 2008).

In protocooperation, dual species biofilms of *Lactococcus lactis* and *P. fluorescens* were more developed than mono species biofilms formed by each species. *L. lactis* showed as much as 20,000 fold increase in attachment while *P. fluorescens* also attached 100 fold better. In addition, *L. lactis* was found to be within the interior of *P. fluorescens* microcolonies as the latter consumed oxygen and created an anaerobic environment where *L. lactis* could grow. In return, *L. lactis* produced lactic acid that was used by *P. fluorescens* as nutrients (Kives *et al.*, 2005).

As an example of synergism, a mixed species biofilm consisting of four epiphytic bacteria was more resistant to hydrogen peroxide and tetracycline compared to the mono species biofilms formed by individual members (Burmolle *et al.*, 2006). In the same report, the mixed species biofilms were also shown to be more resistant to invasion by the marine surface colonizer *P. tunicata*. Another mixed species biofilm made up of a *Variovorax* species, *Comamonas testosteroni* and *Hyphoicrobium sulfonivorans* was also capable of degrading linuron and its metabolic intermediate 3, 4-dichloroaniline more efficiently than their respective mono species biofilms (Breugelmans *et al.*, 2008). In another study, synergism between nine bacterial species and an algal species within a consortium was demonstrated to degrade diclofop methyl, a herbicide that each species in the consortium was unable to degrade alone (Wolfaardt *et al.*, 1994a).

In a syntrophic interaction, H_2, formate and CO_2 produced by fermentative bacteria in the rumen were used by methanogenic bacteria, leading to the reduction of formate and CO_2 to CH_4. This process lowered the partial pressure of H_2 and shifted the equilibrium of fermentation towards the production of more H_2, formate and CO_2, thus generating more ATP yield for the fermentative bacteria (Wolin *et al.*, 1988).

2.2.7 Altruism and cheating

Altruism is defined as a behaviour that increases the fitness of a group relative to other groups, but decreases the fitness of the altruist relative to others within the group (Sober

and Wilson, 1998). On the other hand, individuals that do not pay the cost, but benefit from the presence of public goods produced by others, are cheaters. According to Darwin's theory on natural selection and evolution, altruism does not seem to be possible as cheaters, who are usually fitter than the altruists, will always outcompete and eliminate them. However, the evolution of altruism can be explained by Darwinian evolution when group selection theory (Wilson and Sober, 1994), a multi-level selection approach, is used. While selection at the individual level decreases the proportion of altruists within a group, group level selection increases the number of groups with altruists (fitter than groups without altruists). Hence, the global number of altruists within the community increases and such phenomenon is termed the Simpson's paradox (Blyth, 1972). To counter the gradual decrease in the proportion of altruists within a group, individual altruists leave, multiply and form new groups with a high proportion of altruists (purification). Hence, a biofilm which is spatially structured into different microcolonies (groups) with limited mixing between them (Tolker-Nielsen *et al.*, 2000), disperses and forms new microcolonies from individual bacteria (purification), is suggested to promote altruism (Kreft, 2004b). For example, bacteria with high growth rate, but low growth yield (cheaters) and bacteria with slow growth rate, but high growth yield (altruists) growing together in a biofilm was modeled. It was shown that within each microcolony, the cheaters were selected due to their higher growth rate (individual level selection). However, microcolonies with a higher proportion of the altruists grew better and produced more offspring than microcolonies with either lower proportion, or no altruists (group level selection). As a result, altruists persisted in the biofilm.

In another study, *P. fluorescens* of the wrinkly spreader morphotype (altruists) and smooth morphotype (cheaters) were studied. It was shown that biofilms made up mainly of the altruists could grow at the air-liquid interface due to the overproduction of a cellulosic polymer. Growth at the air-liquid interface ensured that the bacteria have access to oxygen, which became limited in the liquid phase. However, cheaters that grew faster, but did not contribute to the production of cellulosic polymer, always evolved and outcompeted the altruists in the biofilms. As the number of cheaters increased, the

biofilms collapsed. The altruists from the collapsed biofilms would disperse, adhere to one another and grow into a new biofilm (Rainey and Rainey, 2003).

Altruism is also displayed during diversification of a population into various subpopulations (insurers) as an adaptation to fluctuations in environmental conditions. When there is no change in the environmental conditions, these insurers are slow growing or dormant, and are at a fitness disadvantage compared to the wild type strain. However, when the environmental conditions change, the whole population can benefit from the presence of the insurers (Kreft, 2004a). For instance, the insurers were reported to increase the resilience of biofilms to stresses such as treatment with antibiotics and predation by protozoa (Drenkard and Ausubel, 2002; Koh *et al.*, 2012).

2.3 Diversity within a biofilm population

Diversity within a biological system has long been recognized to be the key to combat its decline in survival and functionality during environmental fluctuations. For example, mono species cultivars, such as wheat fields, are more susceptible to environmental perturbations, e.g. drought and insect attack, compared to highly diverse systems such as rain forests. This phenomenon has been termed the "insurance hypothesis", which assumes that a diverse system can thrive in a wider range of conditions (McCann, 2000; Yachi and Loreau, 1999).

A biofilm is a sessile system with limited mobility. Therefore, a bacterium in a biofilm is incapable of escaping from any adverse conditions unless it leaves during dispersal. If it stays, it should have mechanisms that aid in its adaptation to changing environmental conditions. Hence, biofilm may be an ideal system to study the "insurance hypothesis". Indeed, morphotypic variants have been reported for biofilms of *P. fluorescens*, *P. putida*, *Serratia marcescens* and *Streptococcus pneumoniae* (Arciola *et al.*, 2005; Hansen *et al.*, 2007; Koh *et al.*, 2007; Workentine *et al.*, 2010). Further, similar morphotypic variants were also found in biofilms formed in the lungs of cystic fibrosis patients (Kirisits *et al.*, 2005; Woo *et al.*, 2012), indicating the relevance of these variants *in vivo*. Intriguingly, these morphotypic variants were either not found or found at low quantity among the

isolates from planktonic cultures, suggesting that they were biofilm specific (Boles *et al.*, 2004; Koh *et al.*, 2007; Woo *et al.*, 2012). While various mechanisms such as genetic mutation and phase variation have been shown to result in the formation of these morphotypic variants, the reason for their formation specifically in the biofilms were still unreported.

2.3.1 Mechanisms for the generation of variants

Bacteria have one of the highest spontaneous mutation rates among all organisms. The mutation rate of a bacterium ranges from 20 nucleotides to 1.3 x 10^5 nucleotides per replication cycle depending on the size of the genome (Drake *et al.*, 1998) and is believed to generate morphotypic variants with stable and heritable traits (Figure 2-2). For example, the small nonmucoid variant of *S. pneumoniae* evolved from the deletion of a 7 Kbp gene fragment covering *cpuDSU*, part of a capsule biosynthesis operon (*cpuDSUM*) and the *cap3* promoter (Allegrucci and Sauer, 2007). In another study, polymorphism was observed for the gene encoding an autophosphorylating tyrosine protein kinase. Four morphotypic variants of *S. marcescens* were each carrying a single-base, nonsynonymous mutation in the same gene (Koh *et al.*, 2012). These mutations affected the ATP-dependent autophosphorylation function of the tyrosine kinase, in conjunction with its function in EPS biosynthesis (Wugeditsch *et al.*, 2001). Homologous recombination is also another source of genetic modification that can lead to the formation of morphotypic variants (Figure 2-2). It was reported that the formation of mini and wrinkly colony variants from a *P. aeruginosa* biofilm was dependent on the presence of RecA, a 38 KDa protein that was required for the maintenance and repair of DNA (Boles *et al.*, 2004).

Phase variation is the alternative mechanism for the generation of variants and generally occurs at a high frequency of more than 1 in 10^5 cells per generation (Henderson *et al.*, 1999). In contrast to genetic mutation, varying phenotypes formed as a result of phase variation are usually transient and reversible (Figure 2-2). Phase variation can be caused by genomic rearrangement, slipped-strand mispairing and epigenetic regulation (van der Woude and Baumler, 2004).

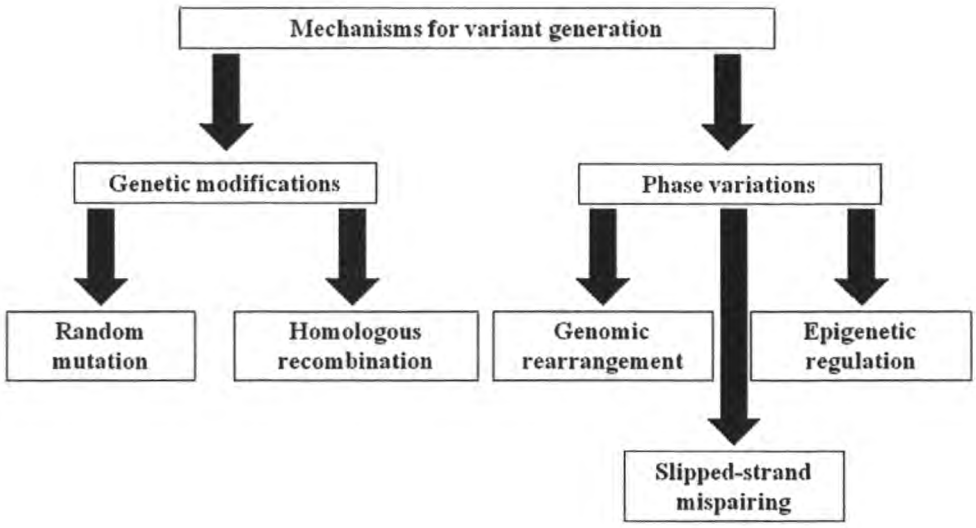


Figure 2-2. Mechanisms for variant generation. Stable variant can be generated by genetic modifications such as random mutation and homologous recombination. Transient variant can be generated by phase variations, which include genomic rearrangement, slipped-strand mispairing and epigenetic regulation.

Phase variation brought about by genomic rearrangement normally involves the inversion of a DNA segment encoding the promoter that controls the gene of interest. The DNA segment is often flanked by short recombining sequences and requires specific recombinase and cofactors during recombination. The phase variation of type 1 fimbriae of *E. coli* and the flagellum of *Salmonella typhimurium* are examples mediated by this mechanism (Abraham *et al.*, 1985; Zieg *et al.*, 1977). The type 1 fimbriae of *E. coli* is encoded by *fimA*, for which transcription is initiated by a promoter that is within a 314 bp chromosomal region bounded by 9 bp inverted repeats. In the "ON" orientation, the promoter can initiate the transcription of *fimA*. In the "OFF" orientation, the chromosomal region is inverted and the promoter will not be in the correct position to initiate transcription of *fimA* (Figure 2-3). The inversion is directed by two integrases encoded by *fimB* and *fimE* (Klemm, 1986) with participation from other factors such as histone-like

protein (H-NS), integration host factor (IHF) and leucine response protein (Lrp) (Blomfield *et al.*, 1997; Dorman and Higgins, 1987). FimE was found to induce only the "ON" to "OFF" inversion while FimB induced both the "ON" to "OFF" and "OFF" to "ON" inversion (McClain *et al.*, 1993). Further, actions of FimB and FimE are modulated by the growth temperature and media (Gally *et al.*, 1993), indicating that the environment has an impact on phase variation, thus an effect on variant formation.

Phase variation can also result from slipped-strand mispairing. Slipped-strand mispairing occurs when repetitive DNA segments known as short sequence repeats (SSRs) result in misalignment between the mother and daughter strands during DNA synthesis or repair. SSRs can either be found upstream of a gene, thus influencing its transcription or within a gene and result in a change to its translational reading frame (van Belkum *et al.*, 1998). An example of transcriptional control by slipped-strand mispairing is the regulation of fimbriae in *Haemophilus influenzae*. The number of tandem repeats (dinucleotide TA) that extends from the -10 to -35 region of the promoter determines the expression of the fimbriae. Expression of the fimbriae is maximum when the number of repeats is 10. When the number of repeats is 11, the expression level is reduced. Expression of the fimbriae is silenced when the number of repeats is 9 (van Ham *et al.*, 1993). Translational control by slipped-strand mispairing involves alteration in the reading frame of a gene when the number of repeats changes. For instance, 6, 9, and 12 copies of the pentamer repeats (5'-CTCTT-3') within the *opa* gene of *Neisseria gonorrhoeae* leads to expression of the mature opacity protein. If the copy of pentamer repeats is 4 or 8, translation will be out of frame and a truncated opacity protein will be produced (Stern *et al.*, 1986).

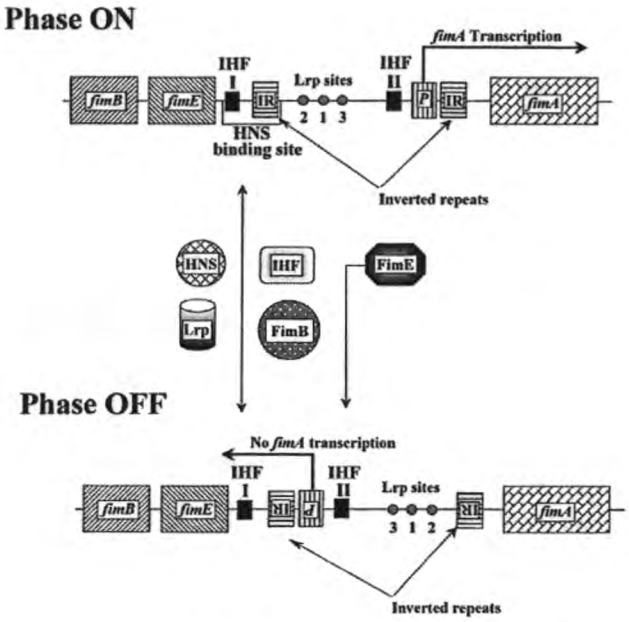


Figure 2-3. Phase variation mediated by genomic inversion. The promoter (P) is within the invertible element flanked by two inverted repeats (IR). Within the invertible element are three other Lrp binding sites (shaded circles) and one IHF binding site II (solid box). IHF binding site I is outside the invertible element. Integrases FimB and FimE, together with H-NS, Lrp and IHF direct the inversion of the promoter (adapted from Henderson *et al.*, 1999).

Epigenetic modification such as differential methylation can also elicit phase variation. In contrast to genome rearrangement and slipped-strand mispairing, genome integrity of the cell is unaffected by methylation. The regulation of P fimbriae expression in uropathogenic *E. coli* is one of the most well studied examples of methylation-mediated phase variation. Expression of the P fimbriae from *papBA* is controlled at the transcriptional level by the differential methylation of two GATC sites (GATC-I and GATC-II). When GATC-I is methylated and GATC-II is unmethylated, *papBA* is not transcribed. On the contrary, *papBA* is transcribed when GATC-I is unmethylated and GATC-II is methylated (Figure 2-4). Methylation of the GATC site by Deoxyadenosine methylase (Dam) is blocked when Lrp binds. Hence, competition between Lrp and Dam determines the methylation, thus expression of P-fimbriae. Other factors such as PapB,

cAMP receptor protein (CRP) and H-NS also participate in the phase variation of P fimbriae (Blyn *et al.*, 1990; Henderson *et al.*, 1999).

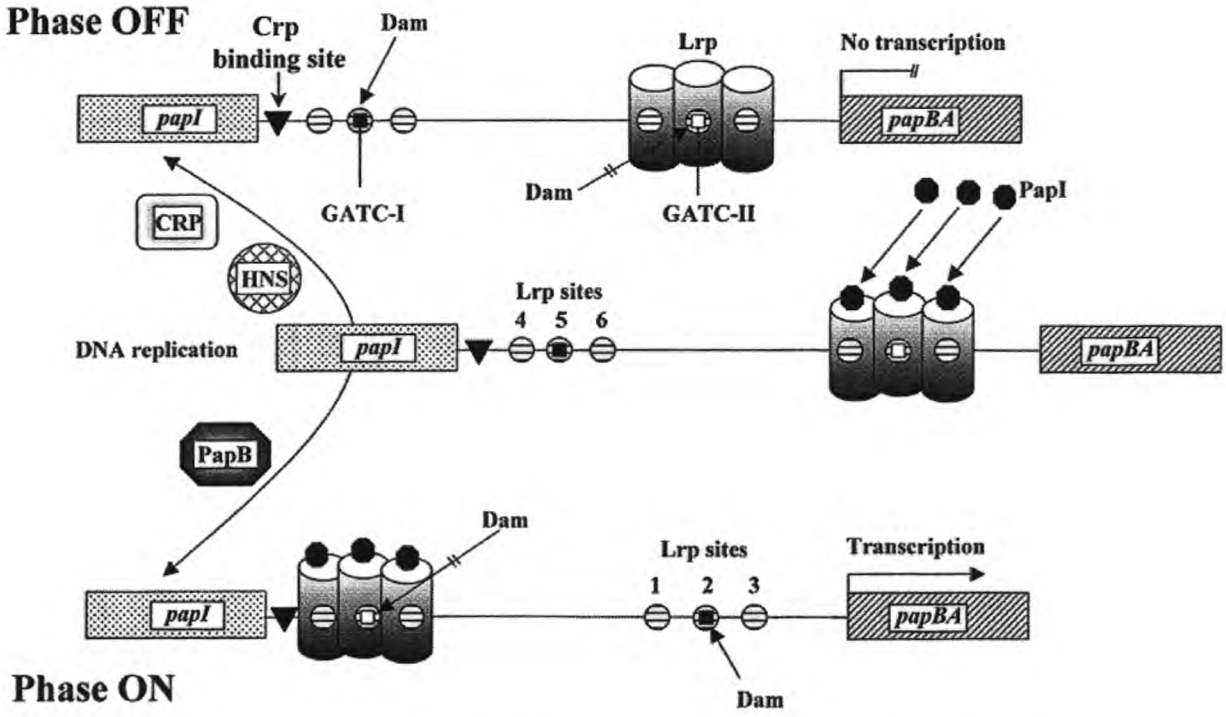


Figure 2-4. Phase variation by differential methylation. The diagram shows the intergenic region between *papI* and *papBA*. Six Lrp binding sites (hatched circles) are present. The GATC site (box) is either methylated (closed box) or unmethylated (opened box). Hatched arrows indicate no interaction (adapted from Henderson *et al.*, 1999).

2.3.2 Importance of diversity for adaptation and survival

Diversity generated by permanent genetic modification and transient phase variation has facilitated the adaptation of bacteria to survive adverse conditions. For example, different *Halobacterium* species are found in the Dead Sea, where the salinity is extremely high with a total dissolved salt content of 322.6 g L^{-1} (Nissenbaum, 1975), while iron-oxidizing bacteria can be found near hydrothermal vents, where the temperature ranges from 10°C to 167°C with a pH of 5.6 (Emerson and Moyer, 2002). In addition to adapting to adverse conditions, diversity is also believed to acclimatize bacteria to rapidly changing environments such as the sudden drop in temperature when bacteria from the gut of a warm-blooded animal are excreted into cold fresh water together with the feces. In

addition to the macroenvironment mentioned above, the production of EPS and the densely packed nature of biofilms also create microenvironments with different gradients of nutrients, oxygen and other required growth factors within the biofilms (de Beer *et al.*, 1994; Sternberg *et al.*, 1999), where subpopulations of bacteria are believed to grow.

As biofilms in nature are typically mixed species consortia, a conflict of interest becomes apparent. Most morphotypic variants are fitter than their wild type strains in various aspects including biofilm formation capacity, resistance to antibiotics, toxic doses of metal ions and hypochlorite (Boles *et al.*, 2004; Kirisits *et al.*, 2005; Workentine *et al.*, 2010). On the other hand, mixed species biofilms have also been shown to be more resilient to antimicrobials and invasion by other organisms (Burmolle *et al.*, 2006; Whiteley *et al.*, 2001a). As a result, a conflict of interest arises between a species (i.e. to generate more variants that will probably outcompete the other species in the community) and the whole community (i.e. to maintain the stability and functionality of the community).

2.3.3 Relationship between intraspecies and interspecies diversity

The relationship between intraspecies and interspecies diversity has been explored intensively through the development of ecological and evolutionary theories. Generally, this relationship can be grouped into three categories: 1) parallel effects of locality characteristics on intraspecies and interspecies diversity; 2) casual effects of intraspecies diversity on interspecies diversity and 3) casual effects of interspecies diversity on intraspecies diversity (Figure 2-5). Parallel effects of locality characteristics on intraspecies and interspecies diversity involve immigration, genetic drift and selection. In most cases, these processes act in parallel and result in moderate to strong positive relationships between intraspecies and interspecies diversity. With some exceptions, these processes can result in either positive, negative or unimodal relationship between the two types of diversity (Vellend, 2005). The casual effects of intraspecies diversity on interspecies diversity can also be divided into two scenarios. First, an increase in intraspecies diversity will result in an increase in interspecies diversity as different species may be selected by different variants of a population. This scenario aligns with the

"diversity begets diversity" hypothesis (Whittaker, 1975). Second, an increase in intraspecies diversity will boost the fitness of the population so that invasion by another species becomes difficult. Hence, interspecies diversity is decreased (Burmolle *et al.*, 2006; Elton, 1958). Similarly, the casual effects of interspecies diversity on intraspecies diversity can also be divided into two scenarios and are in fact, variations of the two hypotheses mentioned above. First, a community with numerous competing species tends to restrict the niche in which each species can grow. Thus, a smaller number of intraspecies variants can coexist in a diverse community, while more variants can coexist in a less diverse community (Van Valen, 1965). Second, different species in a diverse community can favour different intraspecies variants. Therefore, an increase in interspecies diversity will lead to an increase in intraspecies diversity and vice versa (Harper, 1977).

Most ecological and evolutionary theories describing interspecies interactions are mainly developed from studies done on plants and animals. As a result, the genetic recombinations that took place during sexual reproduction are challenges to clearly measure the changes in a population's genetic diversity. In addition, their (relatively) slow reproduction rates and long life cycles would mean that ecological experiments must be carried out over a long period of time that ranges from months to years (Vellend and Geber, 2005). In contrast, bacteria reproduce asexually and give rise to variants that can either be identified by their colony morphologies or molecular markers. Their relatively short life cycles also allow ecological experiments to be completed over a shorter period of time. As a result, bacterial models may be ideal systems to test out ecological theories on interspecies interactions and genetic adaptation.

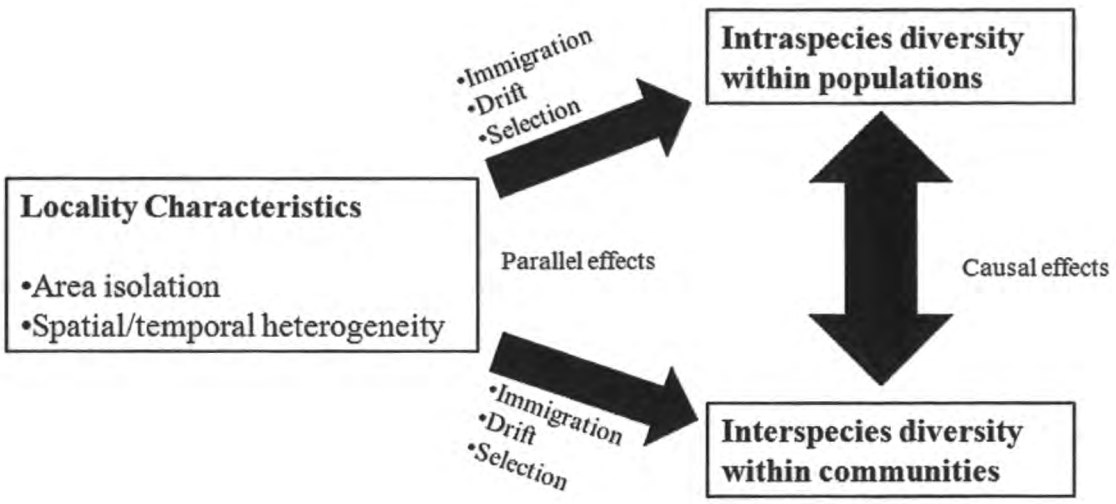


Figure 2-5. Relationships between intraspecies diversity and interspecies diversity (adapted from Vellend and Geber, 2005).

In summary, biofilms in nature are comprised of numerous species. These species often interact, resulting in biofilms with increased resistance to environmental stresses such as oxidative stress, antibiotics and predation. In addition, gene expression profile and intraspecies diversity may also be influenced by interspecies interactions. Clearly, interspecies interactions cannot be experimentally investigated using mono species biofilm systems and it is increasingly evident that observations derived from mono species biofilm studies may not be applicable to a mixed species biofilm. As a result, the effects that interspecies interactions had on a model mixed species biofilm were investigated in this thesis.

CHAPTER 3 . GENOME SEQUENCING AND ANNOTATION OF *KLEBSIELLA PNEUMONIAE*, *PSEUDOMONAS AERUGINOSA* AND *PSEUDOMONAS PROTEGENS*

3.1 Introduction

Interspecies interactions have been shown to affect numerous aspects of a mixed species biofilm (Elias and Banin, 2012; James *et al.*, 1995; Moons *et al.*, 2009; Rendueles and Ghigo, 2012). For example, the spatial organization of *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13 in a biofilm was different when the mixed species biofilm was fed with chlorobiphenyl (with metabolic interaction) and citrate (without metabolic interaction) (Nielsen *et al.*, 2000). In addition, it has been shown that mixed species biofilms display enhanced resistance to numerous stresses (Burmolle *et al.*, 2006), suggesting that they behave differently from mono species biofilms and planktonic cells. To better understand how a mixed species biofilm coordinates various behaviours of its members as well as the genes and proteins involved in driving community phenotypes such as enhanced resistance, a three species biofilm comprised of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Pseudomonas protegens* was used.

K. pneumoniae is a Gram-negative, encapsulated, non-motile, rod shaped facultative anaerobe that ferments lactose. *K. pneumoniae* can be found in plants (Chelius and Triplett, 2000) where it is associated with nitrogen fixation (Mahl *et al.*, 1965). In animals, *K. pneumoniae* is the cause of metritis in mares as well as bovine mastitis (Braman *et al.*, 1973; Platt *et al.*, 1976). *K. pneumoniae* is also the cause of acquired pulmonary infection in humans and can be acquired either from the community or the hospital. Although community acquired *K. pneumoniae* infections have become rare (1-3%) in the United States of America and Europe, it remains a concern in Asian countries such as Taiwan (Wu *et al.*, 2009). In contrast, nosocomial infection by *K. pneumonia* is high globally, representing 3-8% of all nosocomial bacterial infections in hospitals (Podschun and

Ullmann, 1998). Some of the major factors that affect the virulence of *K. pneumoniae* are adhesins, capsular polysaccharides, lipopolysaccharides and siderophores.

Pseudomonas aeruginosa is a Gram-negative, motile, coccobacillus bacterium that is an aerobe. *P. aeruginosa* possesses diverse metabolic and pathogenic mechanisms that allow it to survive in numerous environments and conditions. One of the most unique phenotypic features of *P. aeruginosa* is its green pigmentation. The pigmentation is a result of pigments such as pyocyanin, pyoverdine and pyorubin (Yahr and Parsek, 2006). *P. aeruginosa* is also motile and moves by swimming, swarming and twitching. Each type of motility is driven by either flagella, type IV pili or both (Kohler *et al.*, 2000; Merz *et al.*, 2000). *P. aeruginosa* is commonly found in soil and water, but is also a major opportunistic pathogen of plants, animals and humans. Typically, *P. aeruginosa* infections can be categorized as acute and chronic. Acute infections include ventilator-associated pneumoniae (Crouch Brewer *et al.*, 1996) and chronic lung infections in cystic fibrosis suffers (Hoiby *et al.*, 2010). Factors that are known to affect the virulence of *P. aeruginosa* include adhesins, exotoxins, proteases, hemolysins and type III secretion system (Yahr and Parsek, 2006). In addition, *P. aeruginosa* is one of the best studied model organisms for biofilm biology, where its QS regulated virulence and biofilm development are characterized (Bjarnsholt *et al.*, 2005; Davies *et al.*, 1998).

Pseudomonas protegens is a Gram-negative, motile, rod-shaped bacterium that is an aerobe. It was originally classified as *Pseudomonas fluorescens*, but was recently reclassified as *P. protegens* (Lim *et al.*, 2013a; Ramette *et al.*, 2011). *P. protegens* is a soil bacterium commonly isolated from the roots of numerous plant species. *P. protegens* is recognized as a rhizobacterial strain that can protect roots of the plants against fungal infection through the production of antifungal antibiotics (Haas and Keel, 2003). Recently, *P. protegens* has also been reported to possess insecticidal activity as it produces an insect toxin that kills larvae of major insect pests of agricultural crops, including *Spodoptera littoralis*, *Heliothis virescens* and *Plutella xylostella* (Ruffner *et al.*, 2013).

CHAPTER 3. GENOME SEQUENCING AND ANNOTATION OF *KLEBSIELLA PNEUMONIAE*, *PSEUDOMONAS AERUGINOSA* AND *PSEUDOMONAS PROTEGENS*

To facilitate genomic and transcriptomic analysis of the mixed species biofilms, the three bacteria were sequenced and their genomes were annotated. This was particularly important for *K. pneumoniae* KP-1 used in this study, as its genome has not been previously sequenced. KP-1 was found to be closely related to *K. pneumoniae* strain NTUH-K2044 (GenBank accession number AP006725) and strain MGH 78578 (GenBank accession number CP000647) based on 16S rRNA analysis, but KP-1 possesses 242 unique gene features. In contrast, *P. aeruginosa* PAO1 and *P. protegens* Pf-5 were obtained from the American Type Culture Collection (ATCC), and their genomes have been previously sequenced and annotated. However, insertions and deletions (INDELs) as well as single nucleotide polymorphisms (SNPs) are common between different laboratory strains of the same origin as the spontaneous mutation rate of a bacterium is high (Drake *et al.*, 1998). Therefore, the genomes of both PAO1 and Pf-5 were sequenced to identify possible INDELs and SNPs. A total of 74 and 11 non-synonymous INDELs and SNPs were identified for PAO1 and Pf-5 respectively.

3.2 Materials and Methods

3.2.1 Bacterial strains and genomic DNA extraction

The bacterial strains used in this study are listed in Table 3-1. Genomic DNA was extracted for each organism using the QIAamp DNA Mini Kit (Qiagen, Netherlands) according to the protocols suggested by the manufacturer.

Table 3-1. List of bacterial strains

Species and strain	Genotypic and phenotypic characteristics	Source or reference
Pseudomonas aeruginosa PAO1	Wild type	[1]ATCC BAA-47
[2]***Pseudomonas protegens*** Pf-5	Wild type	[1]ATCC BAA-477
[3]***Klebsiella pneumoniae*** KP-1	Wild type	This study

[1]ATCC number
[2]*Pseudomonas fluorescens* Pf-5 has recently been renamed *Pseudomonas protegens* (Lim *et al.*, 2013a; Ramette *et al.*, 2011)
[3]*K. pneumoniae* is an environmental strain isolated from soil

3.2.2 16S rRNA sequencing and whole genome sequencing

16S rRNA gene sequencing was carried out to verify the identity of KP-1. First, colony PCR amplification was performed using the primers 16S_27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S_1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). A single colony of KP-1 was suspended in 30 µl of sterile water and boiled for 5 min. The lysate (1 µl) was then transferred to a PCR tube containing 9 µl of the reaction mixture (1 x PCR buffer (Invitrogen, USA), 0.5 mM dNTP, 2 µM of each primer and 0.5 unit of *Taq* polymerase (Invitrogen, USA)) and run in a C1000^{TM} thermal cycler (Biorad, USA) with an initial denaturation at 97°C for 3 min followed by 35 cycles of amplification (denaturation at 97°C, 30 s; annealing at 55°C, 30 s; extension at 72°C, 1 min) and a final extension at 72°C for 10 min. Second, the PCR product was purified using Qiagen QIAquick PCR Purification Kit (Qiagen, Netherlands) and 50-100 ng of the purified product was used as a sequencing template using the BigDye sequencing reagents (Applied Biosystems, USA) and 3.2 pmol of primer. The PCR reaction, initial denaturation at 96°C for 1 min followed by 60 cycles of amplification (denaturation at 96°C, 10 s; annealing at 50°C, 5 s; extension at 60°C, 4 min), was performed using a C1000^{TM} thermal cycler (BIO-RAD, USA). The amplified product was purified as described (Tillett and Neilan, 1999) and sequenced. Sequences were aligned using BLASTN against the nucleotide sequence database of National Center for Biotechnology Information (NCBI).

Whole genome sequencing of KP-1 was performed on a 454 GS-FLX sequencing platform (Roche, Switzerland) and on a MiSeq benchtop sequencer (Illumina, USA). The reads from both platforms were trimmed and *de novo* assembly was performed using Newbler (Roche, Switzerland). Subsequently, reference guided assembly was performed by mapping the *de novo* assembled contigs to the complete genome of *K. pneumoniae* strain NTUH -K2044 using OSLay (Richter *et al.*, 2007).

The genomic DNA of PAO1 and Pf-5 were sequenced on a MiSeq benchtop sequencer. Reads obtained were trimmed and *de novo* assembly was performed using Velvet v1.2.10 (Zerbino and Birney, 2008). Reference guided assembly was performed as mentioned

above by mapping the *de novo* assembled contigs of PAO1 and Pf-5 to their respective genome sequences deposited in GenBank of NCBI (PAO1, GenBank accession number AE004091 and Pf-5, GenBank accession number CP000076).

3.2.3 Genome annotation

The open reading frames (ORFs) of KP-1 were predicted using Glimmer v3.02 (Delcher *et al.*, 2007). All ORFs predicted were annotated by performing BLASTX analysis against the non-redundant protein sequence database of NCBI. tRNAs were predicted using tRNAscan-SE (Lowe and Eddy, 1997) while rRNA sequences were identified by RNAmmer (Lagesen *et al.*, 2007).

3.2.4 Genome comparison

The ORFs of *Klebsiella pneumoniae* strain KP-1, NTUH-K2044 and MGH 78578 were predicted using GeneMarkS (Besemer *et al.*, 2001). Subsequently, the ORFs of one strain were compared to the ORFs of another strain in a pair-wise manner using BLASTP analysis (filtered for E-value $< 10^{-5}$; identity > 50%). ORFs of interest were then analyzed by BLASTP against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000).

3.2.5 INDELs and SNPs identification

The INDELs and SNPs present in PAO1 and Pf-5 were identified using CLC genomic workbench 6 (CLC bio, Denmark). Paired-end reads of PAO1 and Pf-5 were mapped against their respective sequences deposited in the GenBank database of NCBI.

3.2.6 Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AVNZ00000000. The version described in this thesis is version AVNZ01000000.

3.3 Results

3.3.1 Identity of KP-1

BLASTN analysis comparing the 16S rRNA gene sequence of KP-1 against the nucleotide sequence database of NCBI showed that it was most closely related to *K. pneumoniae* strain NTUH-K2044 and strain MGH 78578 (both with E-value = 0 and 99% identity, Figure A-1 and Figure A-2).

3.3.2 General features of the KP-1 genome

Using 1,673,246 and 1,840,620 reads from the 454 GS-FLX and MiSeq sequencing platforms respectively, 24 contigs with a total length of 5,131,085 bp and an average GC content of 57.60% were assembled (Table 3-2). Despite the high sequencing coverage (180 fold), the complete genome of KP-1 could not be assembled. Subsequent analyses were performed based on the 24 contigs assembled and a total of 4,748 ORFs were predicted making up 85.9% of the total sequence. In addition, a total of five 5S rRNAs, one 23S rRNA and 73 tRNA clusters were predicted (Table A-1). However, no 16S rRNA clusters were identified from the sequenced data, indicating that the clusters may be in the unsequenced regions between the contigs.

Table 3-2. General features of KP-1 genome

Contig no.	Size (bp)	GC content (%)	No. of predicted ORFs	Coding region (%)	Type and no. of rRNAs	No. of tRNAs
1	159,000	59.35	144	88.9		
2	270,427	57.78	258	83.7		
3	164,153	59.38	157	86.7		
4	326,654	58.38	300	85.3		5
5	685,633	57.19	619	86.9	5S rRNA: 1	16
6	548	52.55	0	0		
7	428,503	58.40	407	87.5		9
8	180,434	57.96	181	85.6		2
9	121,192	56.29	117	85.5	5S rRNA: 1	3
10	26,546	52.29	38	79.2		

11	493,584	57.84	446	87.7		2
12	94,013	57.28	89	87.2		
13	2,928	52.63	0	0	23S rRNA: 1	
14	86,577	57.25	76	88.7		6
15	544	56.25	0	0		
16	34,749	56.44	31	87.1		
17	129,870	57.34	120	86.3	5S rRNA: 1	
18	250,202	55.37	214	83.0		5
19	376,503	58.03	349	90.1	5S rRNA: 1	3
20	763	52.42	0	0		
21	625,120	57.78	577	84.1	5S rRNA: 1	17
22	427,540	57.06	386	84.9		5
23	1,224	62.34	0	0		
24	244,378	56.94	239	82.6		
Total	5,131,085	57.60	4,748	85.9	5S rRNA: 5 23S rRNA: 1	73

3.3.3 Comparison between KP-1 genome and genomes of *K. pneumoniae* strain NTUH-K2044 and MGH 78578

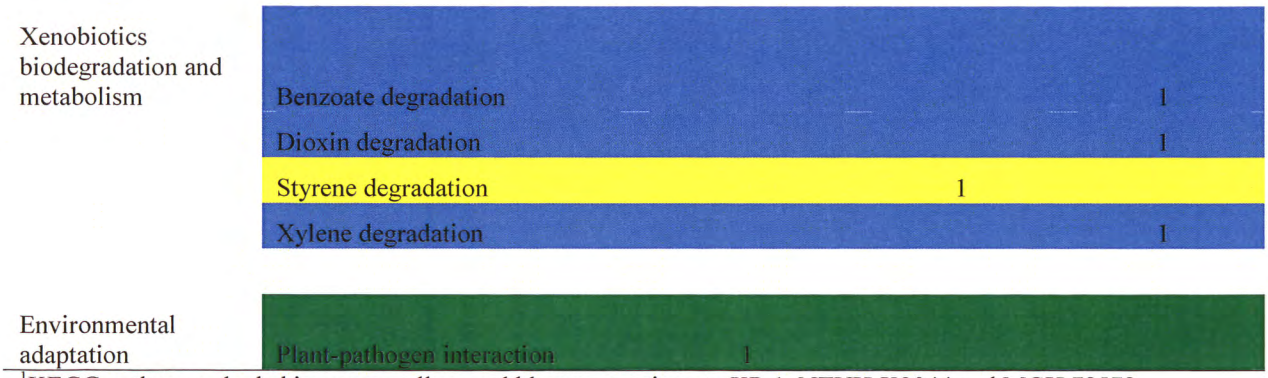
As KP-1 was closest to *K. pneumoniae* strains NTUH-K2044 and MGH 78578, their genomes were compared in this study. It should be noted that NTUH-K2044 and MGH 78578 are medical isolates, collected from patients with nosocomial *K. pneumoniae* infections. From the comparison, KP-1 was found to share at total of 4,589 and 4,634 gene features with NTUH-K2044 and MGH 78578 respectively. Further, KP-1 was found to possess 242 unique gene features whereas strain NTUH-K2044 and MGH 78578 were found to possess 251 and 305 unique gene features respectively. Out of all the unique gene features, only 3, 27 and 8 gene features from KP-1, NTUH-K2044 and MGH 78578 respectively were successfully mapped to either one or more KEGG pathways (Table 3-3). Based on these results, KP-1 possesses a truncated elongation factor Tu-like protein (involved in plant-pathogen interaction pathway) and GDP-L-fucose synthase (involved in fructose and mannose metabolism), suggesting its origin from the environment, especially in association with plants. In contrast, multiple gene features from the bacterial secretion system, epithelia cell signaling, biosynthesis of siderophores and various metabolic

pathways were present in NTUH-K2044 and MGH78578, but not found in the sequenced genome of KP-1. These unique gene features in NTUH-K2044 and MGH78578 were consistent with their origins from nosocomial pneumoniae infections (Wu *et al.*, 2009).

Table 3-3. KEGG pathways with mapped gene features

		Strain		
		KP-1	NTUH-K2044	MGH78578
General function	**[1]KEGG pathway**	**No. of gene features**		
Transport and catabolism	Lysosome		1	
Membrane transport	ABC transporters		2	
	Bacterial secretion system		9	
Signal transduction	HIF-1 signaling pathway		1	
	Two-component system		2	1
Translation	Ribosome		1	
Infectious diseases	Amoebiasis			1
	Epithelial cell signaling in Helicobacter pylori infection		1	
	Legionellosis		1	
Amino acid metabolism	Arginine and proline metabolism	1	1	1
	Biosynthesis of amino acids			2
	Cysteine and methionine metabolism			1
	Tyrosine metabolism		1	
Biosynthesis of secondary metabolites	Biosynthesis of secondary metabolites		3	4
	Phenylpropanoid biosynthesis		1	
	Streptomycin biosynthesis			2

Carbohydrate metabolism	Amino sugar and nucleotide sugar metabolism	1		1
	Butanoate metabolism		1	
	Citrate cycle (TCA cycle)		2	
	Fructose and mannose metabolism	1		
	Glycolysis/Gluconeogenesis		2	1
	Pentose and glucuronate interconversions			1
	Pentose phosphate pathway			2
	Pyruvate metabolism		2	
	Starch and sucrose metabolism		1	1
	Carbon metabolism		3	2
Energy metabolism	Methane metabolism			1
	Nitrogen metabolism		1	
Glycan biosynthesis and metabolism	Other glycan degradation		1	
Metabolism	Metabolic pathway	1	7	7
	Microbial metabolism in diverse environments		7	3
Metabolism of cofactors and vitamins	Riboflavin metabolism		1	
Metabolism of other amino acids	Cyanoamino acid metabolism		1	
Metabolism of terpenoids and polyketides	Biosynthesis of siderophores group nonribosomal peptides		3	
	Polyketide sugar unit biosynthesis			2
Nucleotide metabolism	Purine metabolism		4	



[1]KEGG pathways shaded in green, yellow and blue were unique to KP-1, NTUH-K2044 and MGH 78578 respectively.

3.3.4 INDELs and SNPs for PAO1 and Pf-5

Both PAO1 and Pf-5 were sequenced using the MiSeq benchtop sequencer. A total of 1,946,110 (average length: 468.77 bp) and 1,687,202 paired-end reads (average length: 461.01 bp) were obtained for PAO1 and Pf-5 respectively. The sequencing coverage for PAO1 and Pf-5 were 60 and 48 fold respectively. When the reads were mapped to the genome sequence of PAO1, deposited in GenBank of NCBI (GenBank accession number AE004091), a total of 423 INDELs and SNPs were found. Out of these INDELs and SNPs, 74 were non-synonymous and were found on 30 gene features (Table 3-4). Most of the INDELs and SNPs were found on genes encoding for the coat protein (6 SNPs) and helix destabilizing protein (5 SNPs) of the filamentous bacteriophage Pf1. While some of the gene features affected could be categorized into either genes responsible for encoding metabolic proteins (e.g. urocanate hydratase and enolase) and transcriptional proteins (e.g. transcriptional regulator MexT), the majority (46 INDELs and SNPs) were associated with hypothetical proteins with unknown functions.

Table 3-4. INDELs and SNPs for PAO1

Types of mutation	[1]Coding region changes	[2]Amino acid changes	Gene features
SNP	NP_248849.1:c.930T>G	Cys310Trp	transcriptional regulator
SNP	NP_249409.1:c.146G>A	Gly49Asp	hypothetical protein
SNP	NP_249409.1:c.50C>G	Ala17Gly	hypothetical protein
SNP	NP_249409.1:c.80T>C	Val27Ala	hypothetical protein
SNP	NP_249410.1:c.107C>T	Pro36Leu	hypothetical protein

Deletion	NP_249410.1:c.295delC	Leu99fs	hypothetical protein
SNP	NP_249410.1:c.332C>A	Ser111Tyr	hypothetical protein
SNP	NP_249410.1:c.334C>T	Pro112Ser	hypothetical protein
SNP	NP_249410.1:c.344C>T	Pro115Leu	hypothetical protein
SNP	NP_249411.1:c.320T>C	Met107Thr	helix destabilizing protein of bacteriophage Pf1
SNP	NP_249411.1:c.321G>C	Met107Thr	helix destabilizing protein of bacteriophage Pf1
SNP	NP_249411.1:c.356T>C	Val119Ala	helix destabilizing protein of bacteriophage Pf1
SNP	NP_249411.1:c.357G>A	Val119Ala	helix destabilizing protein of bacteriophage Pf1
SNP	NP_249411.1:c.415A>T	Thr139Ser	helix destabilizing protein of bacteriophage Pf1
SNP	NP_249412.1:c.85G>A	Ala29Thr	hypothetical protein
SNP	NP_249415.1:c.1032C>A	Asp344Glu	coat protein A of bacteriophage Pf1
SNP	NP_249415.1:c.560A>G	Asn187Ser	coat protein A of bacteriophage Pf1
SNP	NP_249415.1:c.561T>C	Asn187Ser	coat protein A of bacteriophage Pf1
SNP	NP_249415.1:c.745A>G	Ser249Gly	coat protein A of bacteriophage Pf1
SNP	NP_249415.1:c.755G>A	Gly252Asp	coat protein A of bacteriophage Pf1
Deletion	NP_249415.1:c.821_823delGCG	Gly274_Asp275del insAsp	coat protein A of bacteriophage Pf1
SNP	NP_249417.1:c.1005C>G	Asp335Glu	hypothetical protein
SNP	NP_249417.1:c.1110G>C	Glu370Asp	hypothetical protein
SNP	NP_249417.1:c.1132A>G	Thr378Ala	hypothetical protein
SNP	NP_249417.1:c.1144C>G	Pro382Ala	hypothetical protein
SNP	NP_249417.1:c.1166T>C	Val389Ala	hypothetical protein
SNP	NP_249417.1:c.1199C>T	Ala400Val	hypothetical protein
SNP	NP_249417.1:c.1269G>C	Gln423His	hypothetical protein
SNP	NP_249417.1:c.761C>G	Ala254Gly	hypothetical protein
SNP	NP_249417.1:c.841A>G	Thr281Ala	hypothetical protein
SNP	NP_249418.1:c.388C>A	Leu130Ile	hypothetical protein
SNP	NP_249418.1:c.390C>T	Leu130Ile	hypothetical protein
SNP	NP_249418.1:c.509A>C	Lys170Thr	hypothetical protein
SNP	NP_249418.1:c.510A>G	Lys170Thr	hypothetical protein

Deletion	NP_249418.1:c.517delC	Arg173fs	hypothetical protein
Insertion	NP_249418.1:c.519_520insT	Asp174fs	hypothetical protein
SNP	NP_249418.1:c.796C>A	Leu266Ile	hypothetical protein
SNP	NP_249418.1:c.809T>A	Phe270Tyr	hypothetical protein
SNP	NP_249418.1:c.810C>T	Phe270Tyr	hypothetical protein
Deletion	NP_249418.1:c.934delA	Ser312fs	hypothetical protein
Insertion	NP_249418.1:c.936_937insA	Phe313fs	hypothetical protein
SNP	NP_249418.1:c.940A>C	Lys314Arg	hypothetical protein
SNP	NP_249418.1:c.941A>G	Lys314Arg	hypothetical protein
SNP	NP_249418.1:c.942G>T	Lys314Arg	hypothetical protein
SNP	NP_249418.1:c.943C>G	Leu315Val	hypothetical protein
Insertion	NP_249418.1:c.954_955insG	Thr319fs	hypothetical protein
SNP	NP_249418.1:c.956C>G	Thr319Arg	hypothetical protein
Deletion	NP_249418.1:c.961delG	Val321fs	hypothetical protein
SNP	NP_249418.1:c.970C>G	Pro324Ala	hypothetical protein
SNP	NP_249418.1:c.972G>C	Pro324Ala	hypothetical protein
Insertion	NP_249720.1:c.1_2insC	Val1fs	hypothetical protein
Insertion	NP_249813.1:c.376_377insG	Asp126fs	peptide deformylase
Deletion	NP_249865.1:c.31delT	Phe11fs	nitrate reductase catalytic subunit
Deletion	NP_250018.1:c.1920delA	Lys640fs	protease
SNP	NP_250150.1:c.101G>C	Gly34Ala	chemotaxis-specific methylesterase
Insertion	NP_250376.1:c.653_654insC	Ser218fs	enolase
SNP	NP_250732.1:c.259T>C	Tyr87His	Serine/threonine transporter SstT
Insertion	NP_250829.1:c.88_89insG	Thr30fs	hypothetical protein
Deletion	NP_250831.1:c.517delC	Leu173fs	hypothetical protein
SNP	NP_251090.2:c.2455C>G	Pro819Ala	protein PvdJ
SNP	NP_251181.1:c.745G>A	Asp249Asn	oxidoreductase
Deletion	NP_251182.1:c.240_247delGCCG GCCA	Gln80fs	transcriptional regulator MexT
SNP	NP_251182.1:c.514T>A	Phe172Ile	transcriptional regulator MexT
Insertion	NP_251358.1:c.39_40insG	Ala14fs	hypothetical protein
Insertion	NP_251417.1:c.3347_3348insG	His1116fs	hypothetical protein
SNP	NP_252449.1:c.1907A>G	His636Arg	N-acetyl-D-glucosamine phosphotransferase system transporter

SNP	NP_252846.1:c.332T>G	Val111Gly	transcriptional regulator
SNP	NP_253031.1:c.474A>C	Glu158Asp	transcriptional regulator
Insertion	NP_253050.1:c.1_2insC	Val1fs	hypothetical protein
SNP	NP_253084.1:c.532G>C	Val178Leu	hypothetical protein
Insertion	NP_253711.1:c.420_421insGGC	Gly140_Trp141insGly	hypothetical protein
SNP	NP_253787.1:c.1292C>G	Thr431Ser	urocanate hydratase
SNP	NP_253787.1:c.1293G>C	Thr431Ser	urocanate hydratase
SNP	NP_254121.1:c.858A>C	Lys286Asn	tryptophan permease

[1]ins: insertion; del: deletion
[2]fs: frame shift; ins: insertion; del: deletion

For Pf-5, a total of 111 INDELs and SNPs were identified of which 11 were non-synonymous and linked to 9 gene features (Table 3-5). Out of the 9 gene features, 4 code for hypothetical proteins. The other 5 code for polynucleotide phosphorylase, drug resistance major facilitator superfamily transporter, signal peptide peptidase SppA, phosphoglycerate mutase and class V aminotransferase. Thus, relative to the PAO1 genome, the Pf-5 genome appeared to be more conserved.

Table 3-5. INDELs and SNPs for Pf-5

Type of mutations	Coding region changes	Amino acid changes	Gene features
Deletion	YP_004842345.1:c.828delC	Pro276fs	hypothetical protein
Deletion	YP_257981.1:c.704_709delACTGGG	Asp235_Ala237delinsAla	polynucleotide phosphorylase
Deletion	YP_258519.1:c.1241delG	Gly414fs	drug resistance major facilitator superfamily transporter, drug: H^+ antiporter-2 family
Deletion	YP_258698.1:c.42delC	Arg14fs	hypothetical protein
SNP	YP_258910.1:c.811G>A	Gly271Arg	signal peptide peptidase SppA, 36K type
Deletion	YP_260770.1:c.538delG	Ala180fs	phosphoglycerate mutase
Insertion	YP_260770.1:c.541_542insC	His181fs	phosphoglycerate mutase
SNP	YP_261421.1:c.646G>T	Val216Leu	hypothetical protein
SNP	YP_261691.1:c.853G>T	Val285Phe	class V aminotransferase
Deletion	YP_262344.1:c.152delA	Lys51fs	hypothetical protein
Insertion	YP_262344.1:c.159_160insA	Asp54fs	hypothetical protein

[1]ins: insertion; del: deletion
[2]fs: frame shift; ins: insertion; del: deletion

3.4 Discussion

In this study, the genome of *K. pneumoniae* KP-1 was sequenced and annotated. Comparison between the genome of KP-1 and the genomes of two closely related *K. pneumoniae* strains (NTUH-K2044 and MGH 78578) revealed that KP-1 possesses 242 unique gene features. One of the most interesting gene features that KP-1 possesses is a N-terminal truncated elongation factor Tu (EF-Tu). It has been shown that a peptide consisting of the first 18 amino acids of *N*-acetylated EF-Tu elicited innate immunity in *Arabidopsis thaliana* (Kunze *et al.*, 2004). Therefore, it is not surprising that KP-1, an environmental isolate, possesses a N-terminal truncated EF-Tu, which may still retain its function as an elongation factor, but will not elicit the innate immunity of plants. In addition, GDP-L-fucose synthase was also identified as a unique gene feature of KP-1. GDP-L-fucose synthase participates in the fructose and mannose catabolism pathway and catalyzes the conversion of GDP-L-fucose and $NADP^+$ to GDP-4-dehydro-6-deoxy-D-mannose, NADPH and H^+ (Lau and Tanner, 2008). As fructose is the main monosaccharide found in plants, one might speculate that KP-1 has close association with plants in the environment. In addition to the two gene features discussed above, most of the other KP-1 unique gene features encode for hypothetical proteins which functions have not been characterized, and thus are not found in the KEGG database that categorizes genes into functional pathways. In addition to the unique gene features that suggest that KP-1 is an environmental isolate that closely associates with plants, it also possesses genes that encode essential components for plant-bacteria interactions such as type IV secretion system and catalase (Soto *et al.*, 2006).

In contrast to KP-1, both NTUH-K2044 and MGH78578 were originally isolated from patients with nosocomial pneumoniae infections. Hence, they possess unique gene features that are consistent with their origins of isolation as pathogens. For example, NTUH-K2044 has multiple genes involved in type IV secretion system while MGH78578 possesses a gene that codes for the enzyme arginase. The type IV secretion system has been demonstrated to secrete and inject virulence factors into the eukaryotic host cells (Backert and Meyer, 2006), thus enabling the pathogens to suppress the host immune response. On the other hand, arginase suppresses the host immune response by breaking

down arginine, an essential amino acid that modulates the host immune response during infection (Das *et al.*, 2010).

The genomes of both PAO1 and Pf-5 were also sequenced to identify possible INDELs and SNPs. A small number of 11 non-synonymous INDELs and SNPs were found on various genes involved in different functions for Pf-5, indicating that the genome of Pf-5 is highly conserved. In contrast, 74 non-synonymous INDELs and SNPs were identified for PAO1. More intriguingly, 6 SNPs were found in the gene coding for the coat protein A of bacteriophage Pf1 (Table 3-4). Alteration of the coat protein was previously reported to affect the infectivity of bacteriophage (Li *et al.*, 2003). Hence, the high number of mutations in this gene can in part be due to the selection for bacteriophages that exhibit either lower or higher infectivity, depending on the environmental conditions. More importantly, these results support the findings reported by Klockgether *et al.* (2010), where 140 and 153 SNPs were identified for two different laboratory strains of PAO1. It is also worth noting that 9 SNPs from one of these strains were found in genes encoding bacteriophage Pf1 proteins, which are consistent with the results from this study. In general, PAO1 has accumulated many mutations throughout its propagation and use in various laboratories around the world. As a result, these changes in genotypes should be identified to improve the accuracy of gene expression studies, such as metatranscriptomic analysis.

In conclusion, a bacterium is constantly undergoing spontaneous mutations. If the mutation results in a phenotypic change, environmental selection pressure may act to either select for or against the mutation. For example, the high number of mutations observed in the gene encoding bacteriophage Pf1 coat protein A (Table 3-4), indicates that these mutations may be selected. In addition, acquisition of genes from other bacteria living in close proximity through horizontal gene transfer is also a mean by which a bacterium can adapt quickly to a new environment (Dzidic and Bedekovic, 2003; Liang *et al.*, 2011). This is probably the case for the presence of unique gene features in the three *K. pneumoniae* strains. Finally, with high-throughput genome sequencing readily available, it

is always advised that genomes of strains used should be resequenced and compared to reference genomes deposited in the database for quality control.

CHAPTER 4 . BACTERIAL TAGGING USING TRANSPOSON 7, ITS STABILITY AND EFFECT ON GROWTH

4.1 Introduction

To study biofilm development in real time, it is optimal to insert a marker that allows the different species to be visualized and discriminated from each other. Although multiple tools such as fluorescence *in situ* hybridization (FISH) have been developed to specifically label a species in a mixed community, there are still several fundamental problems related to their use. For example, most of these techniques involve fixation of the sample, which makes real time studies impossible. In this study, a transposon was used to deliver genes coding for different fluorescent proteins and antibiotic resistance into bacteria of interest. This approach does not involve fixation and the use of toxic fluorophores, thus allowing the performance of real time biofilm imaging, where the community members can be distinguished from each other.

Transposons, a class of mobile genetic elements that can be found in almost every organism, are thus far the most versatile tools for genetic analysis and delivery of small or large fragments of DNA into bacteria (Berg *et al.*, 1989). Unlike the use of plasmids that require maintenance by selection pressure, for example selection for antibiotic resistance, transposons insert into the chromosomes of their hosts. This property has rendered transposons exceptionally useful in situations where a recombinant phenotype is to be stably maintained in the host without any selection pressure. In addition, transposons once inserted into the chromosomes of their hosts are inherited in a stable fashion (de Lorenzo and Timmis, 1994).

Two of the most commonly studied and used transposon systems are Transposon 5 (Tn5) and Transposon 10 (Tn10). Both Tn5 and Tn10 insert randomly into the chromosomes of their hosts and have been used to either generate library of mutants with random gene knockouts or to insert foreign DNA. While the feature of random insertion is beneficial

when screening for genes associated with a specific phenotype, the use of Tn5 and Tn10 for tagging bacterial cells with markers poses a problem. Random insertion of Tn5 and Tn10 may insert into and hence inactivate the host genes and cause unwanted phenotypic changes. Since insertion is random, the site of insertion, thus the potential of gene inactivation, is difficult to control. Even if a random insertion has no obvious phenotype, e.g. change in growth, it may have subtle effects that are not predictable.

While the Tn5 and Tn10 transposons insert randomly, this is not the case for all transposons. Tn7 is an example of a transposon that does not insert randomly, but has a preferred site of insertion. Tn7 is exceptionally complex as it has developed several alternative mechanisms to ensure its continuous propagation. In the first transposition pathway, Tn7 transposes into random sites at low frequency with a preference for conjugal plasmids. The preferential targeting of Tn7 to conjugal plasmids is believed to aid in the dispersal of Tn7 among bacterial populations (horizontal transmission). In the second transposition pathway, Tn7 transposes into a neutral intergenic site downstream of the glucosamine-fructose-6-phosphate (*glmS*) coding region (Tn7 attachment), with high frequency and specific orientation in many Gram-negative bacteria including *E. coli*, *K. pneumoniae*, *Serratia marcescens*, *Desulfovibio desulfuricans* and *Sphingomonas yanoikuyae* (Craig, 1989; Lichtenstein and Brenner, 1982; Wall *et al.*, 1996; Wang and Lau, 1996). This form of site-specific insertion is not deleterious and promotes the coexistence of Tn7 with its host. Hence, Tn7 can be inherited to the daughter cells, ensuring its propagation from one generation to the next (vertical transmission).

As a result, Tn7 is an excellent tool to deliver DNA into a wide range of bacteria without compromising their fitness. Moreover, insertion of Tn7 is easy to verify with primers designed to the *glmS* coding region and the right end of Tn7. Hence, the bacteria used in this study have been tagged using a Tn7 expression system constructed by Choi *et al.* (2005).

4.2 Materials and Methods

4.2.1 Bacterial strains and culture media

All bacterial strains used in this study are listed in Table 4-1. Bacteria were cultured in either M9 minimal medium (48 mM Na_2HPO_4; 22 mM KH_2PO_4; 9 mM NaCl; 19 mM NH_4Cl; 2 mM $MgSO_4$; 0.1 mM CaCl; and 0.04% w/v glucose) supplemented with 0.2% w/v casamino acids (supplemented M9 minimal medium), Luria Bertani (LB_{10}) broth (10 g L^{-1} NaCl; 10 g L^{-1} tryptone; 5 g L^{-1} yeast extract) or Super Optimal Broth (SOB) (10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 g L^{-1} tryptone; 5 g L^{-1} yeast extract).

Table 4-1. List of bacterial strains

Species and strain	Genotypic and phenotypic characteristics[2]	Source or reference
Pseudomonas aeruginosa		
PAO1	Wild type	[1]ATCC BAA-47
Pseudomonas protegens		
Pf-5	Wild type	[1]ATCC BAA-477
Klebsiella pneumoniae		
KP-1	Wild type	This study
Escherichia coli		
JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB*$^+$ Δ(*lac-proAB*) *e14*$^-$ [F' *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *hsdR17*(r_K^- m_K^+)	Yanisch-Perron *et al.*, 1985
HPS1	F$^-$ Δ(*lab-proAB*) *endA1 gyrA96 hsdR17 supE44 relA1 recA1 thi rif*^{R} zzx::mini-Tn5Lac4	Choi *et al.*, 2005
CC118 λpir	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ *pir*	Choi *et al.*, 2005
DH5α λpir	F$^-$,Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1* λ *pir*	Choi *et al.*, 2005

S17-1 λpir	*hsdR recA pro RP4-2* (Tc::Mu; Km::Tn7)(λ pir)	Miller and Mekalanos, 1988
HB101	F⁻, *hsdS20* (rb⁻, mb⁻), *supE44*, *ara14*, *galK2*, *lacY1*, *proA2*, *rpsL20* (Str^R), *xyl-5*, *mtl-1*, l-, *recA13*, *mcrA*⁻, *mcrB*⁻	Lambertsen *et al.*, 2004

[1]ATCC number
[2]Gm^R: Gentamicin resistance; Str^R: Stretomycin resistance

4.2.2 Plasmids and plasmid constructions

Plasmids (

Table 4-2) were purified using Invitrogen PureLink™ HQ Plasmid Purification Kit (Invitrogen, USA) according to the manufacturer's protocols. R6K replicon-based delivery plasmids were constructed. Briefly, purified ColE1 replicon-based delivery plasmids, pUC18T-mini-Tn7T-Gm-GFPmut3, pUC18T-mini-Tn7T-Gm-eYFP, pUC18T-mini-Tn7T-Gm-eCFP and vector plasmid were sequentially digested with *Sal*I and *Kpn*I restriction endonucleases (New England Biolabs, USA). The 2.6 Kbp fragment (containing the gentamicin cassette and fluorescent protein coding gene) digested from the ColE1 replicon-based delivery plasmid and the 3.0 Kbp fragment (containing the R6K *ori*) digested from the vector plasmid were gel purified using Invitrogen PureLink™ Quick Gel Extraction Kit (Invitrogen, USA). Purified fragments were ligated with T4 DNA ligase (Promega, USA). Similarly, the ColE1 replicon-based delivery plasmid, pUC18T-mini-Tn7T-Gm-DsRedExpress, was double digested with *Sal*I and *Sca*I restriction endonucleases followed by a single digestion using the *Sap*I restriction endonuclease (New England Biolabs, USA), while the vector plasmid was sequentially digested with *Sal*I and *Sap*I restriction endonucleases (New England Biolabs, USA). The 2.9 Kbp fragment digested from the ColE1 replicon-based delivery plasmid as well as the 2.8 Kbp fragment digested from the vector plasmid were gel purified and ligated as above. The newly constructed plasmids were verified by double digestion using *Sap*I and *Psi*I restriction endonucleases (New England Biolabs, USA). A schematic drawing illustrating the construction process is shown in Figure 4-1.

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Table 4-2. List of plasmids used in this study

Plasmid/host	Relevant characteristic[3]	Source
Helper plasmid		
pTNS1/CC118 λpir	helper plasmid, providing the Tn7 transposition function. Ap^R, R6K *ori*, *ori*T	Choi *et al.* (2005)
pTNS2/DH5α λpir	helper plasmid, providing the Tn7 transposition function. Ap^R, R6K *ori*, *ori*T	AY884833[1, 2]
pTNS2-ColE1/JM109	helper plasmid, providing the Tn7 transposition function. Ap^R, ColE1 *ori*, *ori*T	This study
ColE1 replicon-based delivery plasmid		
pUC18T-mini-Tn7T-Gm-GFPmut3/HPS1	pUC18 –based delivery plasmid for mini-Tn7-Gm-GFPmut3. Ap^R, Gm^R, R6K *ori*, *ori*T	DQ493877[1, 2]
pUC18T- mini-Tn7T-Gm-DsRedExpress/HPS1	pUC18 –based delivery plasmid for mini-Tn7-Gm-DsRedExpress. Ap^R, Gm^R, R6K *ori*, *ori*T	DQ493880[1, 2]
pUC18T- mini-Tn7T-Gm-eYFP/HPS1	pUC18 –based delivery plasmid for mini-Tn7-Gm-eYFP. Ap^R, Gm^R, R6K *ori*, *ori*T	DQ493879[1, 2]
pUC18T- mini-Tn7T-Gm-eCFP/HPS1	pUC18 –based delivery plasmid for mini-Tn7-Gm-eCFP. Ap^R, Gm^R, R6K *ori*, *ori*T	DQ493878[1, 2]
R6K replicon-based delivery plasmid		
pUC18TR6K-mini-Tn7T-Gm-GFPmut3/S17-1 λpir	pUC18 –based delivery plasmid for mini-Tn7-Gm-GFPmut3. Ap^R, Gm^R, R6K *ori*, *ori*T	This study
pUC18TR6K- mini-Tn7T-Gm-DsRedExpres/S17-1 λpir	pUC18 –based delivery plasmid for mini-Tn7-Gm-DsRedExpress. Ap^R, Gm^R, R6K *ori*, *ori*T	This study
pUC18TR6K- mini-Tn7T-Gm-eYFP/S17-1 λpir	pUC18 –based delivery plasmid for mini-Tn7-Gm-eYFP. Ap^R, Gm^R, R6K *ori*, *ori*T	This study
pUC18TR6K- mini-Tn7T-Gm-eCFP/S17-1 λpir	pUC18 –based delivery plasmid for mini-Tn7-Gm-eCFP. Ap^R, Gm^R, R6K *ori*, *ori*T	This study
Mobilizing plasmid		
pRK600/HB101	mobilizing plasmid, providing the mobilization ability during conjugation. Ap^R, Cm^R, R6K *ori*	N.A.

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Vector

pUC18TR6K-mini-Tn7T[1]/CC118 λpir	pUC18 –based vector plasmid for construction of R6K replicon-based delivery plasmids in this study. Ap^R, R6K *ori*, *ori*T	AY712953[2]

N.A. Not applicable
[1]Plasmids were generously provided by Herbert P. Schweizer (Choi *et al.*, 2005).
[2]GenBank accession number
[3]Ap^R: Ampicillin resistance; Cm^R: Chloramphenicol resistance; Gm^R: Gentamicin resistance

The helper plasmid, pTNS2-ColE1, was constructed by amplifying (using primers ColE1_F and ColE1_R from Table 4-3) the ColE1 *ori* from a ColE1 replicon-based delivery plasmid and subsequently ligating it into the *EcoR*I and *Sma*I restriction endonuclease sites of pTNS2. Successful construction of pTNS2-ColE1 was verified by sequential digestion with *EcoR*I and *Sma*I restriction endonucleases (New England Biolabs, USA).

Table 4-3. List of primers used

Primer	Sequence (5'-3')	Description
ColE1_F	AGGATCCCCGGGGATAACGCAGG AAAGAACAT	Primer used during PCR amplification of ColE1 *ori*. Primer is flanked with *Sma*I site at 5' end.
ColE1_R	GATTACGAATTCCTGTCAGACCAA GTTTACTC	Primer used during PCR amplification of ColE1 *ori*. Primer is flanked with *Eco*RI site at 5' end.
Tn7R	CAGCATAACTGGACTGATTTCAG	Common primer used for checking chromosomal insertion of Tn7.
PAglmS-down	GCACATCGGCGACGTGCTCTC	Primer used with Tn7R to check chromosomal insertion of Tn7 in PAO1
PF_Tn7_R2	TGCCGCACATCCACGACATCCTC	Primer used with Tn7R to check chromosomal insertion of Tn7 in Pf-5
KP_Tn7_R2	GTGGCGCCGAACAACGAACTGCT	Primer used with Tn7R to check chromosomal insertion of Tn7 in KP-1

4.2.3 Transformation of *Pseudomonas* species by electroporation

Electrocompetent PAO1 and Pf-5 were prepared according to Choi *et al.* (2006) with some modifications. Briefly, 50 mL of bacterial cells cultured in SOB were collected at mid-log phase with OD_{600} equal to 0.5-1.0. Collected cultures were chilled to 4°C and subsequent steps were performed at 4°C. The cells were collected by centrifugation (12,850 *g* for 2 min) and washed twice with 300 mM ice cold sucrose solution. Washed cells were resuspended in 400 µl of ice cold 300 mM sucrose solution.

During transformation, 2 µg of ColE1 replicon-based delivery plasmids and 2 µg of helper plasmids, pTNS1, were mixed with 40 µl of electrocompetent cells in a sterile disposable cuvette (BIORAD, USA) with an interelectrode distance of 2 mm. The mixture was incubated on ice for 5 min followed by electroporation (25 µF, 200 Ω and 2.5 kV cm^{-1}) using a Gene Pulser™ apparatus (BIORAD, USA). Transformed cells were recovered by addition of ice cold Super Optimal Broth with Catabolite repression (SOC) (SOB supplemented with 2% w/v glucose) and incubated with shaking for 3 h at 37°C and 30°C for PAO1 and Pf-5 respectively. Recovered cells were plated onto LB_5 agar (5 g L^{-1} NaCl; 10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 1.5% w/v agar) plates supplemented with 100 µg mL^{-1} gentamicin.

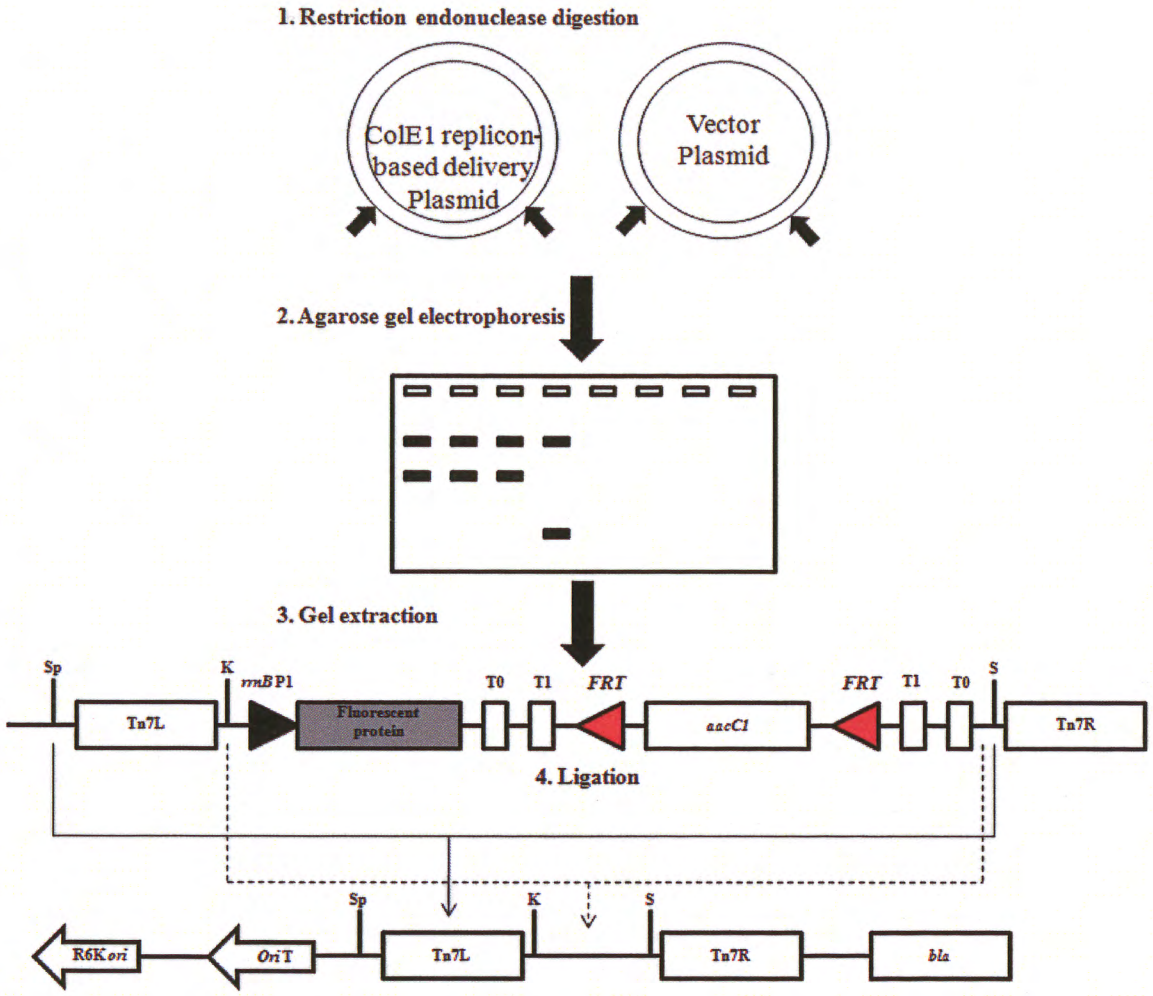

Figure 4-1. Schematic drawing illustrating the construction process for R6K replicon-based delivery plasmids. 1) ColE1 replicon-based delivery plasmid and vector plasmid were digested with restriction endonucleases mentioned in Materials and Methods. 2) Fragments resulting from the restriction endonuclease digestions were separated by agarose gel electrophoresis. 3) The desired fragments were subsequently extracted from the gel and 4) ligated together. The dashed line shows the ligation for pUC18T-mini-Tn7T-Gm-GFPmut3, pUC18T-mini-Tn7T-Gm-eYFP and pUC18T-mini-Tn7T-Gm-eCFP while the solid line shows the ligation for pUC18T-mini-Tn7T-Gm-DsRedExpress. *rrnB*P1, ribosomal promoter; T0 and T1, termination sites; *FRT*, Flippase Recognition Target; *aacC1*, gene encoding for gentamicin resistance; *bla*, gene encoding for ampicillin resistance; Tn7L and Tn7R, Tn7 inverted repeats. Relevant restriction sites: Sp, *Sap*I; K, *Kpn*I; S, *Sal*I.

4.2.4 Transformation of KP-1 by electroporation and conjugation

Electrocompetent KP-1 was prepared as described above with the following modification. KP-1 was grown in SOB supplemented with 0.7 mM of Ethylene Diamine Tetra Acetate (EDTA) to increase transformation efficiency (Fournet-Fayard *et al.*, 1995). Plasmid, pTNS2-ColE1, was added to electrocompetent KP-1 in a sterile disposable cuvette (BIORAD, USA) with an interelectrode distance of 1 mm and electroporated (25 μF, 200 Ω and 1.8 kV cm^{-1}). After recovery for 3 h at 37°C, the cells were plated onto LB_5 agar plates supplemented with 200 μg mL^{-1} ampicillin

KP-1 carrying pTNS2-ColE1 was subsequently transformed with R6K replicon-based delivery plasmids by tri-parental conjugation as described (de Lorenzo and Timmis, 1994). Briefly, overnight cultures (100 μl each) of KP-1 with pTNS2-ColE1 (recipient), *E. coli* with pRK600 (mobilizer) and *E. coli* with R6K replicon-based delivery plasmid (donor) were mixed with 700 μl of 10 mM $MgSO_4$. Cells were collected by centrifugation (7,000 *g* for 2 min), washed twice with 10 mM $MgSO_4$, resuspended in 30 μl of 10 mM $MgSO_4$ and pipetted onto a filter membrane placed on non-selective LB_{10} agar (LB_{10} with 1.5% w/v agar) plate. After overnight incubation at 37°C, cells were recovered and plated onto LB_5 agar plates supplemented with 100 μg mL^{-1} gentamicin.

4.2.5 Determination of Tn7 chromosomal insertion site

Colony PCR as described in Chapter 3 was used to verify chromosomal Tn7 insertion using the common primer (Tn7R) and primers specific for the different species (PAglmS-down for PAO1, PF_Tn7_R2 for Pf-5 and KP_Tn7_R2 for KP-1) (Table 4-3). The PCR product was analyzed on an 1% w/v agarose gel and sequenced to determined the exact site of Tn7 insertion.

4.2.6 Expression stability of the fluorescent protein

Stability of the Tn7 chromosomal insertion and expression of the fluorescent protein in the absence of antibiotic selection was determined by daily passaging (100 x dilution) in supplemented M9 minimal medium without gentamicin (200 rpm at 25 ± 1°C) for 72 h. The final culture was diluted to yield approximately 1 x 10^8 cfu mL^{-1}, stained with 4', 6-

diamidino-2-phenylindole (DAPI) as described (Yu *et al.*, 1995). Briefly, 1 µl of DAPI stock (10 mg mL^{-1}) was added to 1 mL of the diluted culture and incubated at room temperature for 1 h. Stained cells were collected by centrifuging (13,000 *g* for 2 min), washed twice and resuspended with 1 mL of filter sterilized Phosphate Buffer Saline (PBS) before visualization using a confocal laser scanning microscope (LSM 780, Carl Zeiss, Germany). The total number of DAPI and fluorescent protein expressing cells were quantified using ImageJ (http://rsb.info.nih.gov/ij/index.html).

4.2.7 Evaluating the effect of Tn7 chromosomal insertion on growth

Tagged and untagged bacteria were grown in 20 mL of supplemented M9 minimal medium (200 rpm at 25 ± 1°C) for 24 h. Subsequently, the 24 h cultures were diluted 100 x in 70 mL of supplemented M9 minimal medium and growth was monitored by Optical Density (OD_{600}) over a 12 h period using an UV spectrophotometer (UV-1800, Shimadzu, Japan).

4.3 Results

4.3.1 R6K replicon-based delivery plasmids and helper plasmid, pTNS2-ColE1

KP-1 belongs to the family of *Enterobacteriaceae*. Hence, the ColE1 replicon-based delivery plasmids constructed by Choi *et al.* (2005) can replicate in KP-1, making selection for tagged cells that have undergone transposition relative to those that carry the plasmids difficult. Thus, the ColE1 replicon-based delivery plasmids are not suitable for Tn7 based tagging of KP-1.

Plasmids with the R6K *ori* can only replicate in cell where the *pir* gene is supplied in *trans* (Bao *et al.*, 1991). KP-1 lacks the *pir* gene, and thus the R6K replicon-based delivery plasmids were constructed to tag KP-1. Constructed plasmids were verified by restriction endonuclease digestion and agarose gel electrophoresis. For R6K replicon-based plasmid, a 580 bp fragment, which represented the fragment carrying the R6K *ori* (Figure B-1), was observed after digestion (Figure 4-2A). ColE1 replicon-based delivery plasmids were digested as negative controls.

CHAPTER 4. BACTERIAL TAGGING USING TRANSPOSON 7, ITS STABILITY AND EFFECT ON GROWTH

Transformation efficiency is typically low for KP-1 as it possesses abundant capsular polysaccharide that is a barrier to the penetration of plasmid DNA by electroporation (Fournet-Fayard *et al.*, 1995). As a result, KP-1 was tagged by first transforming it with helper plasmids followed by the R6K replicon-based delivery plasmids (sequential transformation). This method requires a replicative helper plasmid that can be maintained in KP-1 after the first transformation before the second transformation with the suicide R6K replicon-based delivery plasmids. Hence, pTNS2-ColE1 (replicative helper plasmid) was constructed by cloning a ColE1 *ori* into the suicide helper plasmid, pTNS2. pTNS2-ColE1 was verified by restriction endonuclease digestion and agarose gel electrophoresis. An 840 bp fragment was observed (Figure 4-2B) for pTNS2-ColE1, indicating the presence of the ColE1 *ori* (Figure B-2). No fragment was observed for pTNS2, which lacks the ColE1 *ori*.

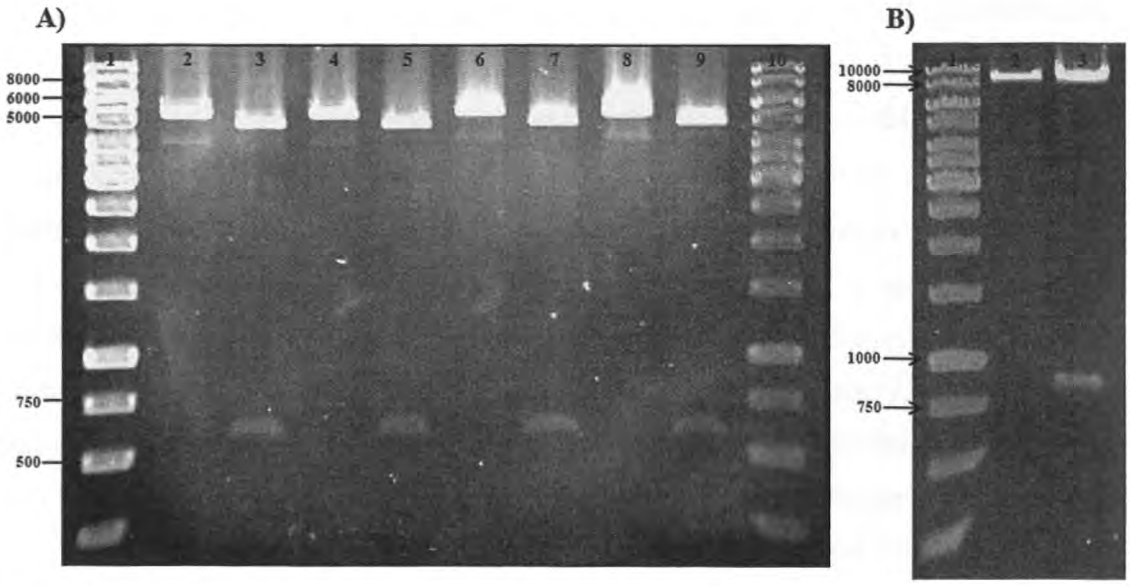

Figure 4-2. Verification of plasmids constructed using restriction endonuclease digestion. A) Verification of R6K replicon-based delivery plasmids by double restriction endonuclease digestion with *Sap*I and *Psi*I. An 1 Kbp DNA ladder with sizes indicated in bp (lanes 1 and 10), pUC18T-mini-Tn7T-Gm-GFPmut3 (lane 2), pUC18TR6K-mini-Tn7T-Gm-GFPmut3 (lane 3), pUC18T-mini-Tn7T-Gm-DsRedExpress (lane 4), pUC18TR6K-mini-Tn7T-Gm-DsRedExpress (lane 5), pUC18T-mini-Tn7T-Gm-eYFP (lane 6), pUC18TR6K-mini-Tn7T-Gm-eYFP (lane 7), pUC18T-mini-Tn7T-Gm-eCFP

(lane8) and pUC18TR6K-mini-Tn7T-Gm-eCFP (lane 9). B) Verification of pTNS2-ColE1 by sequential restriction endonuclease digestion with *EcoR*I and *Sma*I. An 1 Kbp DNA ladder with sizes indicated in bp (lane 1), digested pTNS2 (lane 2) and digested pTNS2-ColE1 (lane 3).

4.3.2 Site of Tn7 chromosomal insertion

PAO1, Pf-5 and KP-1 were each chromosomally tagged with GFPmut3, eYFP, eCFP and DsRedExpress. Site of Tn7 chromosomal insertion was determined by colony PCR using the common primer (Tn7R) and primers specific for the different species (PAglmS-down for PAO1, PF_Tn7_R2 for Pf-5 and KP_Tn7_R2 for KP-1) (Figure 4-3B). PCR products of approximately 250 bp were amplified for tagged PAO1 and Pf-5 whereas PCR products of approximately 400 bp were amplified for tagged KP-1. No PCR product was amplified for the untagged bacteria. In addition, the sizes of the PCR products matched the expected sizes as illustrated in Figure 4-3B, confirming that the PCR products resulted from Tn7 insertion (Figure 4-3A). To determine the precise location at which the Tn7 was inserted for the three bacterial species, the PCR products were sequenced. Results confirmed that the Tn7 had inserted 25 bp downstream the stop codon of the *glmS* coding region for Pf-5 (Figure 4-3C), which was different from the 29 bp reported by Koch *et al.* (2001). Further results also showed that the Tn7 had inserted 25 bp and 24 bp downstream the stop codon of the *glmS* coding region for KP-1 and PAO1 respectively (Figure 4-3C). The site of insertion for each species was the same for every fluorescent protein gene inserted (data not shown). Sequences of the PCR products are shown in Figure B-3.

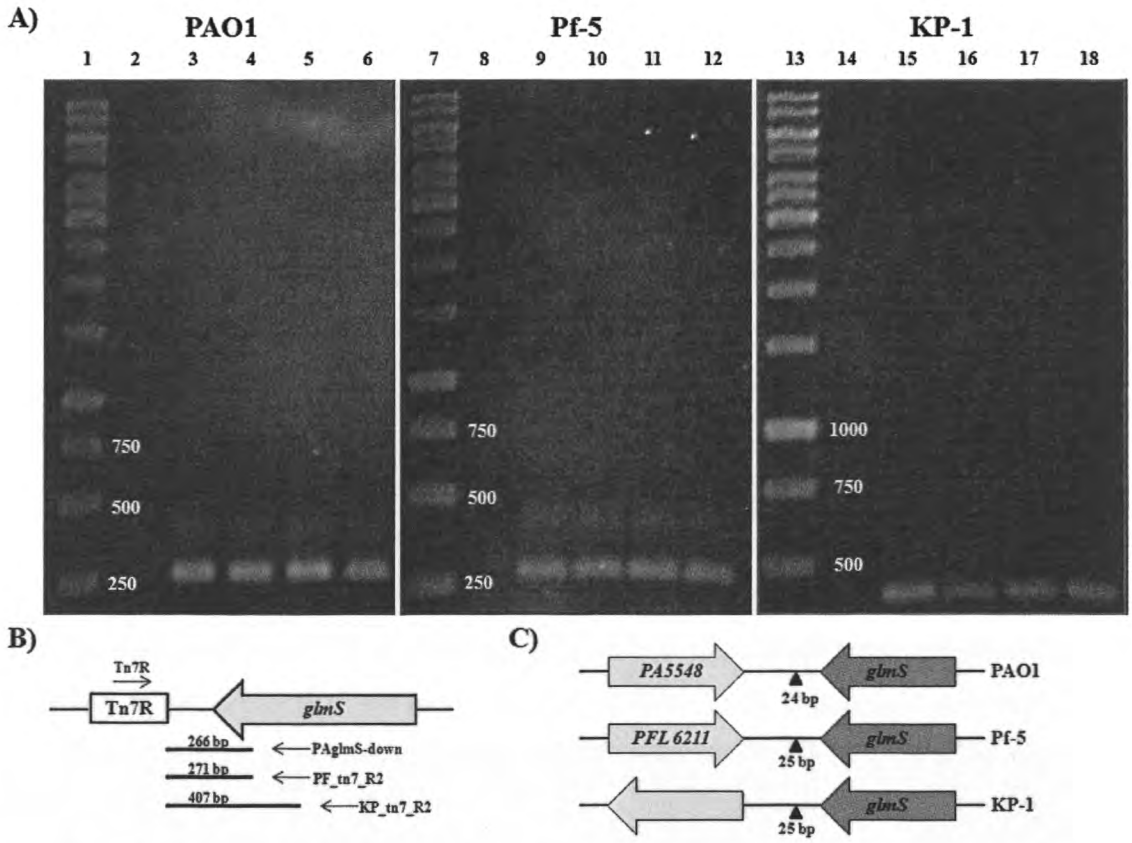

Figure 4-3. Tn7 insertion site in bacteria used in this study. A) Determination of the specific site of Tn7 insertion in the intergenic region between the coding region of *glmS* and its downstream gene. An 1 Kbp DNA ladder with sizes indicated in bp (lanes 1, 7 and 13), untagged bacteria (lane 2, 8 and 14), bacteria tagged with GFPmut3 (lane 3, 9 and 15), bacteria tagged with DsRedExpress (lane 4, 10 and 16), bacteria tagged with eYFP (lane 5, 11 and 17), bacteria tagged with eCFP (lane 6, 12 and 18). B) Schematic diagram illustrating the PCR products that were amplified by the respective primers. The length of each product was also indicated. C) Schematic diagram showing the exact site of insertion (indicated by triangle) for each bacterial species.

4.3.3 The fluorescent proteins were stably maintained without selection pressure

For optimal discrimination of the different species in a mixed species sample by confocal laser scanning microscopy, PAO1 tagged with eYFP, Pf-5 tagged with eCFP and KP-1 tagged with DsRedExpress were used. No fluorescent bleed-through was observed for the three fluorescent protein markers used (Figure B-4). They were also checked for their

respective fluorescent protein expression in the absence of gentamicin selection. Results showed that more than 90% of the tagged cells maintained their respective fluorescence after 72 h. Untagged PAO1, Pf-5 and KP-1 were used as negative controls (Figure 4-4).

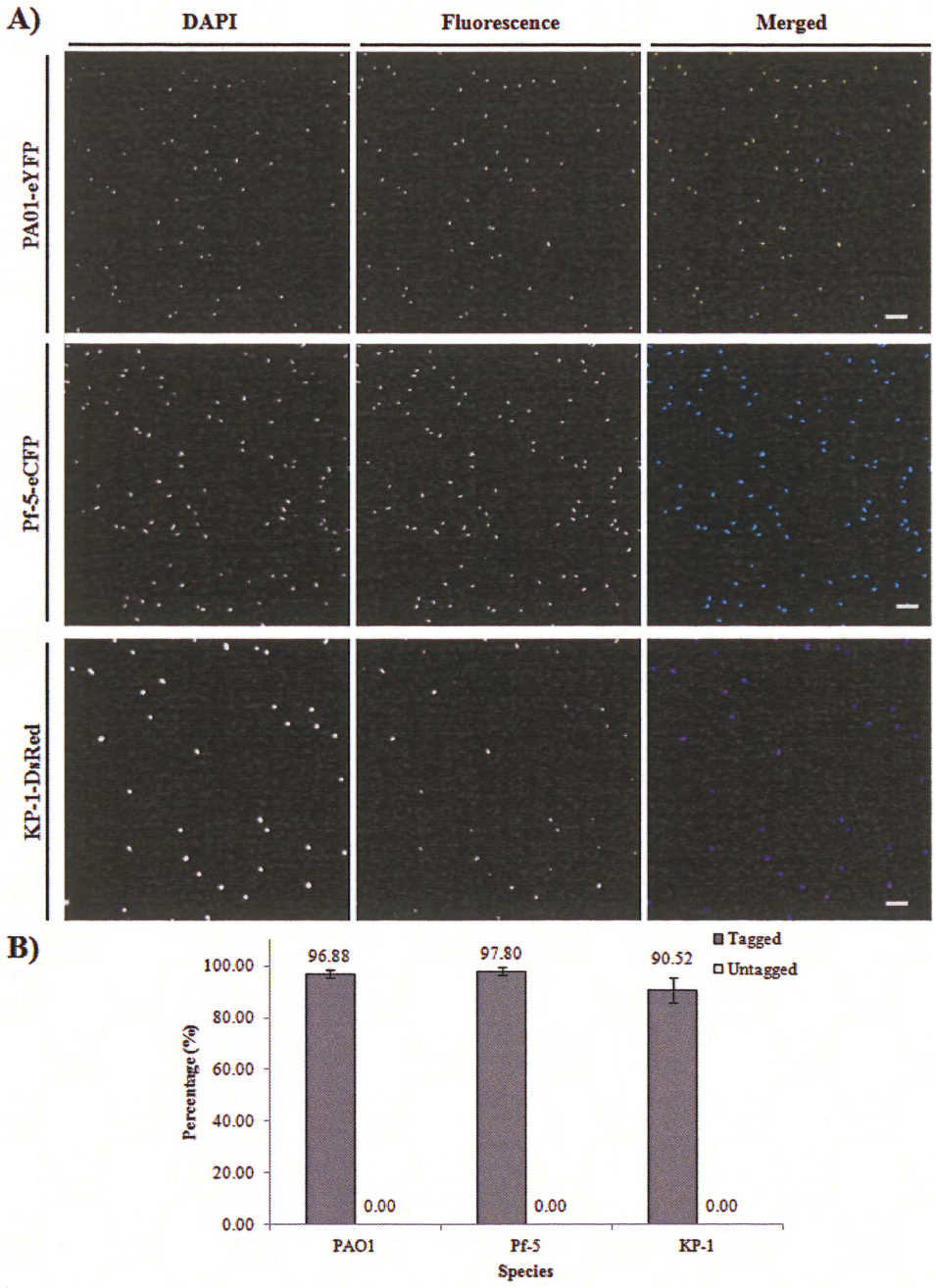

Figure 4-4. Fluorescent protein expression stability. A) Confocal microscopy images of eYFP tagged PAO1 (yellow), eCFP tagged Pf-5 (cyan) and DsRedExpress tagged KP-1

(red) (middle column). Cells were counter stained with DAPI (blue) to image all cells (left column) and the merged DAPI and fluorescent protein images are also shown (right column). Magnification: 63 x. Scale bar: 10 µm. B) The percentages of tagged and untagged cells that showed fluorescence after 72 h without gentamicin selection. Error bars represent standard deviations (n = 3).

4.3.4 Tn7 chromosomal insertion does not affect growth

It has previously been shown that chromosomal insertion and a high level of fluorescent protein expression in the cell can lead to growth defects (Hadjantonakis *et al.*, 2002; Tao *et al.*, 2007). Therefore, the growth of eYFP tagged PAO1, eCFP tagged Pf-5 and DsRedExpress tagged KP-1 were investigated. The growth profiles of tagged PAO1 and Pf-5 were similar to those displayed by untagged PAO1 and Pf-5 while the DsRedExpress tagged KP-1 exhibited a slight, but not significant (*P*-value = 0.569, One-way ANOVA) reduction in growth yield (Figure 4-5). Such growth reduction was not observed when KP-1 was tagged with the other fluorescent proteins (Figure B-5).

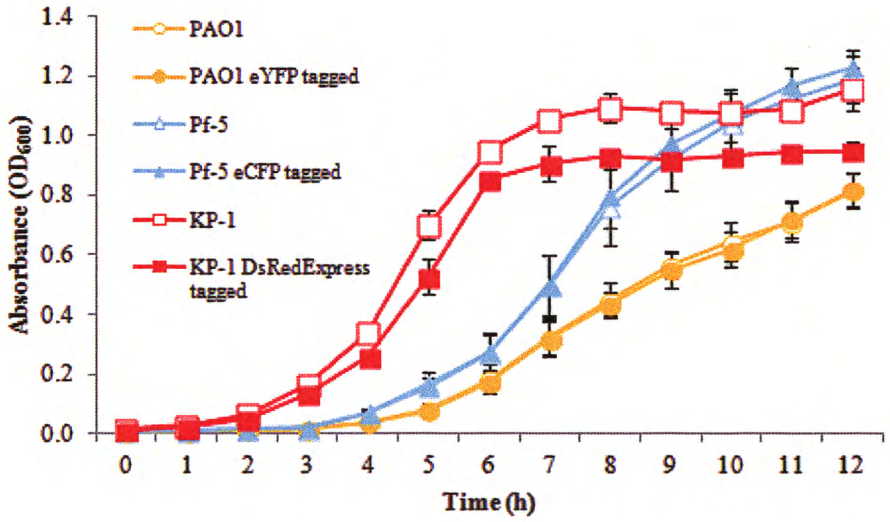

Figure 4-5. Growth profiles of tagged and untagged bacteria. Optical density measured over a period of 12 h. Error bars represent standard deviations (n = 3).

4.4 Discussion

Tn7 is a simple molecular tool that has attracted much attention due to its ability to transpose at high frequency and in specific orientation into a defined and neutral bacterial chromosomal site that can be found in most bacteria. These properties have rendered Tn7 an attractive tool for tagging bacteria with fluorescent markers as well as to insert complement gene into deletion mutant during phenotypic studies.

In this study, the ColE1 replicon-based plasmids provided (Choi *et al.*, 2005) can replicate in KP-1, which is a member of the *Enterobacteriaceae* family. Hence, selection for KP-1 cells that undergo transposition is difficult due to replication of the plasmid in the cytoplasm. In addition, the thick capsular polysaccharide produced by KP-1 results in low efficiency for simultaneous transformation of both delivery and helper plasmids. To counter these problems, R6K replicon-based delivery plasmids and helper plasmid with the ColE1 *ori* (pTNS2-ColE1) were constructed for sequential transformation. In this process, KP-1 was transformed with pTNS2-ColE1 and R6K replicon-based delivery plasmids in two independent transformations (Materials and Methods). Using this method, transformation and Tn7 chromosomal insertion are more succeesful for KP-1 cells, but curing the transformants of pTNS2-ColE1 after tranposition did not occur for KP-1 tagged with all fluorescent proteins. However, the presence of pTNS2-ColE1 in the transformants does not result in any noticeable effects e.g. growth defect (Figure B-5). Alternatively, a plasmid that possesses a temperature-sensitive ColE1 *ori* (McKenzie and Craig, 2006) can be used to chromosomally tagged KP-1 with the fluorescent protein. Growth of transformed cell at a permissive temperature will induce the recombination machinery, leading to chromosomal insertion of Tn7. Once Tn7 has inserted into the chromosome, the transformed cell can be grown at a non-permissive temperature that would inactivate replication, thus leading to the loss of plasmid from the cell.

Although Tn7 chromosomal insertion was reported to be site and orientation specific, the site of insertion may differ for different strains of the same species. The results presented here indicated that Tn7 had inserted 25 bp downstream of the *glmS* stop codon for Pf-5 instead of the 29 bp reported for DR54 (Koch *et al.*, 2001). Such phenomenon can be

explained by the slight variation (between different strains of the same species) in the specific sequence recognized by Transposase D, the transposase subunit responsible for Tn7 transposition into the specific chromosomal site (Waddell and Craig, 1989). While it has been suggested by Craig (1989) that Tn7 has a defined chromosomal insertion site for *K. pneumoniae*, this data has not been published to date. Based on the sequence of the PCR product amplified from the tagged KP-1 in this study, it was determined that the Tn7 inserted 25 bp downstream of the *glmS* stop codon. Further, BLASTN analysis of this sequence against the 28 bp *attn7* recognition site from Waddel and Craig (1989) showed that KP-1 possesses similar sequence (Figure B-6).

Previous reports have shown that Tn7 chromosomal insertion did not affect the growth of the host (Lambertsen *et al.*, 2004). While this was found to be the case for tagged PAO1 and Pf-5, a reduction in growth and final cell density was observed for the DsRedExpress tagged KP-1. The reduction in growth and final cell density was hypothesized to be due to cytotoxicity of DsRedExpress in tagged KP-1 cells (Strack *et al.*, 2008; Tao *et al.*, 2007). Such reduction was not observed when the other fluorescent proteins were used, indicating that the reduction in growth was specific for the DsRedExpress protein.

CHAPTER 5 . BIOFILM DEVELOPMENT AND STRESS RESISTANCE OF MONO SPECIES AND MIXED SPECIES BIOFILMS

5.1 Introduction

Many studies have demonstrated that biofilms are distinct from their planktonic counterparts. For example, biofilms have been found to be more resistant to antibiotics and host immune responses (Arciola *et al.*, 2005; Costerton *et al.*, 1999; Jensen *et al.*, 2007; Mah and O'Toole, 2001). This increased resistance has been hypothesized to in part reflect the expression of biofilm specific genes and phenotypes, as revealed by transcriptomic and proteomic studies. About 1-2% of the entire genome (79 genes in *E. coli* and 73 genes in *P. aeruginosa*) and more than 800 proteins were differentially expressed when biofilms and planktonic bacteria were compared in different studies (Sauer *et al.*, 2002; Schembri *et al.*, 2003; Whiteley *et al.*, 2001a). It is also clear that specific genes and proteins are required for the orderly development of a biofilm. For example, two-component systems encoded by the biofilm initiation sensor and regulator (PA4196-4197), the biofilm maturation sensor and regulator (PA4101-4102) and the microcolony formation sensor and regulator loci (PA5511-5512) were shown to be sequentially phosphorylated, thus controlling the transition of *P. aeruginosa* biofilms into the irreversible attachment, early development and maturation stages respectively (Petrova and Sauer, 2009). Further, the biofilm dispersion locus (PA1423) was also reported to be essential for the dispersal of *P. aeruginosa* biofilms. Deletion of this gene resulted in the formation of biofilms that were not responsive to common dispersal cues such as succinate, glutamate and various heavy metals (Jackson *et al.*, 2002).

Most studies thus far have focused on mono species biofilms to understand the molecular and physiological mechanisms that drive biofilm development (Sauer and Camper, 2001; Sauer *et al.*, 2002). However, biofilms in both natural and engineered environments are highly diverse. For example, 454 pyrosequencing of activated sludge revealed 1,183 to 3,567 operational taxonomic units (OTUs) (Zhang *et al.*, 2012). In addition, about 100 to

CHAPTER 5. BIOFILM DEVELOPMENT AND STRESS RESISTANCE OF MONO SPECIES AND MIXED SPECIES BIOFILMS

200 bacterial species were found to colonize and form biofilms on hard and soft tissues of the oral cavity (Paster *et al.*, 2006).

Given the diversity of biofilms in nature, it is likely that interspecies interactions play important roles in determining the development and function of these biofilms. For example, there may be intense competition for resources, as exemplified by the invasion and over growth of *Hyphomicrobium* sp. biofilms by *P. putida* (Banks and Bryers, 1991). Cooperation also appears to occur within biofilms where *Burkholderia* sp. LB400 feeding on chlorophenyl was observed to excrete chlorobenzoate, which could be metabolized by *Pseudomonas* sp. B13 (FR1) (Nielsen *et al.*, 2000). Similarly, mixed species biofilms of *Variovarax* sp., *Comamonas testosteroni* and *Hyphomicrobium sulfonivorans* degraded the phenylurea herbicide, linuron, and its metabolic intermediate more efficiently than the comparable mono species biofilms (Breugelmans *et al.*, 2008). These results indicate that interspecies interactions within a biofilm can affect its structure, physiology and function and that such interactions are unpredictable from the studies of mono species biofilms.

In this study, a mixed species biofilm comprised of *P. aeruginosa* PAO1, *Pseudomonas protegens* Pf-5 and *Klebsiella pneumoniae* KP-1 was used. This mixed species biofilm has previously been shown to be reproducible with respect to structure and viable cell counts (Jackson *et al.*, 2001; Stoodley *et al.*, 2001b). In addition, these three species are commonly found in soil habitats and were reported to coexist in metalworking fluids, where the growth of *P. protegens* was a prerequisite for the growth of *P. aeruginosa* and *K. pneumoniae* (Chazal, 1995). These three species were also isolated from the gut of the silk moth, *Bombyx mori* (Anand *et al.*, 2010), further supporting the relevance of these three species as a mixed species biofilm model. Here, confocal microscopy and image analysis of biofilms formed by fluorescent protein tagged bacteria were used to investigate the development, structure and resilience of this mixed species biofilm in comparison to mono species biofilms. Development of the biofilms was consistent where the mixed species biofilms displayed a delayed development and distinct structures that were different from all comparable mono species biofilms. Further, the mixed species biofilms were more resilient to stresses compared to the mono species biofilms as well as

the planktonic cultures and the spatial organization of the community members is here shown to directly impart the community level protection.

5.2 Materials and Methods

5.2.1 Bacterial strains and culture media

Bacteria (Table 5-1) were cultured in M9 minimal medium (48 mM Na_2HPO_4; 22 mM KH_2PO_4; 9 mM NaCl; 19 mM NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; and 0.04% w/v glucose) supplemented with 0.2% w/v casamino acids (supplemented M9 minimal medium).

Table 5-1. List of bacterial strains

Species and strain	Genotypic and phenotypic characteristics[1]	Source or reference
Pseudomonas aeruginosa		
PAO1 eYFP	carries the gene encoding eYFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
Pseudomonas protegens		
Pf-5 eCfP	carries the gene encoding eCFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
Klebsiella pneumoniae		
KP-1 DsRed	carries the gene encoding DsRedExpress in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study

[1] Gm^R: Gentamicin resistance

5.2.2 Mono and mixed species planktonic cultures

PAO1, Pf-5 and KP-1 were grown in 20 mL of supplemented M9 minimal medium (200 rpm at 25 ± 1°C) for 24 h. The cultures were then diluted 100 x in 70 mL of supplemented M9 minimal medium and growth was monitored by Optical Density (OD_{600}) over a 12 h period using an UV spectrophotometer (UV-1800, Shimadzu, Japan). At 0, 3, 6, 9 and 12

h, the cfu mL^{-1} were enumerated as described by Miles *et al.* (1938) and used to calculate the generation time for each species.

Mixed species planktonic culture was prepared by diluting the 24 h cultures of PAO1, Pf-5 and KP-1 to yield approximately 1 x 10^8 cfu mL^{-1} each before they were mixed in the ratio of PAO1:Pf-5:KP-1 = 5:5:1 (volume). This ratio was used to account for the faster growth rate of KP-1 and was optimized experimentally. Subsequently, the mixed culture was diluted 100 x in 10 mL of supplemented M9 minimal medium and allowed to grow (200 rpm at 25 ± 1°C) for 7 d with daily passage (100 x dilution). Starting from 0 h, the cfu mL^{-1} of each species was determined at 24 h intervals.

5.2.3 Continuous-culture flow cell experiments

Biofilms were cultivated in three-channel flow cells (channel dimensions, 1 x 4 x 40 mm) made from polycarbonate (Biocentrum-DTU, 2005) (Sternberg and Tolker-Nielsen, 2005). The flow cells were set up and supplied with supplemented M9 minimal medium at 9 mL h^{-1} (mean velocity = 0.625 mm s^{-1}) with a Reynolds number of 1.12 (Figure 5-1). Each channel was injected with 0.5 mL of diluted 24 h culture containing approximately 1 x 10^8 cfu mL^{-1}. Mixed species biofilms were established by inoculating mixed cultures of PAO1, Pf-5 and KP-1 as described above. Three day old biofilms were treated with 0.1% w/v Sodium Dodecyl Sulfate (SDS) in supplemented M9 minimal medium at a flow rate of 9 mL h^{-1} for 2 h. Tobramycin (10 ug mL^{-1} in supplemented M9 minimal medium) was added to 2 d old biofilms at 9 mL h^{-1} for 24 h.

5.2.4 Quantification using 16S rRNA

Biomass of the mixed species biofilm was extracted by flushing ice cold PBS through the flow cell. Total RNA was extracted using RNeasy Protect Bacteria Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocols. Sequencing libraries were prepared from total RNA using the TruSeq RNA Sample Preparation Kit (Illumina, USA) and sequenced on the HiSeq 2500 platform (Illumina, USA) according to the manufacturer's protocols. Subsequently, specific sequence tags for 16S rRNA V4 and V6 regions were used to quantify the relative abundance of the three species. The species-

specific tags were derived from the complete genomes of PAO1, Pf-5 and NTUH-K2044 (close relative of KP-1) deposited in GenBank of NCBI (PAO1, GenBank accession number AE004091; Pf-5, GenBank accession number CP000076 and NTUH-K2044, GenBank accession number AP006725). The list of species-specific tag sequences are shown in Table C-1.

To quantify the relative abundance of the three species, frequency of the species-specific tag sequence was counted. Relative abundance for the three species in each sample was then expressed as percentage of the species-specific tag sequences relative to the total number of tag sequences observed.

5.2.5 Microscopy, image and statistical analysis

Microscopic observation and image acquisition was performed by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Germany) using the multi track mode to minimize fluorescent bleed-through. The excitation wavelengths for eCFP, eYFP and DsRedExpress were 458 nm, 514 nm and 561 nm respectively. The emission wavelengths for eCFP, eYFP and DsRedExpress were 476 nm, 527 nm and 584 nm respectively. For each flow cell channel, 5 image stacks were acquired from the centre of the channel (2 mm from the walls), starting at a distance of 5-10 mm from the inlet (unless otherwise stated) and spaced approximately 5 mm apart (Heydorn *et al.*, 2000a). The 5 image stacks covered a total area of approximately 9×10^5 μm^2, which was more than the minimum of 1×10^5 μm^2 required for representative data (Korber *et al.*, 1993). For image analysis, three biological replicates (flow cells) with a total of 45 image stacks (15 from each flow cell) were quantified for each biofilm type using IMARIS (Bitplane AG, Switzerland). Reproducibility of the biofilms was determined as suggested by Jackson *et al.* (2001). Statistical analysis was performed using IBM SPSS Statistics 20.

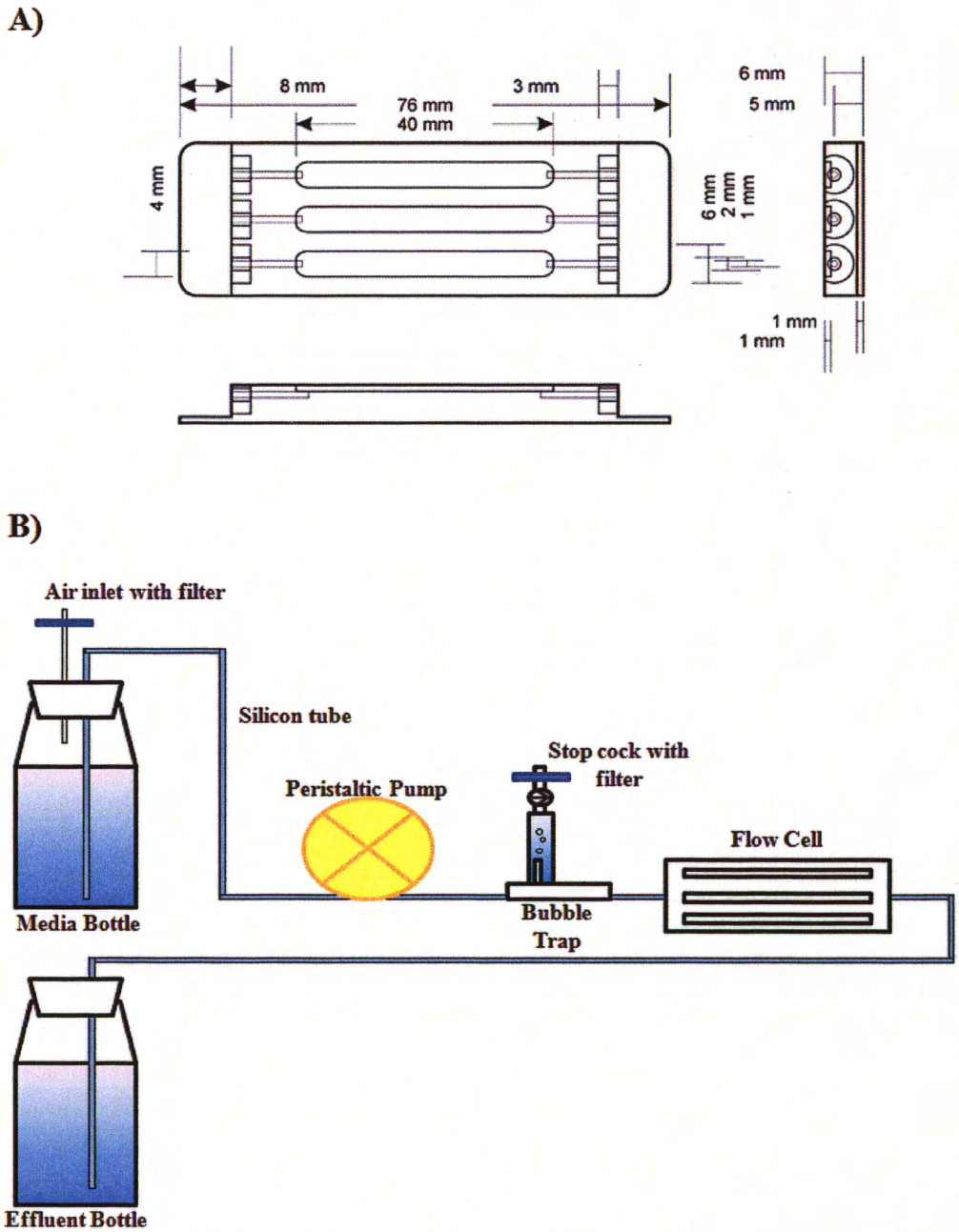

Figure 5-1. Continuous-culture flow cell setup. A) Working drawing of a flow cell (design, copyright Biocentrum-DTU, 2005). B) Schematic drawing illustrating the setup of a continuous-culture flow cell system. The system consists of a medium bottle, a peristaltic pump, bubble trap, flow cell and an effluent bottle. Silicone tubings and connectors (Silastic, Dow Corning, USA) were used to connect the medium, bubble trap, and the flow cell.

5.3 Results

5.3.1 Growth studies of mono species and mixed species planktonic cultures

The growth profile of KP-1 showed that it entered stationary phase 7 h after inoculation as a planktonic culture and had a generation time of 0.77 ± 0.03 h. PAO1 and Pf-5 entered early stationary phase 12 h after inoculation and had longer generation times of 1.33 ± 0.50 h and 1.27 ± 0.24 h respectively (Figure 5-2). After 24 h of growth, PAO1 and Pf-5 reached OD_{600} 1.16 and 1.29, respectively.

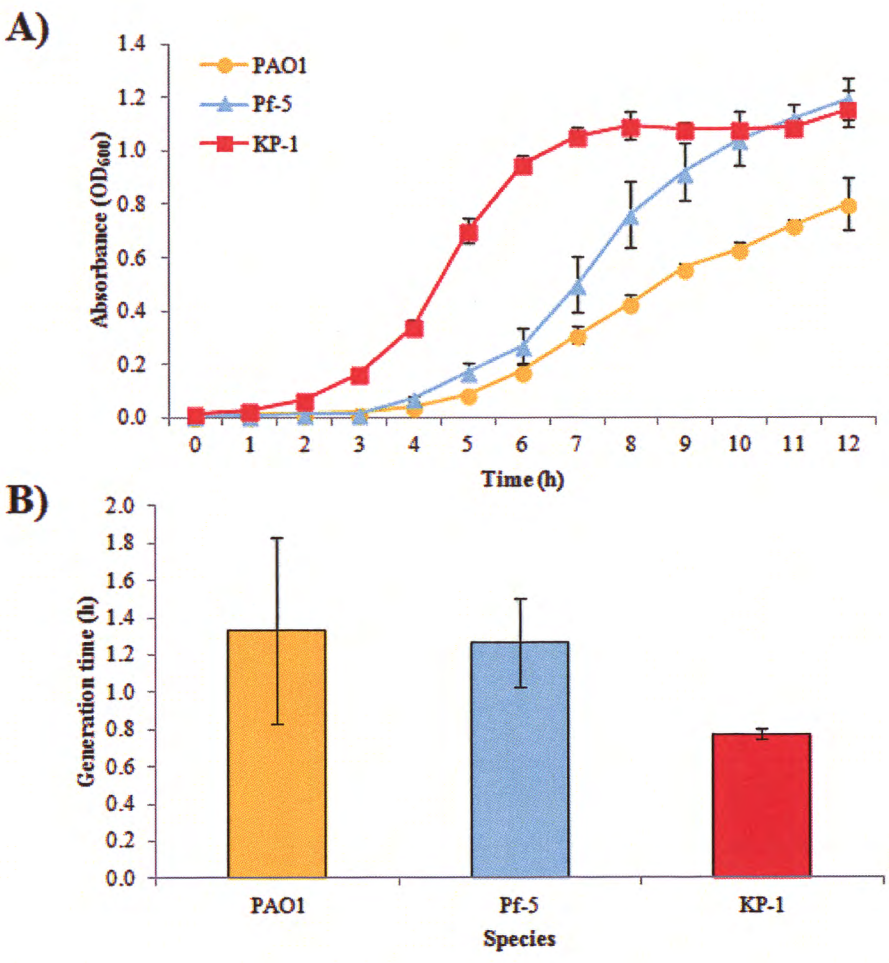

Figure 5-2. Growth of planktonic PAO1, Pf-5 and KP-1. A) Growth profiles of PAO1, Pf-5 and KP-1. Error bars represent standard deviations (n = 3). B) Generation times of PAO1, Pf-5 and KP-1. Error bars represent standard deviations (n = 3).

Although the generation times for PAO1, Pf-5 and KP-1 were different, results from the mixed species planktonic cultures demonstrated that the percentage of each species remained relatively constant throughout the observation period of 7 d. The average percentages of PAO1, Pf-5 and KP-1 throughout the 7 d was on average 46.6 ± 9.2%, 41.1 ± 9.8% and 12.3 ± 2.8% respectively (Figure 5-3).

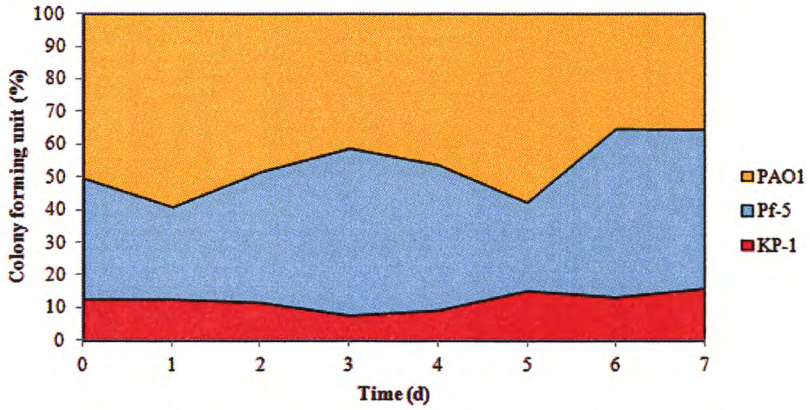

Figure 5-3. Proportions of mixed species planktonic cultures. The proportions of the three species within the mixed species planktonic cultures, derived by quantifying colony forming units over the 7 d observation period.

5.3.2 Development and structures of mono and mixed species biofilms

Comparison of the development of mono and mixed species biofilms demonstrated that KP-1 mono species biofilms matured on day 3 when the biovolume per unit base area first peaked at 15.0 ± 1.5 $\mu m^3\ \mu m^{-2}$ (Figure 5-4A). The mature KP-1 biofilms were normally flat with no distinct structure (Figure C-1). By comparison, PAO1 and Pf-5 mono species biofilms first peaked in biomass on day 4, with the appearance of mushroom and tower-like microcolonies respectively (Figure C-1). Their biovolume per unit base area were 25.1 ± 3.0 $\mu m^3\ \mu m^{-2}$ and 34.7 ± 1.9 $\mu m^3\ \mu m^{-2}$ respectively, which were both higher than that observed for the KP-1 biofilms (Figure 5-4A). Biovolume per unit base area of the mixed species biofilms increased steadily up to day 7, with a slight, but not significant decrease on day 6. However, this insignificant decrease on day 6 was consistent across multiple experiments. The biovolume per unit base area of the mature mixed species

biofilms first peaked at 31.8 ± 8.1 $\mu m^3\ \mu m^{-2}$ (Figure 5-4A) and exhibited very different structures compared to those exhibited by the mono species biofilms. For example, PAO1 and Pf-5 did not form any mushroom or tower-like microcolonies in the mixed species biofilms but were distributed randomly (Figure 5-5). Surprisingly, KP-1 was observed to form microcolonies that were shaped either as isolated mound-like structures, which measured up to 100 µm in diameter or that were similarly thick but flat, presumably due to the merging of multiple microcolonies. As noted above, such distinct structures of KP-1 were seldom observed when KP-1 was grown alone.

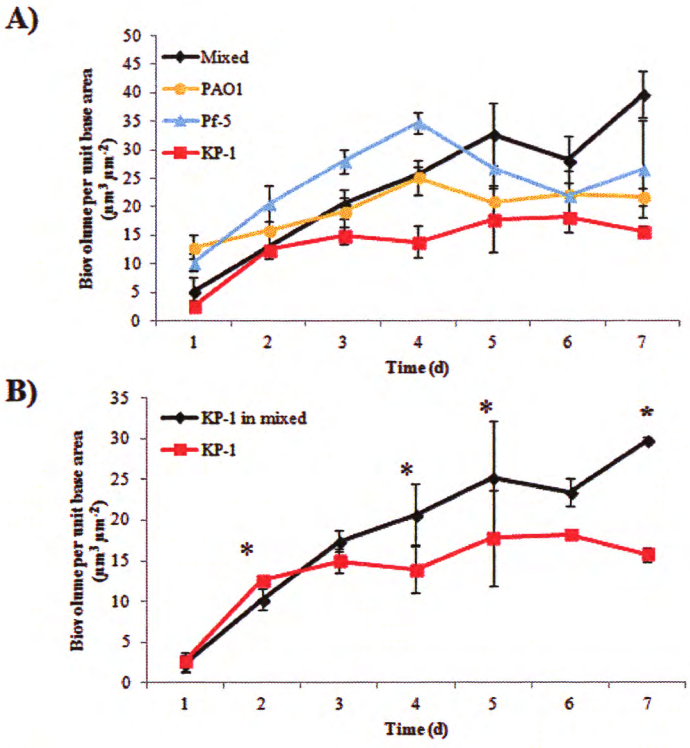

Figure 5-4. Development of mono species and mixed species biofilms. A) Biovolume per unit base area ($\mu m^3\ \mu m^{-2}$) of PAO1, Pf-5, KP-1 and mixed species biofilms. Error bars represent standard deviations (n = 3). B) Biovolume per unit base area ($\mu m^3\ \mu m^{-2}$) of KP-1 biofilms and KP-1 within the mixed species biofilms. Error bars represent standard deviations (n = 3). * denotes significant difference between the biovolume per unit base area of KP-1 biofilms and KP-1 in the mixed species biofilms (One-way ANOVA: *P*-value < 0.1).

It was interesting to note that the biovolume per unit base area for KP-1 within the mixed species biofilms was generally higher than the biovolume per unit base area of KP-1 when grown as a mono species biofilm (Figure 5-4B), even though the amount of glucose used for both types of experiment were identical. Quantitative image analysis for the mixed species biofilms indicated that KP-1 made up 70-80% of the biofilm biomass after day 2, Pf-5 accounted for 10-15% of the biomass and PAO1 was present at 1-5% (Figure 5-6). This was in contrast to the relative proportion of the three species when grown as planktonic mixed cultures (Figure 5-3). It should be noted that similar quantitative results were obtained irrespective of which fluorescent protein was used to tag the individual species (data not shown). The biofilm composition was also verified by quantifying the 16S rRNA extracted from the mixed species biofilms. The 16S rRNA genes were sequenced from total RNA extracted from the flow cell grown biofilms on days 4, 5 and 6. The results showed that the mixed species biofilms were mainly comprised of KP-1 and small populations of PAO1 and Pf-5 (Table C-2). One-way ANOVA comparisons between the percentages of KP-1, PAO1 and Pf-5 obtained from quantitative image analysis and 16S rRNA sequencing indicated that there was no significant difference between the percentage of each species obtained by the two methods (Table C-3). Similarly, the number of colony forming units in the effluents for both mono and mixed species biofilms were determined (Figure C-2) and closely reflected the changes in biofilm biomass as determined by quantitative image analysis. Thus, quantitative image analysis appears to give an accurate reflection of the biofilm community.

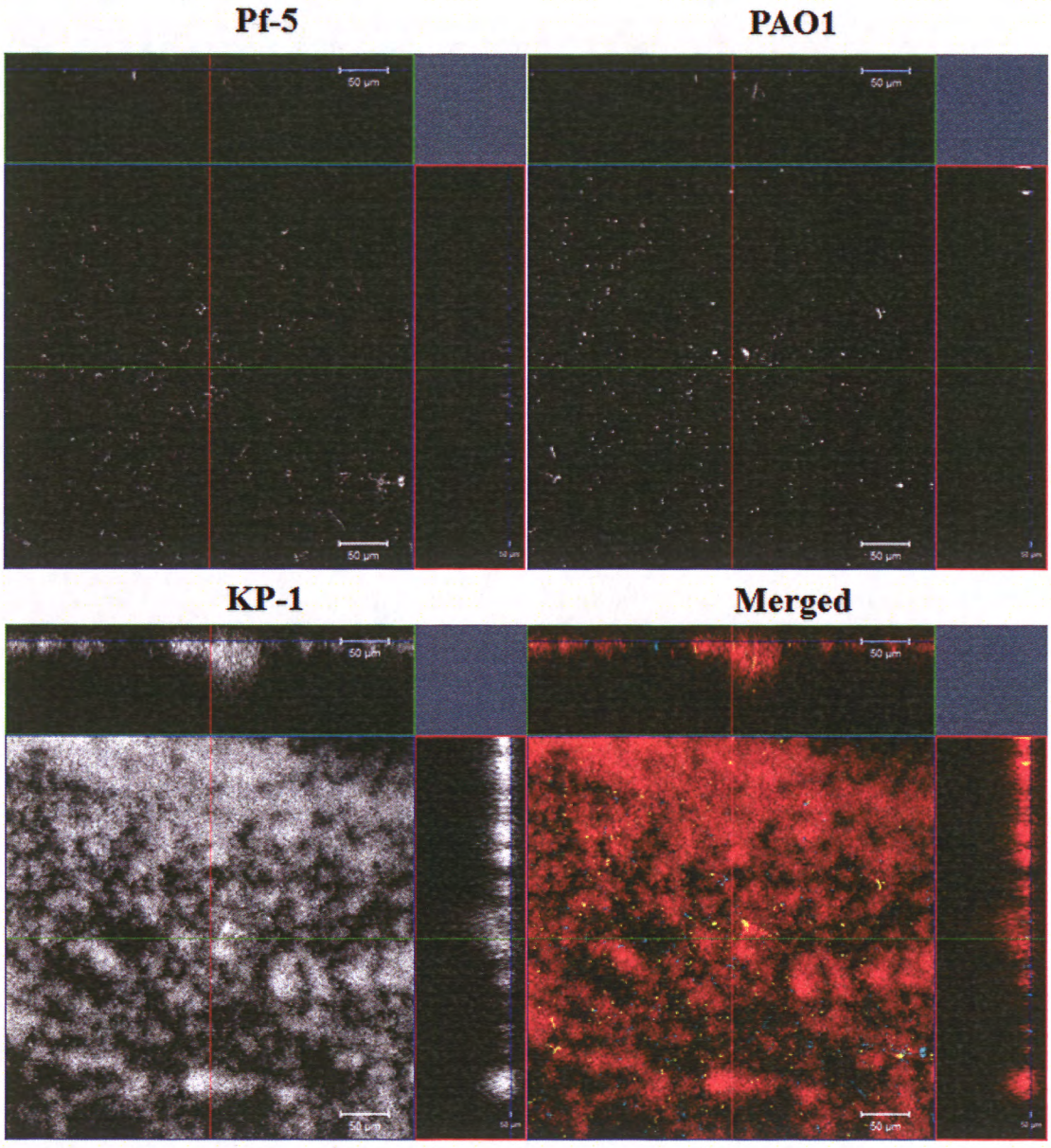

Figure 5-5. Spatial localization and structure of a mixed species biofilm. Confocal microscopy images showing the spatial localization of a mixed species biofilm. The formation of KP-1 (red) microcolonies surrounded by sparsely distributed PAO1 (yellow) and Pf-5 (cyan). Top left panel: Pf-5; Top right panel: PAO1; Bottom left panel: KP-1; Bottom right panel: merged image. The top and side images of each panel represent the *x-z* and *y-z* planes respectively. The green and red lines indicate the positions corresponding to the *x-z* and *y-z* cross sections respectively. The blue line indicates the *x-y* plane of the main panel. Magnification: 20 x. Scale bar: 50 µm.

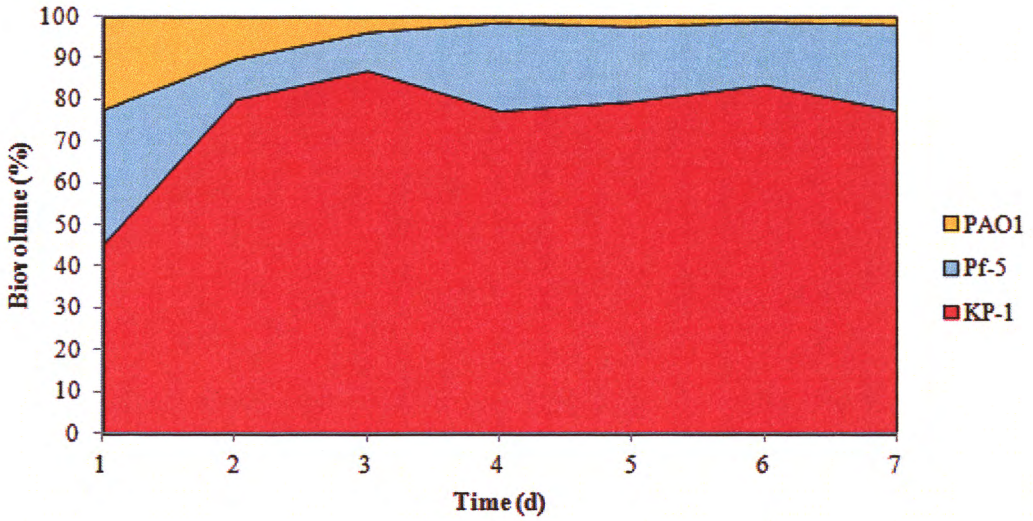

Figure 5-6. Proportions of individual species in the mixed species biofilm. The proportions of the three species within the mixed species biofilm, derived from quantitative image analysis over the 7 d observation period.

5.3.3 Biofilms were reproducible

The biovolume per unit base area of all mono and mixed species biofilms were found to decrease from day 4 onwards. Hence, 3 d biofilms were selected for subsequent studies to ensure that any change in biovolume and structure was due to the treatment applied. Further, reproducibility of 3 d biofilms was tested as described (Jackson *et al.*, 2001). Results showed that almost all of the data for the three biological replicates fell within the 95% confidence limit, suggesting that the mono and mixed species biofilms were reproducible (Figure 5-7).

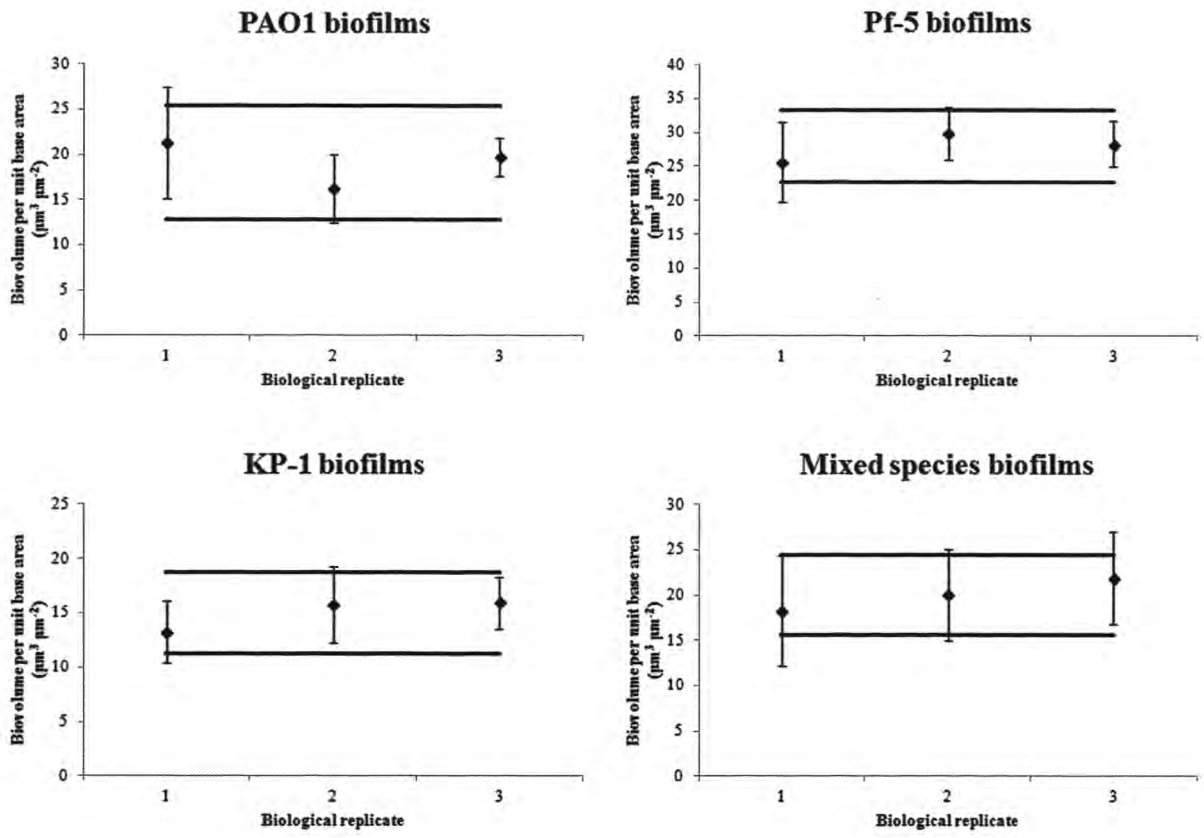

Figure 5-7. Reproducibility of mono and mixed species biofilms. Each point (average of 15 image stacks, 5 from each channel of a flow cell) represents the average biovolume per unit base area for one biological replicate of PAO1, Pf-5, KP-1 and mixed species biofilm. The two horizontal lines in each panel demark the 95% confidence intervals of the data.

5.3.4 Resource competition and biofilm structure

When imaging the mixed species biofilms, it was observed that biofilms growing at the inlet and outlet ends of a flow cell differed (Figure 5-8). The proportion of PAO1 increased from 7.8 $\pm$ 3.0% (inlet) to 29.4 $\pm$ 5.0% (outlet) whereas the proportion of KP-1 decreased from 65.7 $\pm$ 6.2% (inlet) to 36.2 $\pm$ 11.5% (outlet). Interestingly, the proportions of Pf-5 at the inlet and outlet ends of the flow cells were not significantly different. The specific growth rates for all three species at different glucose concentrations were thus determined. The growth rate of KP-1 decreased from a maximum of 0.058 $\pm$ 0.001 OD_{600} h^{-1} to 0.007 $\pm$ 0.002 OD_{600} h^{-1} as the glucose concentration decreased from 20,000 μg mL^{-}

^{1} to 4 μg mL^{-1}, while the growth rates of PAO1 and Pf-5 did not change (Figure C-3). Given its higher growth rate, it was not surprising that KP-1 dominated the mixed species biofilm when glucose concentration was 400 μg mL^{-1} at the inlet. The glucose concentration in the medium collected from the outlet end was approximately 138 ± 15 μg mL^{-1} on day 3 (Figure C-4). Thus, slower growth of KP-1 at the lower glucose concentrations might result in the observed reduction of its biomass at the outlet. When mixed species biofilms were grown in the presence of 10 fold more glucose (4,000 μg mL^{-1}), it was observed that the three species were present in almost equal proportions, furthering illustrating that resource limitation, and thus competition, might affect the composition of a mixed species biofilm. However, competition for carbon alone cannot account for all of the results observed. For example, when grown in pair wise combinations as planktonic cultures, Pf-5 was observed to outgrow both PAO1 and KP-1 (~ 1-2 $\log_{10}$ cfu mL^{-1} difference) while KP-1 and PAO1 grew together in equal proportions, but achieved a significantly lower growth yield (~ 2 $\log_{10}$ cfu mL^{-1} lower) than when grown individually. Thus, the accumulation of waste products and change in flow dynamics in a biofilm may also impact the assembly of biofilms at the inlet and outlet ends. It should be noted that for consistency, only image stacks of biofilms grown at the inlet ends were used for the stress experiments that follow.

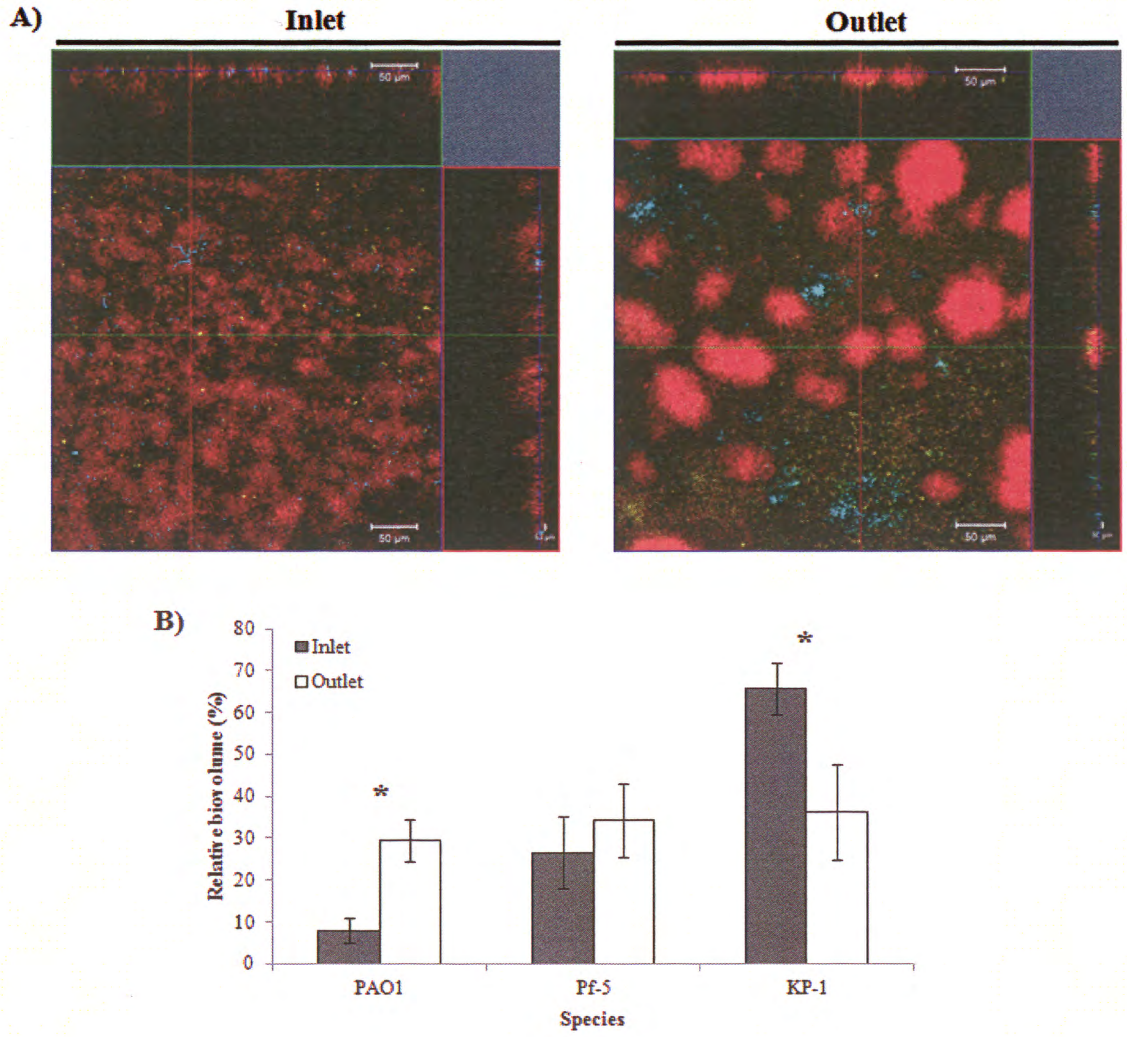

Figure 5-8. Differences in composition and structure of mixed species biofilms at different positions of the flow cell. A) Confocal microscopy images showing mixed species biofilms of PAO1 (yellow), Pf-5 (cyan) and KP-1 (red) growing at the inlet and outlet ends of a flow cell. The top and side images represent the *x-z* and *y-z* planes respectively. The green and red lines indicate the positions corresponding to the *x-z* and *y-z* cross sections respectively. The blue line indicates the relative position of the *x-y* plane in the main panel. Magnification: 20 x. Scale bar: 50 µm. B) Bar chart depicting the percentages of PAO1, Pf-5 and KP-1 within the mixed species biofilms growing at the inlet and outlet ends of the flow cells. Error bars represent standard deviations (n = 3). * denotes significant differences (One-way ANOVA: P-value < 0.05).

5.3.5 Mixed species biofilms have enhanced stress resistance

Many studies have shown that biofilms are more resistant to stresses compared to their planktonic counterparts (Mah and O'Toole, 2001; Nickel *et al.*, 1985). However, limited studies have been carried out to determine the relative resistance of mixed species biofilms compared to mono species biofilms. Here, mono and mixed species biofilms were treated with 10 μg mL^{-1} tobramycin, an aminoglycoside antibiotic. When grown on their own, mono species biofilms of PAO1, Pf-5 and KP-1 were reduced by 33.6 ± 14.0, 8.8 ± 2.3 and 37.2 ± 8.4% in biovolume respectively, while the mixed species biofilms were reduced by 7.6 ± 7.0% (Figure 5-9A). This was similarly reflected in the number of colony forming units in the effluents, where the counts decreased by 89.4 ± 8.2 (PAO1), 32.8 ± 16.0 (Pf-5), 99.6 ± 0.2 (KP-1) and 19.0 ±16.3% (mixed species biofilm). To determine if the increased tobramycin resistance of the mixed species biofilms was a consequence of either a) selection for the tobramycin resistant Pf-5 or b) Pf-5 providing protection to the two sensitive species, the proportion of each species was determined for the control and treated mixed species biofilms. Interestingly, there was no significant difference between the proportions of PAO1, Pf-5 and KP-1 in the control and treated mixed species biofilms (Figure 5-9B). This would suggest that protection against tobramycin for the mixed species biofilms was extended to all three species despite the fact that only Pf-5 was resistant to tobramycin. When dual species biofilms of two sensitive species, PAO1 and KP-1, were treated, the biofilms were severely affected with a reduction of 42.5 ± 1.4% in biovolume (Figure C-6A). Moreover, when mixed planktonic cultures of PAO1, Pf-5 and KP-1 were treated with 10 μg mL^{-1} tobramycin, only the more resistant Pf-5 was able to grow while both PAO1 and KP-1 were completely eradicated (Figure C-7).

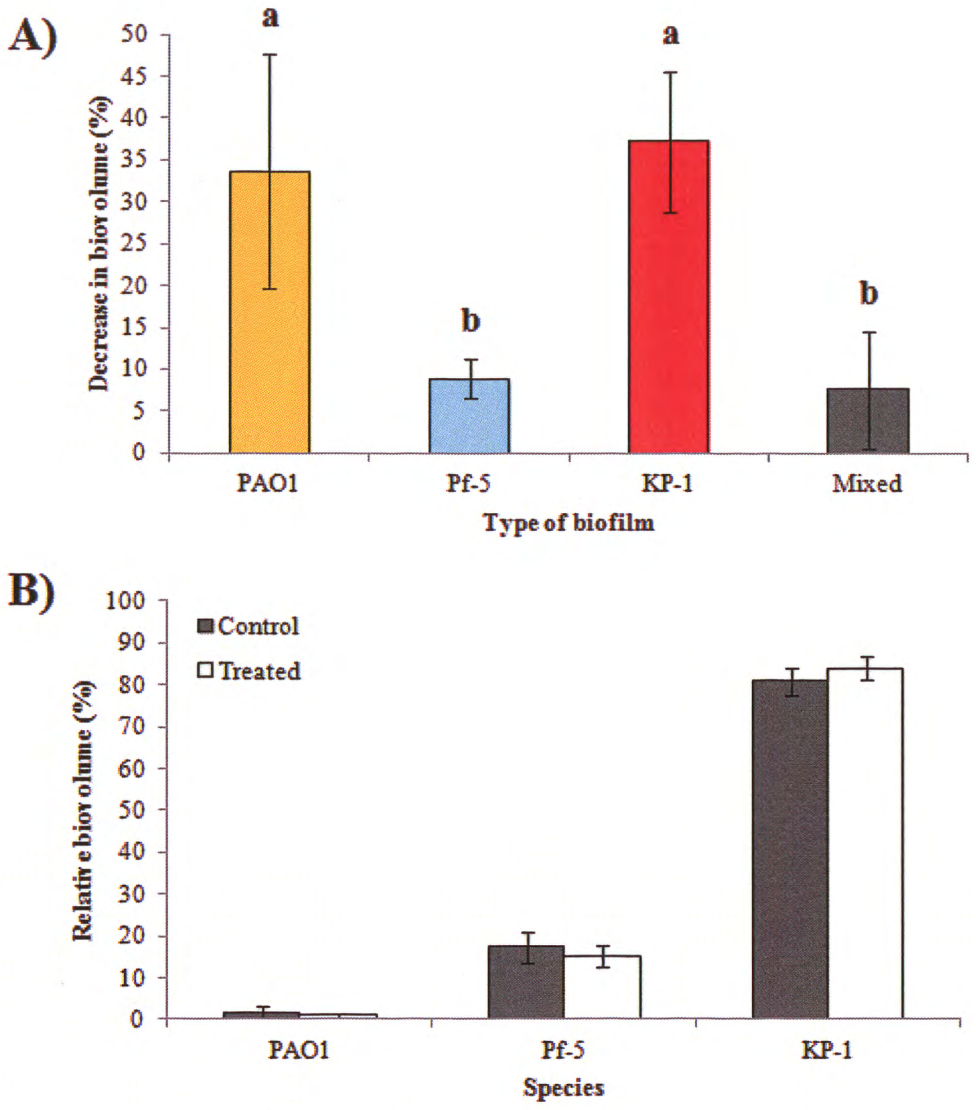

Figure 5-9. Tobramycin treatment of mono species and mixed species biofilms. A) Percentage decrease in biovolume for PAO1, Pf-5, KP-1 and mixed species biofilms after treatment with 10 μg mL^{-1} tobramycin. Error bars represent standard deviations (n = 3). a and b denote significant differences (One-way ANOVA: *P*-value < 0.05). B) Relative biovolume of PAO1, Pf-5 and KP-1 within the control and treated mixed species biofilms. Error bars represent standard deviations (n = 3).

To determine if this protective effect exhibited by the mixed species biofilms was specific to tobramycin treatment or a more general feature of the mixed biofilm communities,

mono and mixed species biofilms were also treated with 0.1% w/v SDS, an anionic surfactant. The mono species biofilms responded differently to SDS compared to tobramycin, where the biovolume of Pf-5 biofilms was significantly reduced (61.9 ± 8.8%) upon SDS exposure, whereas the biovolume of PAO1 and KP-1 biofilms were only marginally reduced (9.7 ± 2.3% and 15.9 ± 2.0% respectively) (Figure 5-10A). However, similar to the result observed for tobramycin treatment, the mixed species biofilms were resistant to SDS (11.5 ± 4.2% reduction in biovolume). When individual species within the mixed species biofilms was quantified for the control and treated biofilms, there was again no significant difference between the proportions for any of the community members (Figure 5-10B), despite Pf-5 being more sensitive to SDS. Dual species biofilms comprised of a sensitive and an insensitive species, i.e. Pf-5 and KP-1 as well as Pf-5 and PAO1, also exhibited increased resistance to 0.1% w/v SDS with a reduction of 2.8 ± 1.7% and 46.9 ± 6.0% in biovolume respectively (Figure C-6). Thus, growth as a mixed biofilm community is protective against SDS and this protection is inclusive of the most sensitive member of the community. Additionally, such community level resistance was not exclusive to a particular stress but may be a general phenomenon.

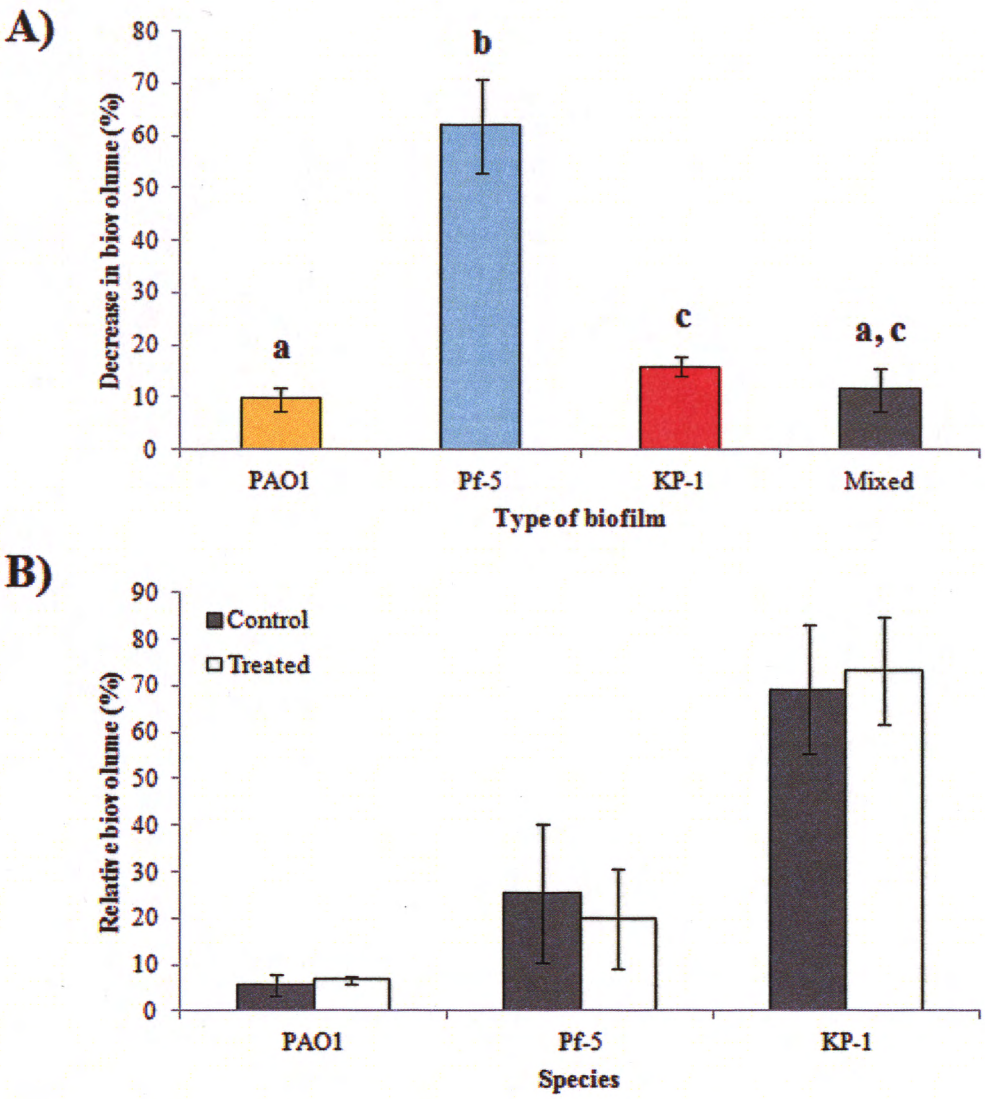

Figure 5-10. SDS treatment of mono species and mixed species biofilms. A) Percentage decrease in biovolume for PAO1, Pf-5, KP-1 and mixed species biofilms after treatment with 0.1% w/v SDS. Error bars represent standard deviations (n = 3). a, b and c denote significant differences (One-way ANOVA: *P*-value < 0.05). B) Relative biovolume of PAO1, Pf-5 and KP-1 within the control and treated mixed species biofilms. Error bars represent standard deviations (n = 3).

5.3.6 Composition and spatial organization affected the resistance of mixed species biofilms to SDS and tobramycin

As noted above, the proportion of individual species within the mixed species biofilms growing at the inlet and outlet ends of a flow cell differed. PAO1, Pf-5 and KP-1 in the mixed species biofilms were also observed to form separate microcolonies at the outlet (Figure 5-11B and D) rather than being closely associated as seen at the inlet of a flow cell (Figure 5-11A and C). The reduction in biovolume after SDS treatment was greater at the outlet (Figure 5-11F) than at the inlet (Figure 5-11E). Treatment with 10 $\mu g\ mL^{-1}$ tobramycin also decreased the biovolume of mixed species biofilm growing at the inlet of the flow cell. However, the proportion of each species remained unchanged (Figure 5-11G). In contrast, tobramycin treatment resulted in the dominance of PAO1 and Pf-5 at the outlet. Further, PAO1 and Pf-5 also formed prominent, individual microcolonies (Figure 5-11H) instead of being arranged around the microcolonies of KP-1 (Figure 5-11G). These results suggest that close association and interaction between species within the structured environment of a biofilm are essential in enhancing the survival of the community and its members when challenged with a stress.

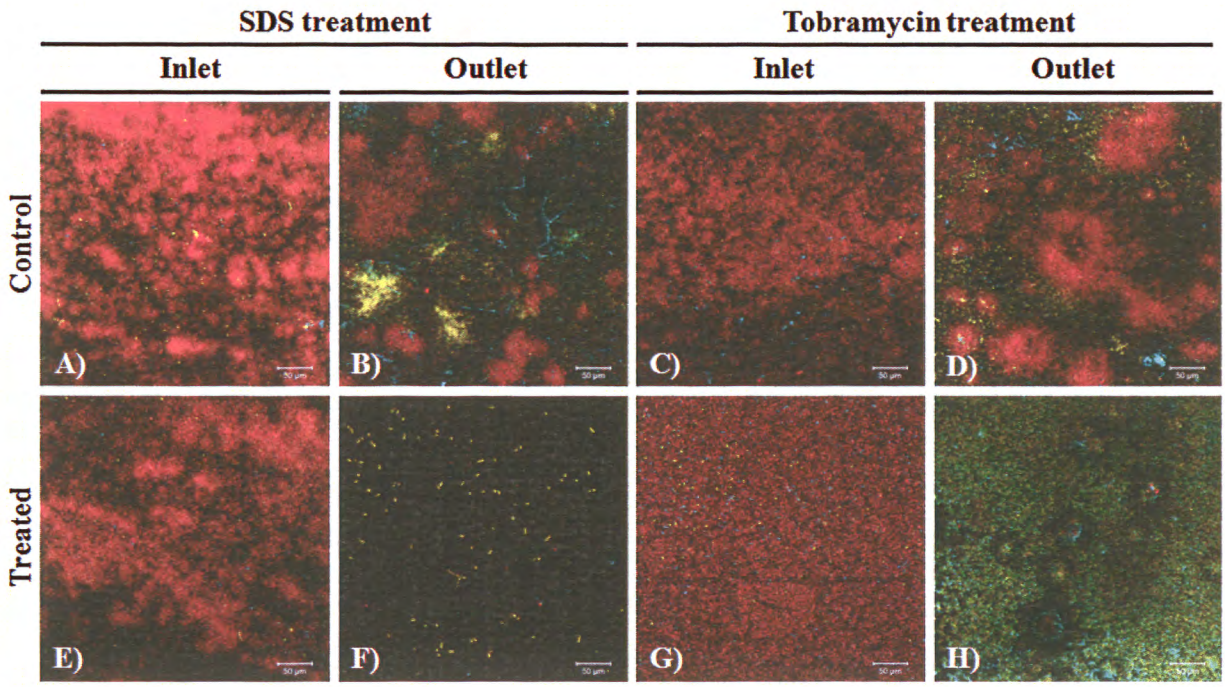

Figure 5-11. Composition and spatial organization affected the resilience of mixed species biofilms. A and C) Confocal microscopy images of control mixed species biofilms growing at the inlets of the flow cells. B and D) Confocal microscopy images of control mixed species biofilms growing at the outlets of the flow cells. E and G) Confocal microscopy images of treated mixed species biofilms growing at the inlets of the flow cells. F and H) Confocal microscopy images of treated mixed species biofilms growing at the outlets of the flow cells. PAO1 (yellow), Pf-5 (cyan) and KP-1 (red). Magnification: 20 x. Scale bar: 50 μm.

5.4 Discussion

Biofilms in the environment are typically comprised of multiple species living together in communities. While such biofilms can be dominated by a specific species, secondary species are invariably present. For example, while *P. aeruginosa* dominated biofilms found in the lungs of cystic fibrosis patients, other species such as *Abiotrophia adiacens*, *Bacteroides gracilis*, *Prevotella salivae*, *Rothia mucilaginosa* and *Staphylococcus hominis* were also present (Lim *et al.*, 2013b; Rogers *et al.*, 2004). Interactions between different species in the mixed biofilm communities are believed to affect their development, structure and function (Harrison, 2007; James *et al.*, 1995; Moons *et al.*, 2009). For

instance, *Pseudomonas putida*, a *p*-cresol-degrading organism, was found to surround and protect sensitive *Pseudomonas* species when the mixed species biofilms were exposed to *p*-cresol (Cowan *et al.*, 2000). In contrast, competition arose as the marine bacterium *Pseudoalteromonas tunicata*, produced an antibacterial protein AlpP against other bacteria on the surface of the marine alga *Ulva lactuca* (Rao *et al.*, 2005). Thus, studies on interspecies interactions that occur in mixed species biofilms will be of utmost importance to understand how such communities function and will ultimately improve strategies for the manipulation of these biofilms.

In this study, the development of a mixed species biofilm (community) was found to be altered relative to the development of comparable mono species biofilms (populations). The biomass of *K. pneumonia* KP-1 within a mixed species biofilm was significantly higher than the biomass of a KP-1 biofilm (Figure 5-4B), despite having access to less nutrients as a consequence of competition with PAO1 and Pf-5. This potentially suggests metabolic cooperation that allows KP-1 to achieve higher biomass with no additional input of carbon. Metabolic cooperation has been shown where the metabolic waste, chlorobenzoate, excreted by *Burkholderia* sp. LB400 could be metabolized by *Pseudomonas* sp. B13 (FR1) (Nielsen *et al.*, 2000). In doing so, the mixed species biofilm persisted as the metabolic waste produced by *Burkholderia* sp. LB400 was constantly removed while *Pseudomonas* sp. B13 (FR1) grew on alternative nutrients. Such interaction illustrates that a mixed species biofilm community is better at maximizing and optimizing the use of nutrients to enhance its growth and persistence. Thus, an assembled community can be at a physiological advantage when a single population becomes resource limited. Alternatively, the higher biomass of KP-1 in the mixed species biofilms can result from physical effects such as increased cohesion, mediated by the extracellular matrix provided by either *P. aeruginosa* PAO1, *P. protegens* Pf-5 or both.

Just as biofilms are more resilient than planktonic cells, mixed species biofilms were found here to be more resilient than mono species biofilms. The increased community level resistance was observed when treated with tobramycin and SDS, both of which have very different mechanisms of action. Further, the increased community level resistance

was not a result of selection for the insensitive species over the other more sensitive species, but was rather a cross protection offered by the resistant species to all members in the community. While the specific mechanism of protection is currently unknown, the cross protection can be mediated by the sharing of individually produced defenses. For example, PAO1 encodes SdsA1, a secreted SDS hydrolase, that can degrade and metabolize SDS, thus protecting all three organisms from the anionic surfactant (Hagelueken *et al.*, 2006). In addition, KP-1 is highly encapsulated and its extensive extracellular matrix may sequester SDS and thus reduce its effect on the biofilm. The combined effect of SdsA1 and the extracellular matrix may therefore offer protection to the SDS sensitive Pf-5 within the mixed species biofilm. SDS treated dual species biofilms comprised of Pf-5 and KP-1 as well as Pf-5 and PAO1 have also indicated that either KP-1 or PAO1 alone was capable of offering protection to the SDS sensitive Pf-5 (Figure C-6). The decreases in biovolume for both types of dual species biofilm were significantly lower than the decrease in biovolume observed for Pf-5 mono species biofilms. Nonetheless, the Pf-5 and PAO1 dual species biofilms were not as resistant to SDS as the Pf-5 and KP-1 dual species biofilms, and this may in part be due to the different mechanisms of shared resistance. Hence, it will be interesting to determine the mechanisms of shared resistance to better understand how mixed communities resist stresses.

The community level resistance to tobramycin can similarly be the consequence of shared defenses that are beneficial to the whole community. For example, Pf-5 may produce aminoglycoside-modifying enzymes such as *N*-acetyltransferase. Aminoglycoside-modifying enzymes catalyze the covalent modification of either amino or hydroxyl functional group, resulting in a modified aminoglycoside that binds inefficiently to ribosomes (Mingeot-Leclercq *et al.*, 1999). The community level resistance against tobramycin was particularly striking as Pf-5, which normally contributed to approximately 15% of the total biofilm biomass was able to protect both PAO1 and KP-1, where only 10% of the mixed species biofilm was removed. Thus, the role each species can play in a community may not be proportional to its abundance. Nevertheless, close association and interaction between different species within a mixed species biofilm are essential for the

community level resistance to both tobramycin and SDS (Figure 5-11). It is clear that community level resistance is important for bacterial biofilms and similar effects have been observed for a marine biofilm system, where the mixed species biofilm was more resistant to oxidative stress and tetracycline exposure than the mono species biofilms (Burmolle *et al.*, 2006).

Intriguingly, when the mixed community was grown in planktonic culture and challenged with 10 μg mL^{-1} tobramycin, only the tobramycin resistant Pf-5 survived (Figure C-7). Hence, the effects on development and resilience are unique to the structured environment of a biofilm, where the presence of extracellular matrix and distinct spatial organization may mediate these effects. In addition, the mixed microbial community may express specific genes and proteins, which are responsible for the protection of a biofilm community, but are either not expressed effectively or not present in a mono species biofilm population. This increased community level resilience has further implications for the control and manipulation of microbial communities in the medical, industrial and environmental settings since their behaviours cannot be predicted from the studies of mono species biofilms.

In conclusion, this study clearly shows that there are new lessons in microbial cell-cell interactions to be learned from mixed biofilm community studies, which are more representative of how bacteria exist in nature and in infections. This model system was shown to be reproducible and demonstrated unique features of a mixed species biofilm such as altered development and increased resistance to stresses. Hence, it is an ideal model for unraveling microbial cell-cell interactions within a mixed species biofilm, where the individual species can be tracked by fluorescent protein tag and confocal laser scanning microscope. Further, all three species are genetically tractable and amenable to molecular techniques such as mutagenesis and 'omics' based approaches. Therefore, this model system offers a unique opportunity to investigate detailed questions on the molecular mechanisms facilitating community assembly and behaviour of a community that is greater than the sum of their members.

CHAPTER 6 . INTERSPECIES VARIATION IS MORE IMPORTANT THAN INTRASPECIES VARIATION DURING MIXED SPECIES BIOFILM DEVELOPMENT

6.1 Introduction

Bacterial biofilms can be found in most engineered settings including drinking water distribution systems and the food industry (Batté *et al.*, 2003; Kumar and Anand, 1998), where their eradication represents a major challenge. In addition, biofilms pose significant health concerns where they are the primary cause for catheter-associated urinary tract infection (Trautner and Darouiche, 2004). Similarly, biofilms that form in the lungs of cystic fibrosis patients are the primary cause of mortality (Govan and Deretic, 1996). In all cases, the biofilms are difficult to eradicate due to their increased resistance to antibiotics, biocides (Costerton *et al.*, 1999; Drenkard and Ausubel, 2002; Gordon *et al.*, 1988; Stewart and William Costerton, 2001) and cell-mediated host defenses (Bjarnsholt *et al.*, 2005; Jensen *et al.*, 2007). Enhanced resilience is in part due to the presence of a matrix of extracellular polymeric substances (EPS), which either sequesters or inactivates antibiotics (Arciola *et al.*, 2005; Farber *et al.*, 1990; Gordon *et al.*, 1988). In addition, slower growing cells that arise in the presence of nutrient and oxygen gradients are also less susceptible to antibiotics that target actively growing cells (Evans *et al.*, 1991; Gilbert *et al.*, 1990; Tuomanen *et al.*, 1986). However, a recent hypothesis has suggested that this high, intrinsic resistance is the result of the differentiation and generation of a subpopulation of cells within a biofilm that are more resistant to various stresses (Drenkard and Ausubel, 2002; Mah and O'Toole, 2001; Stewart and William Costerton, 2001). It is increasingly clear that the establishment of morphotypic variants in biofilms plays an important role in biofilm resilience.

Ecologists have long recognized that diversity within a biological system enhances its stability. For example, mono species cultivars, such as wheat fields, are more susceptible to environmental perturbations, e.g. drought and insect attack, compared to highly diverse

systems such as rain forests. This phenomenon has been termed the "insurance hypothesis", which assumes that a diverse community, and its associated genetic diversity, widens the range of conditions within which the community can thrive as a whole (McCann, 2000; Yachi and Loreau, 1999).

Similar observations have been made for bacterial biofilms where the generation of morphotypic variants within mono species biofilms have been reported to be essential for their resilience to oxidative stress and predation (Boles *et al.*, 2004; Koh *et al.*, 2012). Morphotypic variants generated in biofilms could be transient such as those modulated by regulatory factors, such as phenotype variant regulator (PvrR) found in *Pseudomonas aeruginosa* (Deziel *et al.*, 2001; Drenkard and Ausubel, 2002). In contrast, stable morphotypic variants could arise from genetic recombination (Boles *et al.*, 2004) and mutation, as was observed for the single base mutation in the exopolysaccharide tyrosine kinase gene in *Serratia marcescens* (Koh *et al.*, 2012). The phenomenon of variant formation during biofilm development is widespread and has been described for other species such as *Pseudomonas fluorescens*, *Pseudomonas putida*, *S. marcescens* and *Streptococcus pneumoniae* (Arciola *et al.*, 2005; Hansen *et al.*, 2007; Koh *et al.*, 2007; Workentine *et al.*, 2010). These reports have clearly highlighted that variants formation within a biofilm is essential, spontaneous and occurs in almost all biofilm forming bacterial species. Further, reports that morphotypic variants detected in laboratory biofilms were also found in cystic fibrosis biofilms (Kirisits *et al.*, 2005), suggest that variants are relevant *in vivo* and may contribute to the high level of resistance exhibited by biofilms when exposed to either antibiotics or the host immune system.

While most studies of morphotypic variants, their frequencies and diversities have concentrated on mono species biofilms, the effect of species richness and interspecies interaction on variant formation in a mixed species biofilm is unclear. In this study, the frequency and diversity of variants were compared across mono, dual and triple species biofilms comprised of *P. aeruginosa*, *P. protegens* and *K. pneumoniae*. A total of four morphotypic variants were observed for *P. protegens*, one for *P. aeruginosa* and one for *K.*

pneumoniae. The morphotypic variants were stable and differed from their respective parent strains in a range of phenotypes such as attachment, biofilm formation, swimming motility, swarming motility and the production of pyoverdine and pyocyanin. Further, the variants were found to be more competitive than their respective parent strains during biofilm formation. Despite the increased fitness of the morphotypic variants, variants formation in the mixed species biofilms was strongly attenuated, suggesting that interspecies variation is more important than intraspecies variation. Additionally, supernatants from the *K. pneumoniae* and *P. aeruginosa* biofilms reduced the frequency of variants produced by mono species biofilms of *P. protegens*, suggesting that variants production may be moderated by extracellular cues or signals.

6.2 Materials and Methods

6.2.1 Bacterial strains and culture media

Bacteria (Table 6-1) were cultured in M9 minimal medium (48 mM Na_2HPO_4; 22 mM KH_2PO_4; 9 mM NaCl; 19 mM NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; and 0.04% w/v glucose) supplemented with 0.2% w/v casamino acids (supplemented M9 minimal medium).

Table 6-1. List of bacterial strains

Species and strain	Genotype and/or phenotype[1]	Source or reference
Pseudomonas aeruginosa		
PAO1 eYFP	encodes eYFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
PAO1 eCFP	encodes eCFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
PAO1 SCV	self-generated small colony variant of PAO1; tagged with eYFP; Gm^R	This study
Pseudomonas protegens		
Pf-5 eCFP	encodes eCFP in the intergenic region between the coding region of *glmS* and its downstream	This study

	gene; Gm^R	
Pf-5 eYFP	encodes eYFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
Pf-5 SCV	self-generated small colony variant of Pf-5; tagged with eCFP; Gm^R	This study
Pf-5 CCV	self-generated cauliflower colony variant of Pf-5; tagged with eCFP; Gm^R	This study
Pf-5 RCV	self-generated rough colony variant of Pf-5; tagged with eCFP; Gm^R	This study
Pf-5 WCV	self-generated wrinkled colony variant of Pf-5; tagged with eCFP; Gm^R	This study
Klebsiella pneumoniae		
KP-1 DsRed	encodes DsRedExpress in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
KP-1 eYFP	encodes eYFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
KP-1 NM	self-generated non-mucoid colony variant of KP-1; tagged with DsRedExpress; Gm^R	This study

[1] Gm^R: Gentamicin resistance

6.2.2 Isolation and enumeration of morphotypic variants

Aliquots (100 mL) of biofilm effluent (spent culture media collected from the flow cell effluent) were collected daily throughout the development (7 d) of the biofilms. Planktonic cultures were also passaged (100 x dilution) and sampled daily for 7 d. The samples were sonicated in a water bath sonicator (37 KHz, 100% power, Elmansonic P) for 5 min, vortexed for 3 periods of 6 s and plated onto LB_{10} agar (10 g L^{-1} NaCl; 10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 1.5% w/v agar) plates and *Pseudomonas* isolation agar (Becton Dickinson, USA) plates in triplicate. The plates were incubated at room temperature (25$\pm$ 1°C) for 2 d, after which time, the morphotypic variants were identified and classified by their colony morphologies. The frequency of morphotypic variants was determined while the Shannon-Wiener and evenness indices were also calculated for each sample as previously described (Magurran, 1988).

6.2.3 Verification of *morphotypic variants* by 16S rRNA gene sequencing

16S rRNA gene sequencing as described in Chapter 3 was performed to verify the identities of all morphotypic variants isolated.

6.2.4 Whole genome sequencing, INDELs and SNPs identification for morphotypic variants

The DNA of each morphotypic variant was extracted using QIAamp DNA Mini Kit (Qiagen, Netherlands) according to the manufacturer's protocol. Sequencing libraries were prepared using TruSeq DNA Sample Preparation Kit (Illumina, USA) and sequenced on the MiSeq benchtop sequencer (Illumina, USA) according to the manufacturer's protocols. The paired-end reads were mapped to the reference genomes of PAO1, Pf-5, NTUH-K2044 and MGH78578 (GenBank accession number: AE004091 (PAO1), CP000076(Pf-5), AP006725 (NTUH-K2044) and CP000647 (MGH78578)). The INDELs and SNPs present in the morphotypic variants were identified using CLC genomic workbench 6 (CLC bio, Denmark). Paired-end reads obtained from the sequencing of PAO1, Pf-5 and KP-1 parent strains were used as controls to omit INDELs and SNPs that were already present in the parent strains.

6.2.5 Growth assay

Self-generated morphotypic variants and their respective parent strains were grown in supplemented M9 minimal medium (200 rpm at 25 ± 1 °C). Growth was monitored by Optical Density (OD_{600}) over a 12 h period using an UV spectrophotometer (UV-1800, Shimadzu, Japan).

6.2.6 Attachment assay

A 100 µl aliquot of a 24 h culture was distributed into a 96 well microtitre plate (Nunclon Delta-treated polystyrene, Thermo Scientific, USA) in triplicate, incubated at room temperature (25 ± 1°C) without agitation for 2 h and assessed by crystal violet staining. Briefly, the supernatant was removed from each well, washed twice with 150 µl of PBS and stained with 125 µl of 0.1% w/v crystal violet for 20 min. The crystal violet was

subsequently removed, washed thrice with 150 µl of PBS before 200 µl of 95% v/v ethanol was added to each well, mixed and 100 µl was transferred into a clean plate for quantification (OD_{600}).

6.2.7 Biofilm formation assay

Quantification of biofilm formation was performed as described by O'Toole and Kolter (1998b) with some modifications. Briefly, a 24 h culture was diluted 25 x before 50 µl was added to a 96 well microtitre plate (Nunclon Delta-treated polystyrene, Thermo Scientific, USA) containing an additional 50 µl of supplemented M9 minimal medium in triplicate. The plate was incubated at 30°C (Pf-5) and 37°C (PAO1 and KP-1) without agitation. Biofilm formation was quantified hourly using the crystal violet staining method as described above.

6.2.8 Motility assays

Motility assays were carried out as described by Deziel *et al.* (2001) with some modifications described below.

Swimming motility. A tryptone swim plate (10 g L^{-1} tryptone; 5 g L^{-1} NaCl; 0.3% w/v agar; dried for 2 h) was point inoculated with a 24 h culture and incubated at 30°C (Pf-5) and 37°C (PAO1 and KP-1) for 16 h. Swimming motility was assessed quantitatively by measuring the diameter of the turbid circular zone.

Swarming motility. A swarm plate (8 g L^{-1} nutrient broth; 5 g L^{-1} glucose; 0.5% w/v agar; dried for 2 h) was point inoculated with a 24 h culture and incubated at 30°C (Pf-5) and 37°C (PAO1 and KP-1) for 16 h. Swarming motility was assessed quantitatively by measuring the diameter of the swarmed zone.

6.2.9 Production of Pyoverdine and Pyocyanin

Bacteria were cultured in 10 mL of King's B medium (King *et al.*, 1954) at 30°C (Pf-5) and 37°C (PAO1) for 24 h. Pyoverdine in the supernatant was semi-quantified by

measuring the emission fluorescence at 460 nm after excitation at 400 nm. Similarly, bacteria were cultured in 10 mL of King's A medium (King *et al.*, 1954) and pyocyanin in the supernatant was semi-quantified by measuring the absorbance (OD_{695}).

6.2.10 Continuous-culture flow cell and planktonic co-culture experiments

Mono and mixed species biofilms were cultivated in three-channel flow cells as described in Chapter 5. Triple species biofilms were established by inoculating a mixed culture of PAO1, Pf-5 and KP-1 in the ratio of 5:5:1 while dual species biofilms were established by inoculating a mixed culture of the indicated species (PAO1:Pf-5 = 1:1; PAO1:KP-1 = 5:1; Pf-5:KP-1 = 5:1). Mono species biofilms of Pf-5 were also grown in the presence of KP-1 and PAO1 effluents. The effluents were collected from 3 d old KP-1 and PAO1 mono species biofilm, centrifuged (5,000 *g*, 10 min), filtered (0.2 µm) and added to the supplemented M9 minimal medium.

For planktonic co-cultivation, the 24 h cultures were diluted to approximately 1 x 10^8 cfu mL^{-1} and mixed accordingly (PAO1:Pf-5:KP-1 = 5:5:1; PAO1:Pf-5 = 1:1; PAO1:KP-1 = 5:1; Pf-5:KP-1 = 5:1). After which time, 100 µl of the mixed culture was inoculated into 10 mL of supplemented M9 minimal medium. Sub-culturing (100 x dilution) was performed daily while sampling was done on days 0, 1 and 3.

6.2.11 Microscopy, image and statistical analysis

Microscopic observation and image acquisition was performed by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Germany) using the multi track mode to minimize fluorescent bleed-through. The excitation wavelengths for eCFP, eYFP and DsRedExpress were 458 nm, 514 nm and 561 nm respectively. The emission wavelengths for eCFP, eYFP and DsRedExpress were 476 nm, 527 nm and 584 nm respectively. For each flow cell channel, five image stacks were acquired from the centre of the channel (2 mm from the walls), starting at a distance of 5-10 mm from the inlet (unless otherwise stated) and spaced approximately 5 mm apart (Heydorn *et al.*, 2000a). The five image stacks covered a total area of approximately 9 x 10^5 μm^2, which was more than the

minimum of 1 x 10^5 μm^2 required for representative data (Korber *et al.*, 1993). For image analysis, three biological replicates (flow cells) with a total of 45 image stacks (15 from each flow cell) were quantified for each biofilm type using IMARIS (Bitplane AG, Switzerland). Statistical analysis was performed using IBM SPSS Statistics 20.

6.3 Results

6.3.1 Isolation, classification and verification of self-generated morphotypic variants from mono species biofilms

Effluents collected from the Pf-5 biofilms were observed to contain, in addition to the parent strain (Figure 6-1A), four morphotypic variants a) small colony variant (Pf-5 SCV); b) cauliflower colony variant (Pf-5 CCV); c) rough colony variant (Pf-5 RCV) and d) wrinkled colony variant (Pf-5 WCV) (Figure 6-1B-E respectively). A small colony variant (PAO1 SCV) and a non-mucoid colony variant (KP-1 NM) were also isolated from the effluents of PAO1 and KP-1 biofilms respectively (Figure 6-1F and G). The parents and morphotypic variants were further descibed in Table 6-2. The total proportion of biofilm derived variants for Pf-5 ranged from 5-60%, while the non-mucoid variant of KP-1 first appeared on day 2 and peaked at 80%. The PAO1 SCVs typically appeared after day 4 and steadily increased to represent 40% of the total population (Figure 6-2A). In contrast, few or none of these morphotypic variants were found in planktonic cultures of PAO1, Pf-5 and KP-1 even after 7 d of cultivation (Figure 6-2A).

All morphotypic variants were verified by 16S rRNA gene sequencing confirming that they were morphotypic variants of their respective parent strains and daily passage of all isolates showed that their morphotypes were stable, suggesting that they were the result of permanent genetic modifications (Boles *et al.*, 2004; Koh *et al.*, 2012) rather than transient phenotypes that resulted from phase variations (Deziel *et al.*, 2001; Drenkard and Ausubel, 2002).

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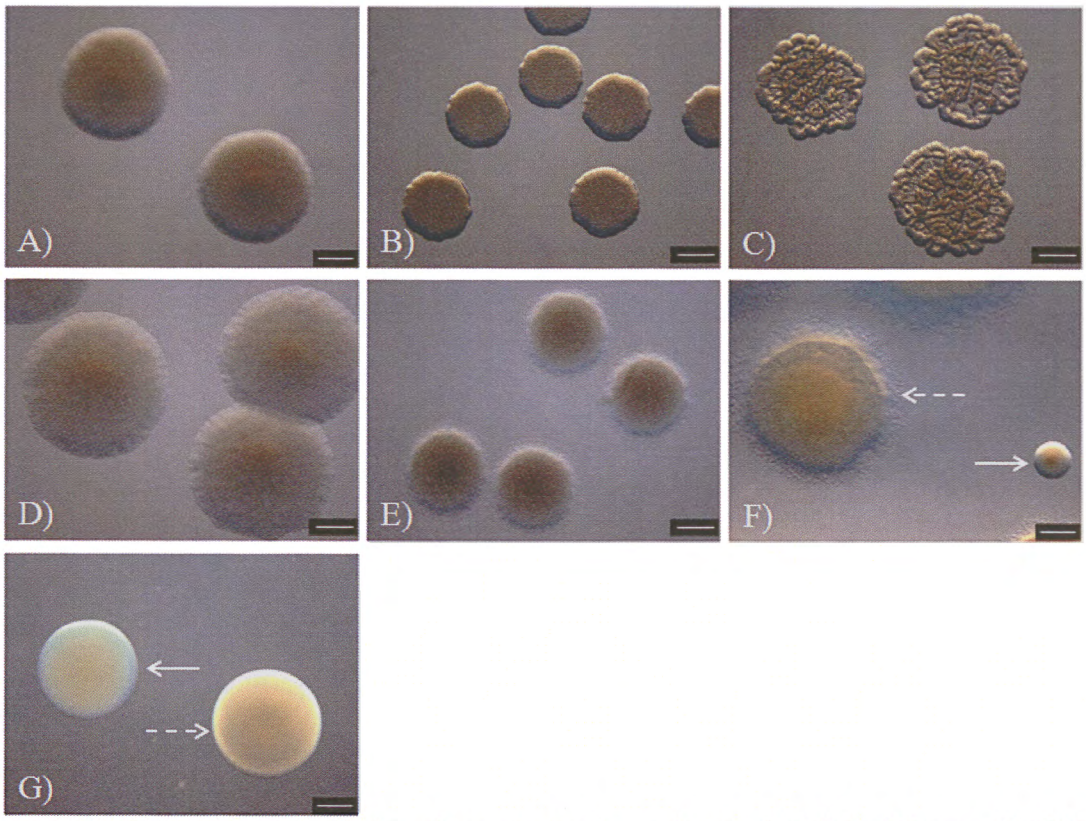

Figure 6-1. Colony morphologies of morphotypic variants. A) Pf-5 wild type, B) Pf-5 small colony variant, C) Pf-5 cauliflower colony variant, D) Pf-5 rough colony variant, E) Pf-5 wrinkled colony variant, F) PAO1 wild type (dashed arrow) and PAO1 small colony variant (solid arrow), G) KP-1 wild type (dashed arrow) and KP-1 non-mucoid colony variant (solid arrow). Scale bar: 1,000 µm.

Table 6-2. Physical decriptions of parents and variants

Parents and variants	Size[1]	Form[2]	Elevation[3]	Surface texture[4]
PAO1	Regular	Filamentous	Flat	Rough
PAO1 SCV	Smaller	Circular	Flat	Smooth
Pf-5	Regular	Circular	Flat	Smooth
Pf-5 SCV	Smaller	Circular	Flat	Smooth
Pf-5 CCV	Regular	Irregular	Flat	Undulating
Pf-5 RCV	Bigger	Circular	Convex	Rough
Pf-5 WCV	Regular	Filamentous	Flat	Smooth

KP-1	Regular	Circular	Convex	Mucoid
KP-1 NM	Regular	Circular	Flat	Smooth

[1]Parent is always considered as "regular" while the variants were either "smaller", "regular" or "bigger" than the parent.
[2]Form described the shape of the colony when viewed from its top.
[3]Elevation described the shape of the colony when viewed from its side.
[4]Surface texture described the deviation of the colony surface from a flat plane.

6.3.2 INDELs and SNPs found in each morphotypic variant

Sequencing of the three wild type strains and their associated variants generated an average of 1,428,181 mapped paired-end reads per genome with a 46-60 fold coverage (Table 6-3). In summary, a deletion that resulted in a frame shift mutation at Thr210 of the twitching motility protein PilT was identified in PAO1 SCV (Table 6-4). Three SNPs were found in the KP-1 NM variant, where an amino acid substitution was each observed for an ATP-dependent RNA helicase HrpB, an exonuclease subunit SbcC and a putative UDP-glucose lipid carrier transferase (Table 6-4).

Table 6-3. Number, average length and coverage of paired-end reads

Strain	No. of mapped paired-end reads	Average length (bp)	Coverage (fold)
PAO1	1,946,110	468.77	60
PAO1 SCV	1,311,756	513.43	52
Pf-5	1,687,202	461.01	48
Pf-5 SCV	1,207,398	667.02	46
Pf-5 CCV	1,372,426	371.13	48
Pf-5 RCV	1,433,126	668.72	50
Pf-5 WCV	1,446,318	347.28	51
KP-1	1,398,780	440.67	56
KP-1 NM	1,050,514	640.43	49

For the variant Pf-5 SCV, an insertion that resulted in a frame shift mutation at Asp78 of a putative flagellar motor switch protein, FliM, was observed. The Pf-5 CCV was also

observed to possess four deletions relative to the parent strain. Three were consecutive deletions at positions 40, 41 and 42, which resulted in a deletion of Glu14 of a chemotaxis-specific methylesterase, while the fourth deletion resulted in a frame shift mutation at Arg17 of the same gene. Two SNPs, each resulting in an amino acid substitution in the putative cell division protein FtsW and 50S ribosomal protein L2, were detected for Pf-5 RCV. Lastly, a SNP that resulted in a nonsense mutation at Glu64 of a group 1 family glycosyltransferase was observed for Pf-5 WCV (Table 6-4). Since the frequency (number of reads supporting the mutation divided by the total number of reads) and probability (the probability that this mutation exits in the sample) of each mutation reported here were > 90% and = 1 respectively, the mutations were unlikely to be the result of sequencing errors. However, the mutations should still be verified by PCR, where multiple isolates with the same morphotype would be checked to determine if these INDELs and SNPs were consistent across all isolates of the same morphotype, thus indicating that they were involved in the morphotype observed.

Table 6-4. List of INDELs and SNPs in morphotypic variants

Strains	Types of mutation	[1]Coding region changes	[2]Amino acid changes	[3]Gene features
PAO1 SCV	Deletion	NP_249086.1:c.628delA	Thr210fs	twitching motility protein PilT
KP-1 NM	SNP	YP_002917861.1:c.1112A>T	Asp371Val	ATP-dependent RNA helicase HrpB
	SNP	YP_001334020.1:c.1953G>T	Gln651His	exonuclease subunit SbcC
	SNP	YP_002920354.1:c.367T>A	Phe123Ile	putative UDP-glucose lipid carrier transferase
Pf-5 SCV	Insertion	YP_258774.1:c.233_234insA	Asp78fs	flagellar motor switch protein FliM
Pf-5 CCV	Deletion	YP_258265.1:c.40delG	Glu14fs	chemotaxis-specific methylesterase
	Deletion	YP_258265.1:c.41delA	Glu14fs	chemotaxis-specific methylesterase
	Deletion	YP_258265.1:c.42delG	Glu14fs	chemotaxis-specific

				methylesterase
	Deletion	YP_258265.1:c.49delC	Arg17fs	chemotaxis-specific methylesterase
Pf-5 RCV	SNP	YP_262141.1:c.464G>T	Trp155Leu	cell division protein FtsW
	SNP	YP_262647.1:c.205C>T	Arg69Cys	50S ribosomal protein L2
Pf-5 WCV	SNP	YP_257663.1:c.190G>T	Glu64*	group 1 family glycosyltransferase

[1]ins: insertion; del: deletion
[2]fs: frame shift; ins: insertion; del: deletion; *: stop codon
[3]Only gene feature with a frequency of > 90 and probability = 1 was shown

6.3.3. Sequential appearance and evolution of Pf-5 *morphotypic variants*

Previous studies have shown that some morphotypic variants are derived in a predictable order and that this was in part due to secondary mutations in some variants (Koh *et al.*, 2007). Since more than one morphotypic variant was isolated from the Pf-5 biofilms, the order of their appearance was investigated. Results showed that the Pf-5 SCV appeared after 1 d (0.6 ± 0.6%) with its proportion peaking at 46.7 ± 10.0% on day 5 before decreasing gradually to 36.6 ± 17.8% on day 7. The Pf-5 CCV appeared (0.5 ± 0.8%) on day 2 with its proportion peaking at 10.6 ± 8.6% on day 6 while Pf-5 RCV and Pf-5 WCV (1.5 ± 1.6% and 0.7 ±1.2% respectively) appeared on day 3 with their proportions peaking at 3.6 ± 3.5% and 19.4 ± 14.3% respectively on day 7 (Figure 6-2B). This pattern of appearance was consistent across multiple experiments.

Biofilms formed by either Pf-5 RCV or Pf-5 WCV were also observed to generate other Pf-5 morphotypic variants. For example, both Pf-5 SCVs and Pf-5 WCVs were found in the effluent of Pf-5 RCV biofilms on day 4 (Figure D-1A). Similarly, Pf-5 SCVs were observed in the effluent of Pf-5 WCV biofilms on day 5 (Figure D-1B). In contrast, neither the Pf-5 SCV nor the Pf-5 CCV biofilms gave rise to other morphotypic variants. While these data suggest that some variants can be derived from others, e.g. Pf-5 RCV biofilms can give rise to Pf-5 WCVs and Pf-5 SCVs, the mutational analysis does not show any common mutation in presumably related variants. For example, the two SNPs

found in the Pf-5 RCV were neither present in the Pf-5 SCV nor in the Pf-5 WCV (Table 6-4).

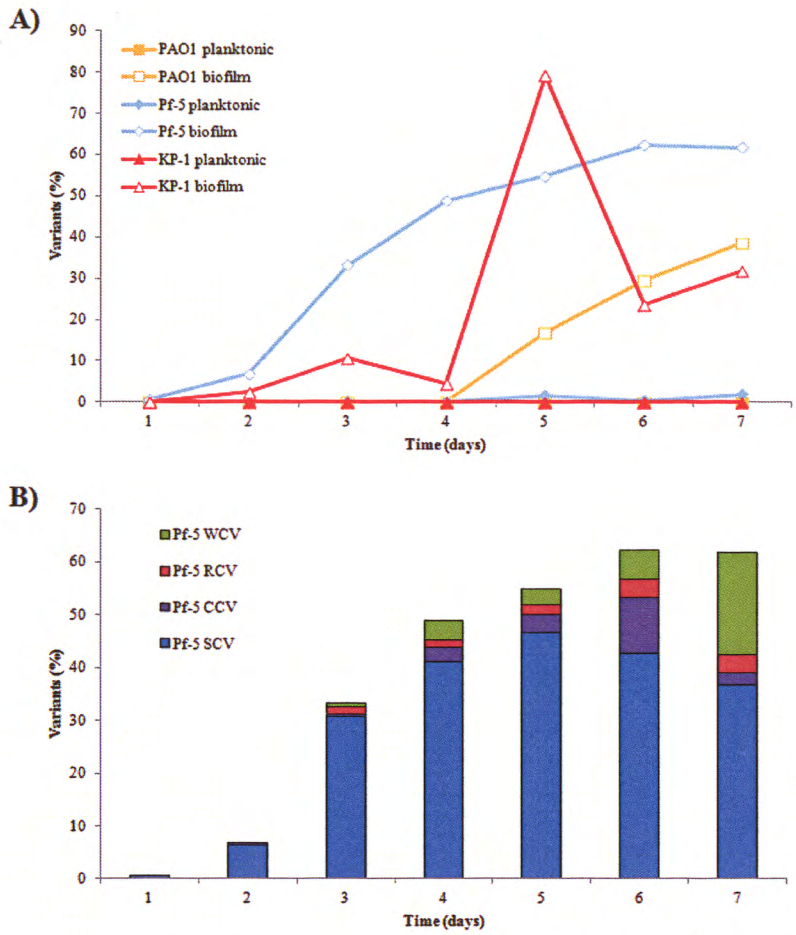

Figure 6-2. Frequencies and proportions of morphotypic variants. A) Frequencies of morphotypic variants in planktonic cultures and effluents of PAO1, Pf-5 and KP-1 biofilms. B) Proportions of the four Pf-5 morphotypic variants in the effluents of Pf-5 biofilms over 7 d.

6.3.4 Phenotypic characterization of morphotypic variants

Growth rate. The growth rates for all of the variants were not significantly different from the parent strains (Table 6-5). Both PAO1 and PAO1 SCV were found to have average doubling times of 1.18 h and the wild type Pf-5 and its four morphotypic variants

had average doubling times of 1.14 h. Although Pf-5 WCV had a longer doubling time of 1.40 ± 0.13 h, this was not significantly different from that of Pf-5 and its other morphotypic variants. KP-1 and KP-1 NM had average doubling times of 0.87 h.

Swimming and swarming motilities. PAO1 was observed to swim (3.60 ± 0.36 cm) and swarm, while PAO1 SCV demonstrated a significant reduction in swimming motility (1.92 ± 0.16 cm) and no swarming motility. Pf-5 showed a swimming zone of 3.66 ± 0.21 cm, while all of its morphotypic variants exhibited significant reductions in swimming motility, especially Pf-5 SCV, which was immotile. Both the wild type Pf-5 and the Pf-5 RCV swarmed, but the swarming diameter for the Pf-5 RCV was significantly larger than that for wild type Pf-5. None of the other Pf-5 morphotypic variants demonstrated swarming motility (Table 6-5). Both KP-1 wild type and KP-1 NM were non-motile (swimming and swarming) (Table 6-5).

Attachment. The PAO1 SCV attached and formed biofilm with approximately 2 fold more biomass (as determined by crystal violet staining) than the wild type PAO1. Similarly, the Pf-5 CCV attached and formed biofilm with 3.5 fold more biomass than the wild type Pf-5, and the other Pf-5 morphotypic variants. Lastly, the attachment abilities of KP-1 and KP-1 NM were not significantly different from each other (Table 6-5).

Biofilm formation. The PAO1 SCV formed biofilm at a rate that was approximately three times faster than the rate for wild type PAO1. In addition, the biomass of the PAO1 SCV biofilm, as quantified by crystal violet staining, was significantly more than the biomass of the wild type PAO1 biofilm after 3 h. Likewise, the Pf-5 CCV formed significantly more biofilm than the Pf-5 parent strain and its other morphotypic variants, all of which did not form biofilm in this assay. Similarly, KP-1 and KP-1 NM were also incapable of forming any biofilm in this assay (Table 6-5).

Production of pyoverdine and pyocyanin. Pyoverdine is a siderophore used by both PAO1 and Pf-5 to scavenge iron under iron limiting conditions (Meyer, 2000; Mirleau *et*

al., 2001). In this study, the levels of pyoverdine produced by PAO1 and PAO1 SCV were similar. In contrast, Pf-5 WCV produced a significantly higher level of pyoverdine than Pf-5. Pyocyanin is an antimicrobial and may play an important role in competition against other microorganisms (Baron *et al.*, 1989; Lau *et al.*, 2004). Here, the PAO1 SCV produced a significantly higher level of pyocyanin than its parent strain. In contrast, no significant difference was observed for the levels of pyocyanin produced by Pf-5 and its morphotypic variants. KP-1 and KP-1 NM were not assayed as *K. pneumoniae* is not known to produce either pyoverdine or pyocyanin (Table 6-5).

6.3.5 Comparison of biofilm structures for the parent strains and morphotypic variants

The results above show that the morphotypic variants differed phenotypically from their respective parent strains and could form more biofilms than their parent strains. Further investigation showed that the structures of biofilms grown in flow cells by the morphotypic variants and their respective parent strains were also different. PAO1 biofilms were compact and possessed microcolonies approximately 85 μm in height (Figure 6-3A). In contrast, PAO1 SCV biofilms had numerous hollow colonies that were significantly taller at 245 μm (Figure 6-3D). Both KP-1 (Figure 6-3B) and KP-1 NM (Figure 6-3E) biofilms were flat and undifferentiated. However, KP-1 NM biofilms were thicker (~ 70 μm) than the KP-1 biofilms (~ 32 μm). Pf-5 biofilms were comprised of intertwined short chains and had tower-like microcolonies that were > 50 μm in height (Figure 6-3C). Pf-5 SCV biofilms were flat and dense (Figure 6-3F) probably due to its lack of motility, while Pf-5 CCV biofilms formed multiple microcolonies (~ 25 μm in diameter) (Figure 6-3G). Pf-5 RCV biofilms were made up of intertwined long chains and were porous (Figure 6-3H), while Pf-5 WCV biofilms were made up of intertwined short chains similar to Pf-5 biofilms (Figure 6-3I).

Table 6-5. Phenotypic characteristics of parents and morphotypic variants

Parents and variants	Mean ± SD						
	Doubling time (h)	Swimming (cm)	Swarming (cm)	Attachment (OD_{600})	Biofilm formation ($OD_{600}\ h^{-1}$)	Pyoverdine (RFU x 10^3)	Pyocyanin (OD_{695})
PAO1	1.19 ± 0.27	3.60 ± 0.36	4.18 ± 0.36	0.40 ± 0.02	0.11 ± 0.02	14.08 ± 2.44	0.03 ± 0.01
PAO1 SCV	1.17 ± 0.07	1.92 ± 0.16*	Nil	0.73 ± 0.06**	0.31 ± 0.02*	13.63 ± 6.12	0.05 ± 0.01*
Pf-5	1.06 ± 0.20	3.66 ± 0.21	1.18 ± 0.16	0.25 ± 0.04	Nil	0.96 ± 0.57	0.03 ± 0.01
Pf-5 SCV	1.12 ± 0.11	Nil	Nil	0.25 ± 0.03	Nil	1.47 ± 0.29	0.03 ± 0.03
Pf-5 CCV	1.09 ± 0.40	2.03 ± 0.16*	Nil	0.87 ± 0.17**	0.42 ± 0.12	1.45 ± 0.43	0.03 ± 0.01
Pf-5 RCV	1.01 ± 0.11	3.05 ± 0.36*	1.92 ± 0.37*	0.22 ± 0.04	Nil	0.57 ± 0.55	0.03 ± 0.01
Pf-5 WCV	1.40 ± 0.13	2.15 ± 0.07*	Nil	0.22 ± 0.05	Nil	2.01 ± 0.77*	0.05 ± 0.02
KP-1	0.90 ± 0.32	Nil	Nil	0.23 ± 0.04	Nil	N. A.	N. A.
KP-1 NM	0.84 ± 0.04	Nil	Nil	0.19 ± 0.02	Nil	N. A.	N. A.

** significantly different from the parent strain with *P*-value < 0.05, Nested ANOVA
*significantly different from the parent strain with *P*-value < 0.05, One-way ANOVA
Nil: the strain did not exhibit the phenotype tested
N. A.: the strain is not tested for the phenotype

6.3.6 Morphotypic variants were more competitive than their respective parent strains

The fitness of each morphotypic variant was studied by co-culturing with its respective parent strain. During planktonic growth, PAO1 and the PAO1 SCV as well as KP-1 and KP-1 NM grew in equal proportions over the 3 d tested (Figure D-2). Pf-5 typically out grew all of its morphotypic variants, with the exception of Pf-5 SCV, which accounted for almost 70% of the CFUs after 3 d of planktonic growth (Figure D-2). However, when co-cultured as biofilms, it was observed that all of the morphotypic variants of PAO1, Pf-5 and KP-1 outcompeted their respective parent strains, making up more than 65-99% of the total biovolume after 1 d of biofilm growth (Figure 6-4A). Further, each morphotypic variant also achieved a higher percentage composition than its parent strain in the triple species biofilms with the exception of KP-1 NM (Figure 6-4B). For example, the biovolume of wild type PAO1 in the triple species biofilms was typically 3.5 ± 2.6% of the total biovolume in contrast to the PAO1 SCV, which comprised 29.1 ± 8.1% of the mixed species biofilm biovolume. Similarly, the morphotypic variants of Pf-5 also achieved 3-5 fold greater biovolume during triple species biofilm growth than their Pf-5 parent. These results suggest that each morphotypic variant was better adapted to biofilm growth than its parent strain, but such fitness did not provide a competitive advantage during planktonic growth.

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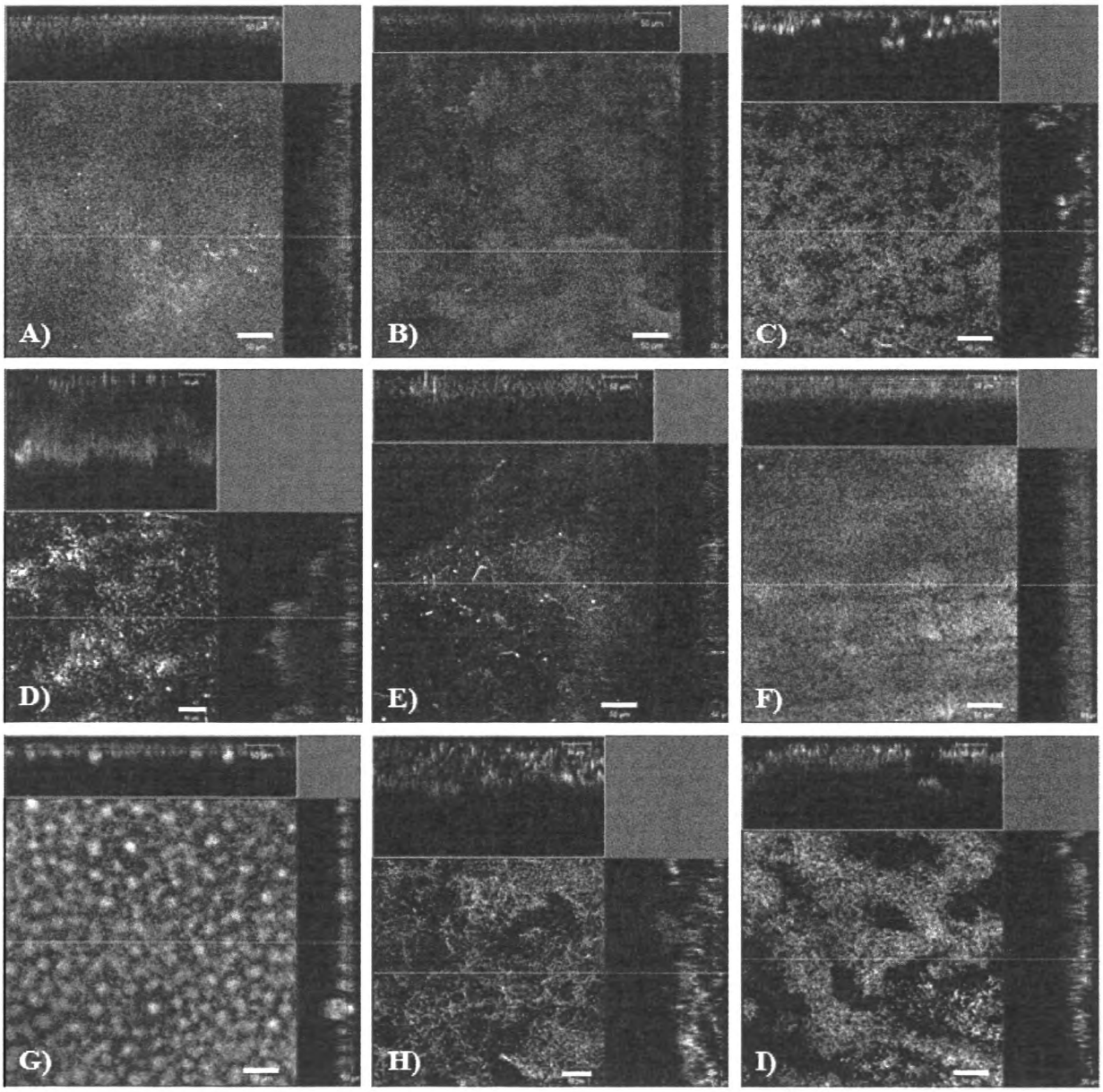

Figure 6-3. Structure of parent and morphotypic variant biofilms. Confocal microscopy images showing the structures of A) PAO1; B) KP-1; C) Pf-5; D) PAO1 SCV; E) KP-1 NM; F) Pf-5 SCV; G) Pf-5 CCV; H) Pf-5 RCV; and I) Pf-5 WCV biofilms. The top and side images represent the *x-z* and *y-z* planes respectively. Magnification: 20 x. Scale bar: 50 μm.

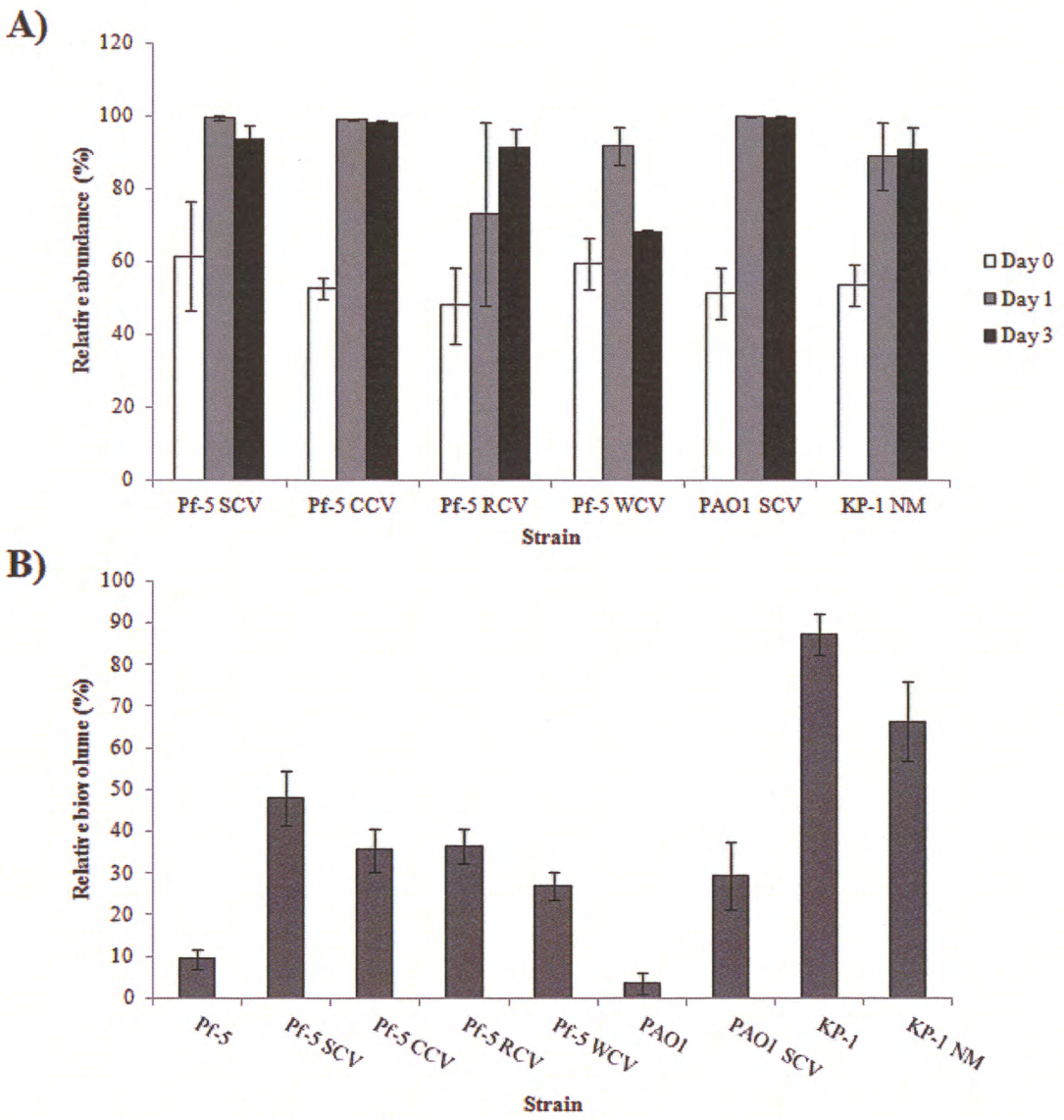

Figure 6-4. Competitive fitness of morphotypic variants over parent strains in co-culture biofilms. A) Relative abundance (%) of morphotypic variants co-cultured with their respective parent strains in mono species biofilms. B) Relative biovolume (%) of morphotypic variants and their respective parent strains in the triple species biofilms. Error bars represent standard deviations of three independent experiments.

6.3.7 Reduction in morphotypic variants during mixed species biofilm growth

While it was observed above that the morphotypic variants of PAO1 and KP-1 during mono species biofilm development represented up to 40% and 80% of the population respectively (detection limit: ~1 x 10^{-2} variants per sample), neither species was observed to generate morphotypic variants when grown as a triple species biofilm (detection limit: ~1 x 10^{-1} variants per sample for PAO1; ~2 x 10^{-2} variants per sample for KP-1). The average frequency of Pf-5 morphotypic variants was 38.4 ± 25.7% (detection limit: ~2 x 10^{-2} variants per sample) during mono species biofilm development, but decreased to 20.6 ± 17.8% when grown in the triple species biofilm. This effect was most pronounced on days 3-6 (*P*-value < 0.05, One-way ANOVA), while by day 7, the percentage of variants in the three species consortia (52.9 ± 10.0%) was no longer statistically significantly different from the percentage of variants (61.9 ± 5.9%) in the mono species biofilms (Figure 6-5A). To determine the minimum species richness required during biofilm development to alter the formation of morphotypic variants, dual species biofilms were grown for every combination of the three different species. For all combinations, the percentage of morphotypic variants was either significantly reduced or completely eliminated. The average frequencies of Pf-5 morphotypic variants from the dual species biofilms of Pf-5 and PAO1 as well as Pf-5 and KP-1 were 26.5 ± 19.2% (detection limit: ~2 x 10^{-2} variants per sample) and 31.3 ± 24.1% (detection limit: ~2 x 10^{-2} variants per sample) respectively. Both were higher than the frequency of variants observed for the triple species biofilms, but lower than the same observed for the mono species biofilms (Figure 6-5A). Similarly, the average frequency of PAO1 SCV in PAO1 mono species biofilms (12.2 ± 16.4%, detection limit: ~1 x 10^{-2} variants per sample) was higher than its frequency in dual species biofilms of Pf-5 and PAO1 (0%, detection limit: ~3 x 10^{-1} variants per sample) as well as in KP-1 and PAO1 (1.3 ± 1.9%, detection limit: ~1 x 10^{-2} variants per sample). In addition, the average frequency of KP-1 NM in KP-1 mono species biofilms (13.4 ± 12.2%, detection limit: ~1 x 10^{-2} variants per sample) was also higher than its frequency in Pf-5 and KP-1 (3.7 ± 3.1%, detection limit: ~5 x 10^{-2} variant per sample) as well as PAO1 and KP-1 (0%, detection limit: ~1 x 10^{-1}) dual species

biofilms (Figure D-3). These observations suggest that variant frequency exhibits an inverse correlation with species richness within the biofilm community.

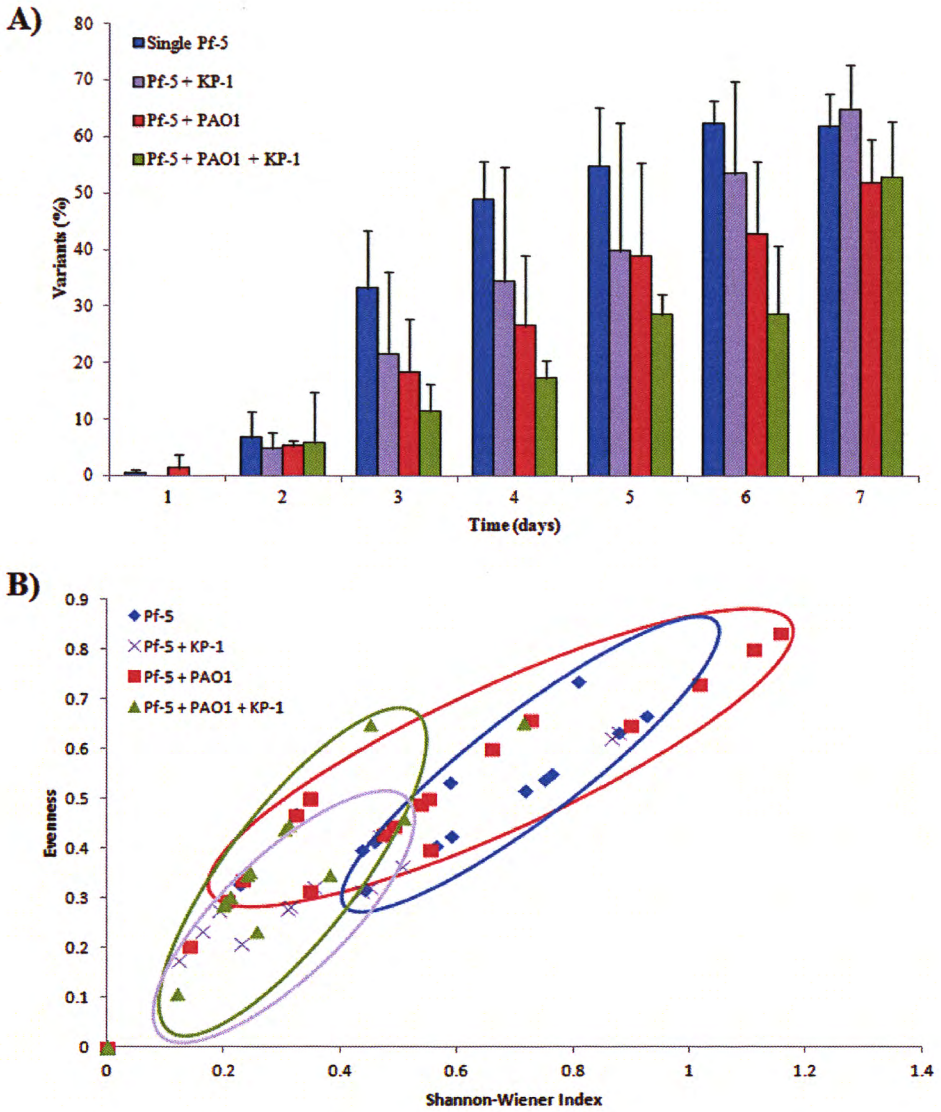

Figure 6-5. Species richness, frequency and diversity of Pf-5 morphotypic variants. A) Frequencies of Pf-5 morphotypic variants in the effluents of mono, dual and triple species biofilms. Error bars represent standard deviations (n = 3). B) Plot of the Evenness Index against the Shannon-Wiener Index depicting the diversity of Pf-5 morphotypic variants in the effluent of each biofilm type across 7 d. Each data point represents a biological replicate from each day of the 7 d experiment.

In addition to the reduction in percentage of morphotypic variants, the diversity of Pf-5 variants from dual (Pf-5 and KP-1) and triple species biofilms were also reduced relative to the diversity of Pf-5 variants from mono species biofilms. For example, the Pf-5 SCV appeared only on day 2 while the Pf-5 WCV was either absent or present at low frequency from day 5 to 7 (Figure D-4). A plot of the Evenness Index against the Shannon-Wiener Index (Figure 6-5B) indicated that the diversity of Pf-5 morphotypic variants from dual (Pf-5 and KP-1) and triple species biofilms were similar, but were less diverse compared to those from the Pf-5 mono species biofilms as well as the Pf-5 and PAO1 dual species biofilms. These results imply that KP-1 may affect the formation of variants by Pf-5.

6.3.8 Effluents of KP-1 and PAO1 biofilms affected the frequency and diversity of Pf-5 morphotypic variants in Pf-5 biofilms

To determine if KP-1 and PAO1 produce signals or cues that may alter variant formation by Pf-5, cell free effluents from KP-1 and PAO1 biofilms were collected, added (1 and 10% v/v) to supplemented M9 minimal medium and used to grow mono species biofilms of Pf-5. The frequency of Pf-5 morphotypic variants decreased in the presence of both KP-1 and PAO1 biofilm effluents in a concentration dependent manner (Figure 6-6A and Figure 6-7A). Surprisingly, the diversity of morphotypic variants from Pf-5 biofilms grown with and without KP-1 biofilm effluent were similar, but different from the diversity observed for Pf-5 morphotypic variants from Pf-5 and KP-1 dual species biofilms (Figure D-5). A plot of the Evenness Index against the Shannon-Wiener Index indicated that the diversity of Pf-5 morphotypic variants from the biofilms grown with 1 and 10% v/v KP-1 biofilm effluent covered the range of diversity exhibited by both the mono and dual species biofilms (Figure 6-6B). Intriguingly, the addition of 1 and 10% v/v PAO1 biofilm effluent to Pf-5 mono species biofilms decreased the diversity of Pf-5 morphotypic variants drastically. More than 90% of all variants were Pf-5 SCVs. At 10% v/v PAO1 biofilm effluent, Pf-5 WCV was not observed while the relative abundance of Pf-5 RCV increased (Figure D-6). A plot of the Evenness Index against the Shannon-Wiener Index showed that the diversity of Pf-5 morphotypic variants from the biofilms growth with 1 and 10% v/v PAO1 biofilm effluent were lowered (Figure 6-7B).

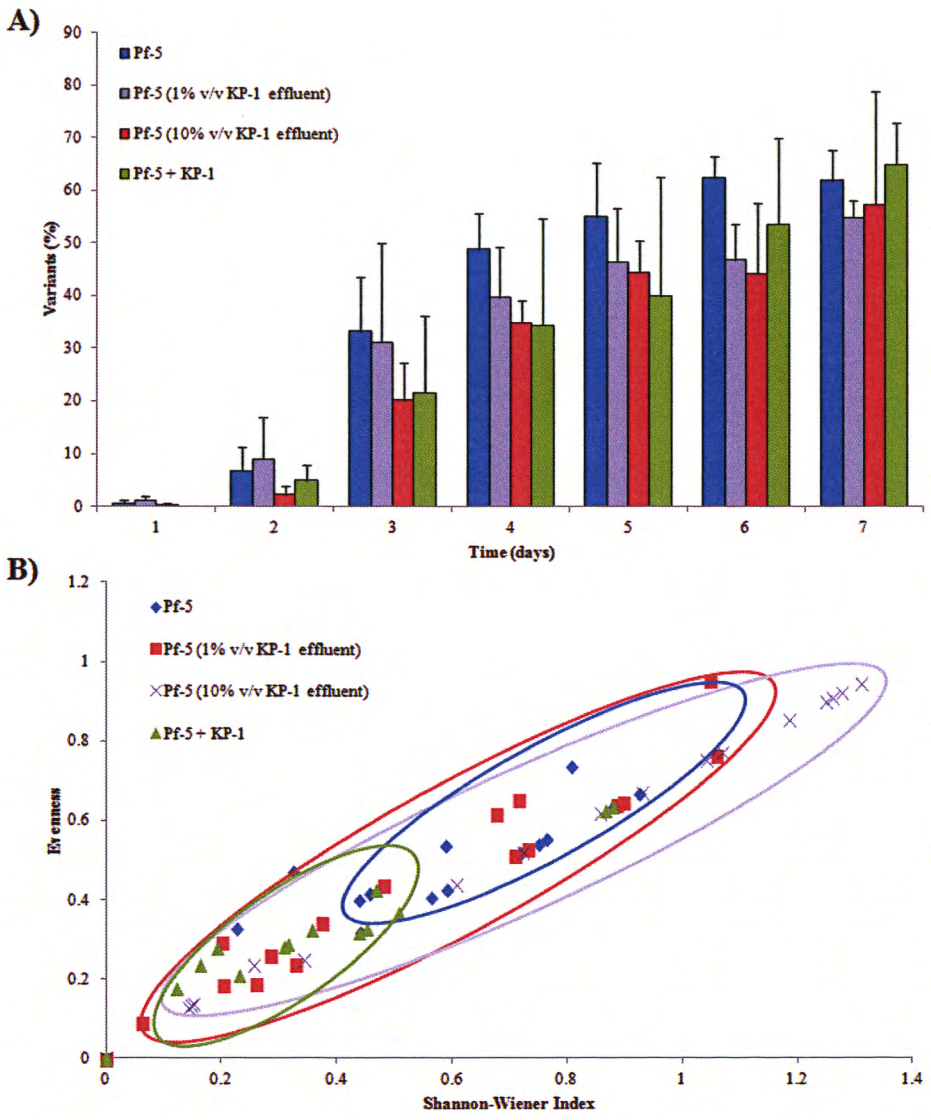

Figure 6-6. Effect of KP-1 effluent on the frequency and diversity of Pf-5 morphotypic variants. A) Frequency of Pf-5 morphotypic variants in the effluent of Pf-5 biofilms, Pf-5 biofilms grown with 1% v/v KP-1 effluent, Pf-5 biofilms grown with 10% v/v KP-1 effluent and dual species biofilms of Pf-5 and KP-1. Error bars represent standard deviations (n = 3). B) Plot of Evenness Index against Shannon-Wiener Index depicting the diversities of Pf-5 morphotypic variants in the effluents of each biofilm type across 7 d. Each data point represents a biological replicate from each day of the 7 d experiment.

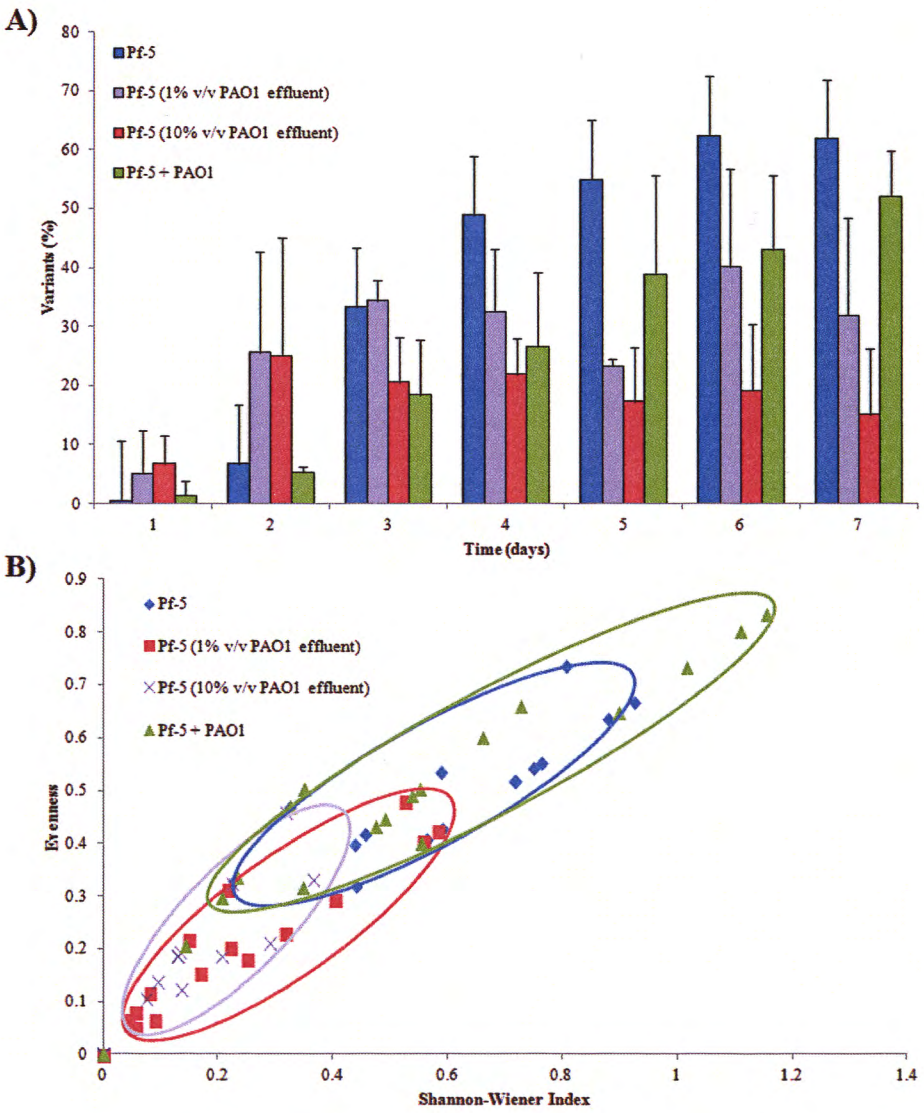

Figure 6-7. Effect of PAO1 effluent on the frequency and diversity of Pf-5 morphotypic variants. A) Frequency of Pf-5 morphotypic variants in the effluent of Pf-5 biofilms, Pf-5 biofilms grown with 1% v/v PAO1 effluent, Pf-5 biofilms grown with 10% v/v PAO1 effluent and dual species biofilms of Pf-5 and PAO1. Error bars represent standard deviations (n = 3). B) Plot of Evenness Index against Shannon-Wiener Index depicting the diversities of Pf-5 morphotypic variants in the effluents of each biofilm type across 7 d. Each data point represents a biological replicate from each day of the 7 d experiment.

6.4 Discussion

Morphotypic variants have typically been divided into two categories, phase variants which are transient and controlled by specific regulatory factors (Deziel *et al.*, 2001; Drenkard and Ausubel, 2002) and stable variants that arise from permanent genetic modifications (Allegrucci and Sauer, 2007; Boles *et al.*, 2004; Koh *et al.*, 2012). Here, the morphotypic variants isolated were stable upon passage. Genome sequencing of these variants also identified a few INDELs and SNPs, which may be linked to the phenotypic and morphotypic changes observed for these isolates.

Surprisingly, each morphotypic variant had either one or only a few mutations that led to non-synonymous substitutions. For example, the PAO1 SCV had a frame shift mutation at Thr210 of a twitching motility protein PilT. Twitching motility is important for the gliding of bacterial cells on surfaces (Merz *et al.*, 2000; Wall and Kaiser, 1999), and may thus explain the reduced motility observed for the PAO1 SCV (Table 6-5). In *K. pneumonia* and other enterobacteria, UDP-glucose lipid carrier transferase is responsible for the production of colanic acid, an exopolysaccharide that leads to the formation of mucoid colonies (Ratto *et al.*, 2006). Hence, it was interesting to note that the non-mucoid variant of KP-1 (KP-1 NM), had a non-synonymous substitution that resulted in a change of phenylalanine to isoleucine at position 123 of this enzyme. Although both phenylalanine and isoleucine are nonpolar amino acids, isoleucine (hydropathy index = 4.5) is more hydrophobic than phenylalanine (hydropathy index = 2.8) and can thus change the three dimensional structure and hence the function of UDP-glucose lipid carrier transferase (Kyte and Doolittle, 1982). Similar to the mutation observed in PAO1 SCV, the Pf-5 SCV possessed an insertion that led to a frame shift mutation at Asp78 of the flagellar motor switch protein FliM, a protein that is responsible for the reversal of flagellar rotation. Frame shift mutations often lead to the translation of non-functional flagellar motor switch proteins that can severely affect motility (Paul *et al.*, 2011). This is consistent with the Pf-5 SCV being impaired in swimming and swarming motilities (Table 6-5) as reflected by the formation of colonies that were smaller in size than colonies formed by the Pf-5 wild type. Mutation of the chemotaxis-specific methylesterase, as

observed in the Pf-5 CCV isolate, has also been shown to be involved in the regulation of exopolysaccharide expression. Indeed, mutation of this gene in *P. fluorescens* was previously shown to result in a colony morphotype that was similar to the colony morphotype of the Pf-5 CCV (Spiers *et al.*, 2003). Additionally, genome sequencing has also identified a SNP that changed a tryptophan (hydropathy index = -0.9) to leucine (hydropathy index = 3.8) at position 155 of the cell division protein FtsW, thereby affecting the folding of this protein and its function (Kyte and Doolittle, 1982). As a membrane protein responsible for the assembly of peptidoglycan at the cell septum during cell elongation, division and sporulation, a non-functional FtsW may inhibit septation and lead to filamentous growth (Pastoret *et al.*, 2004). Elongated cells of *E. coli* have previously been shown to possess higher number of flagella (Maki *et al.*, 2000), which can explain the increased swarming motility observed for Pf-5 RCV in this study (Table 6-5) and can be confirmed by microscopy. Lastly, Pf-5 WCV was identified to have a SNP that resulted in the translational termination of a group 1 family glycosyltransferase at Glu64. Group 1 family glycosyltransferase is involved in the transfer of a sugar moiety from a donor to an acceptor, thus resulting in the synthesis of various exopolysaccharides. Its mutation in *Pseudomonas alkylphenolia* has been shown to reduce surface motility and biofilm formation capability (Veeranagouda *et al.*, 2011). Similarly, the translation of a non-functional group 1 family glycosyltransferase could have led to the reduced motility (both swimming and swarming) and biofilm formation capability observed for Pf-5 WCV (Table 6-5). It is worth noting that all morphotypic variants in this study were isolated based on their distinct colony morphologies. Hence, it is not surprising that all variants have mutations in genes that are either directly or indirectly involved in surface property and cell motility traits. In addition, variants with no morphological change were not identified in this study, although such variants might be present. For example, it was reported that biofilms of *P. tunicata* generated variants which varied in biofilm formation, growth and motility, but exhibited no morphological difference from the parent (Mai-Prochnow *et al.*, 2006).

CHAPTER 6. INTERSPECIES VARIATION IS MORE IMPORTANT THAN INTRASPECIES VARIATION DURING MIXED SPECIES BIOFILM DEVELOPMENT

The appearance and prevalence of self-generated morphotypic variants, and thus diversity in biofilms, have been proposed by Boles *et al.* (2004) as an "insurance" for biofilms to adapt to environmental changes. Aligned with the "insurance hypothesis", all of the morphotypic variants isolated in this study were shown to be more competitive than their respective parent strains in co-cultured biofilms (Figure 6-4A). In addition, each morphotypic variant also performed better (higher relative biovolume) than its respective parent strain in the triple species biofilms (Figure 6-4B). Despite being fitter than their parent strains, the frequencies of all morphotypic variants *in situ* decreased with increased species richness in the biofilm communities (Figure 6-5 and Figure D-3). As a result, the formation of fitter variants, as observed in mono species biofilms, seemed to not take place within a mixed biofilm community. This was most surprising as these observations appeared to oppose the "insurance hypothesis". However, taking these observations together with results presented in Chapter 5 and in other studies (Burmolle *et al.*, 2006), which showed that increased species richness was associated with enhanced resilience to antibiotics, hydrogen peroxide, SDS and invasion by other bacterial species, it is proposed that individual species within a mixed species biofilm cease the formation of fitter morphotypic variants in the presence of other species. While the reason for such a response is unclear, it is tempting to speculate that the community level resilience offered by other species is more effective than the resilience offered through self-generated diversity. In addition, the reduction of intraspecies diversity in the presence of interspecies diversity requires a mechanism for the community members to determine whether or not they are in a diverse community. The reduction in variant production in the presence of spent supernatant would suggest that some form of signaling or cue recognition is important in the regulation of variant formation. Here, it was shown that Pf-5 biofilms grown in the presence of 1 and 10% v/v KP-1 and PAO1 biofilm effluents exhibited decreases in the frequencies of morphotypic variants (Figure 6-6 and Figure 6-7). Intriguingly, this is not the first observation that bacteria have been shown to react differently upon contact with other bacterial species. In fact, studies have suggested that *P. fluorescens* could determine the type of competitor present through a specific cue that regulated the two-component signal transduction system and fine tuned its competitive

strategy accordingly. For example, *P. fluorescens* co-cultured with *Pedobacter* was reported to produced a broad-spectrum antibiotic, which was not produced when *P. fluorescens* was co-cultured with *Bacillus* (Garbeva *et al.*, 2011). It is also well established that bacteria can communicate through the secretion and perception of signaling molecules that are involved in cross-talk between different species (McDougald *et al.*, 2007; Williams, 2007)

Although the frequency of Pf-5 morphotypic variants was reduced in the presence of both PAO1 and KP-1, the diversity of Pf-5 morphotypic variants were affected by their abundance within the mixed species biofilms. As shown by the dual species (KP-1 and Pf-5) and triple species biofilms, a high abundance of KP-1 (~50% of the biovolume) decreased the diversity of Pf-5 morphotypic variants (Figure 6-5), while addition of KP-1 biofilm effluent at 1 and 10% v/v (significantly lower than the 50% biovolume of KP-1 in the biofilms) did not change the diversity of Pf-5 morphotypic variants substantially (Figure 6-6). Similarly, the low abundance of PAO1 (~5% of the biovolume) in the dual species biofilms of PAO1 and Pf-5 did not change the diversity of Pf-5 morphotypic variants extensively (Figure 6-5). In contrast, the presence of 1 and 10% v/v PAO1 biofilm effluent (higher than the 5% biovolume of PAO1 in the biofilms) reduced the diversity of Pf-5 morphotypic variants significantly (Figure 6-7). Therefore, the abundance of PAO1 and KP-1 in the mixed species biofilms could potentially determine the level of cue that is regulating the types of morphotypic variant formed by Pf-5.

In conclusion, these results suggest that the development of genetic variability within a species can be altered, most likely in response to the presence of another species through a signal or cue. In addition, the morphotypic variants generated were fitter than their parent strains in the structured environment offered by the biofilm, but not a homogeneous environment, e.g. planktonic culture. While each bacterial species could enhance its individual fitness by generating morphotypic variants that outcompeted the other species in a mixed biofilm community, this intraspecies diversity was observed to be reduced in the mixed species biofilms. When viewed in the context of other studies (Burmolle *et al.*,

2006; Cowan *et al.*, 2000), which demonstrated that a mixed species biofilm was more resistant to stress, the results here suggested that interspecies diversity could substituted intraspecies diversity during mixed species biofilm development. Based on these results, it is proposed that the mixed species biofilm model established here demonstrated a number of properties that are common to macro ecological systems and hence may be an instructive system for testing various ecological theories relating to interspecies interactions.

CHAPTER 7 . EXTRACELLULAR MATRIX AND STRESS RESPONSES PROTECT BIOFILMS FROM SODIUM DODECYL SULFATE

7.1 Introduction

The biofilm represents a lifestyle that is distinct from the well studied planktonic growth, where biofilms exhibit advantageous traits that are responsible for their prevalence in natural settings. Therefore, much research has focused on identifying the specific genes involved in biofilm growth and enhanced resistance. Some studies have been aimed at deciphering the genes involved in biofilm specific characteristics such as increased resistance to antibiotics (Beloin *et al.*, 2004; Schembri *et al.*, 2003; Whiteley *et al.*, 2001b) while others have identified genes responsible for the transition of the biofilm from one developmental stage to the next (Domka *et al.*, 2007; Waite *et al.*, 2005). To date, metatranscriptomic analyses performed by the use of microarray gene chip and RNA sequencing have identified genes related to bacteriophage Pf1, virulence factors such as rhamnolipids and elastase, two-component systems, respiration and the biosynthesis of extracellular polysaccharides, to be up regulated during *P. aeruginosa* biofilm formation relative to planktonic growth. In contrast, genes responsible for the synthesis of flagella and pili, stress response (*rpoS*) and type III secretion system were found to be down regulated (Dotsch *et al.*, 2012; Waite *et al.*, 2005; Whiteley *et al.*, 2001b). In addition, non-coding sequences such as small regulatory RNAs were also observed to play an important role in the control of global gene expression in *P. aeruginosa* biofilms (Dotsch *et al.*, 2012). When the *P. aeruginosa* biofilms were treated with aminoglycosides such as gentamicin and tobramycin, genes that affected the lipopolysaccharide structure (*tolA*), stress responses (*dnaK* and *groES*) and probable efflux systems (PA1541 and PA3920) were induced and suggested to be the key mechanisms that lead to enhanced antibiotic resistance of the biofilms (Whiteley *et al.*, 2001b).

Hence, metatranscriptomic analysis has the potential to identify genes that elicit a particular response in biofilm. Between the various techniques that are available,

metatranscriptomic analysis using a next-generation sequencing platform has several advantages over focused mRNA analysis techniques such as quantitative reverse transcriptase PCR and global analysis platforms such as microarray. For example, RNA sequencing does not require prior knowledge of genes that may be regulated (as needed in quantitative reverse transcriptase PCR). In addition, paralogous sequences that cross hybridize on a microarray platform can be distinguished by next-generation sequencing (Moran, 2009).

In Chapter 5 of this thesis, the PAO1 and KP-1 mono species biofilms as well as the mixed species biofilms were shown to be more resilient to SDS stress than the Pf-5 mono species biofilms. To unravel the molecular mechanisms that rendered both PAO1 and KP-1 biofilms more resistant to SDS than Pf-5 biofilms, transcriptomic analyses were performed. The stress responses of the mono species biofilms were first defined to provide a basis for comparison with the mixed species biofilms, for which transcriptomic analyses were also performed to understand how interspecies interactions could protect the mixed species biofilms, especially the protection of sensitive Pf-5 within the biofilms from SDS.

In this chapter, a total of 24 RNA samples were sequenced and the number of genes that were found to be differentially expressed (Log_2 fold change > 1, P-value < 0.05) in SDS treated and untreated PAO1, Pf-5 and KP-1 mono species biofilms were 323, 83 and 259 genes respectively. Genes involved in EPS production and export as well as stress responses were proposed to be the key mechanisms that were protecting the PAO1 and KP-1 biofilms from SDS. The SDS stress response of the mixed species biofilms was also investigated and an average of 43,141,246 reads were identified for each sample. Preliminary analyses indicated that transcripts from all three species could be identified, despite the low abundance of PAO1 in the mixed species biofilms. However, many of the transcripts obtained from the mixed species biofilms were found to map to more than one species, probably due to the presence of highly conserved genes in all three species. As a result, more computational analysis will be required to differentiate these transcripts before any conclusion can be drawn from the transcriptomes obtained from the mixed

species biofilms. In addition, the low abundance of PAO1 in the mixed species biofilms decreases the sensitivity and detection limit for PAO1 transcripts. Hence, more sequencing runs, at a deeper depth of coverage, may be required for the complete interpretation of the mRNA expression profile for PAO1 within the mixed species biofilms relative to the mono species biofilm data presented in this thesis.

7.2 Materials and Methods

7.2.1 Bacterial strains and culture media

Bacteria (Table 7-1) were cultured in M9 minimal medium (48 mM Na_2HPO_4; 22 mM KH_2PO_4; 9 mM NaCl; 19 mM NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; and 0.04% w/v glucose) supplemented with 0.2% w/v casamino acids (supplemented M9 minimal medium).

Table 7-1. List of bacterial strains

Species and strain	Genotypic and phenotypic characteristics[1]	Source or reference
Pseudomonas aeruginosa		
PAO1 eYFP	carries the gene encoding eYFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
Pseudomonas protegens		
Pf-5 eCFP	carries the gene encoding eCFP in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study
Klebsiella pneumoniae		
KP-1 DsRed	carries the gene encoding DsRedExpress in the intergenic region between the coding region of *glmS* and its downstream gene; Gm^R	This study

[1] Gm^R: Gentamicin resistance

7.2.2 Continuous-culture flow cell experiments and biomass extractions

Mono and mixed species biofilms were cultivated in three-channel flow cells and treated with 0.1% w/v SDS as described in Chapter 5. The samples from pre and post-SDS

treatment were collected on day 4 (Table 7-2). Biomass from each flow cell was extracted by first flushing ice cold PBS through the channels several times followed by a validation of biomass removal efficiency by confocal laser scanning microscopy. Subsequently, the RNAprotect Bacteria reagent (Qiagen, Netherlands) was added immediately to the extracted biomass according to the protocols suggested by the manufacturer to stabilize the RNA before extraction.

Table 7-2. List of biofilm samples

Age of biofilms (d) / treatment	Types of biofilm and number of replicates				
	Mono PAO1	Mono Pf-5	Mono KP-1	Mixed	Sub-total
4 d / untreated	3	3	3	3	12
4 d / treated with 0.1 w/v SDS	3	3	3	3	12
				Grand-total	24

7.2.3 RNA extraction and next-generation sequencing

Stabilized RNA from the biofilm was extracted from the cells using the RNeasy Protect Bacteria Mini Kit (Qiagen, Netherlands). Extracted RNA was treated with TURBO DNA-*free*™ Kit (Ambion, USA) to remove any DNA before purification by RNA Clean & Concentrator™-5 (ZYMO Research Corporation, USA). All procedures were performed according to the protocols suggested by the manufacturers. Libraries for total RNA were prepared using the TruSeq RNA Sample Preparation Kit (Illumina, USA) and sequenced on the HiSeq 2500 sequencer (Illumina, USA) according to the manufacturer's protocols.

7.2.4 Computational analysis

The sequences were mapped to the *P. aeruginosa* PAO1, *P. protegens* Pf-5 and *K. pneumoniae* NTUH-K2044 reference genomes (GenBank accession numbers: AE004091, CP000076 and AP006725 respectively) using Bowtie 2 (Langmead and Salzberg, 2012). These reference genomes were used as the genomes that were sequenced in Chapter 3 were not complete. Transcript assembly and abundance estimation were subsequently performed by Cufflinks (Trapnell *et al.*, 2010) followed by differential analysis by Cuffdiff 2 (Trapnell *et al.*, 2013). Differentially expressed genes (Log_2 fold change > 1, *P*-

value < 0.05) were then mapped to the KEGG pathways for interpretation at the system level (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2012).

7.3 Results

To understand the genetic basis for the enhanced stress resistance exhibited by the mixed species biofilms relative to the mono species biofilms, the transcriptomes from SDS treated and untreated mono species as well as mixed species biofilms were analyzed. The analyses were performed by first comparing SDS treated and untreated mono species biofilms, followed by comparison with the results obtained from the mixed species biofilms.

7.3.1 Genes that were differentially expressed in SDS treated PAO1 biofilms

An average of 35,631,188 reads with a 121 fold coverage of the coding regions were obtained for each sample and a total of 323 genes were found to be differentially expressed (Log_2 fold change > 1, *P*-value < 0.05) when the transcriptomes of SDS treated and untreated PAO1 mono species biofilms were compared (Table 7-3).

Table 7-3. Overview on the number of differentially expressed genes

Species	[1]SDS treated vs SDS untreated
PAO1	
Up regulated	87
Down regulated	236
Total	323
Pf-5	
Up regulated	40
Down regulated	43
Total	83
KP-1	
Up regulated	118

Down regulated	141
Total	259

[1]The table shows genes that have a Log_2 fold change > 1 and *P*-value < 0.05

Specifically, 87 and 236 genes were up and down regulated respectively in the SDS treated PAO1 biofilms relative to the untreated PAO1 biofilms (Table 7-3). Of these 323 genes, 126 code for conserved hypothetical and hypothetical proteins of unknown functions. The other 197 genes were categorized into functional groups such as metabolism, biosynthesis of siderophores, DNA replication and repair, motility and attachment, protein expression, regulatory and signaling pathways, stress response, surface component, secretion and transport as well as virulence. Metabolically, genes involved in cellular energy metabolism as well as starch and sucrose metabolism, e.g. *coxA* (cytochrome c oxidase, subunit I) and *glgP* (glycogen phosphorylase), were down regulated, while genes involved in amino acid biosynthesis, e.g. *proC* (pyrroline-5-carboxylate reductase) and *dapA* (dihydrodipicolinate synthase), were up regulated. The up regulation of amino acid biosynthesis was also supported with the increase in protein expression activity. For example, ribosomal proteins encoded by *rplI*, *rplP* and *rpm*C were induced (Table 7-4) by a Log_2 fold change of 1.14 to 1.24 (Table E-1) in SDS treated PAO1 biofilms. In addition, the biosynthesis of pyochelin, a siderophore normally synthesized during growth in iron-limited conditions, was up regulated (Table 7-4) by a Log_2 fold change of 1.04 (Table E-1). Furthermore, genes that are involved in DNA recombination and repair were also up regulated (Table 7-4) in SDS treated PAO1 biofilms, where *ssb* (homologous recombination) was induced by a Log_2 fold change of 1.01 (Table E-1). Interestingly, genes involved in the assembly of flagella and pili were mostly up regulated in the SDS treated PAO1 biofilms. For example, genes such as *flgB* and *pilE*, along with a two-component response regulator (encoded by *fleR*) that was involved in flagella assembly (Dasgupta *et al.*, 2003), were up regulated (Table 7-4) by a Log_2 fold change of 1.02 to 1.11 (Table E-1). However, multiple chemotaxis and fimbriae genes were down regulated in the SDS treated PAO1 biofilms (Table 7-4), suggesting that flagella and pili may have a role other than motility during response to SDS. While, various regulatory and signaling pathways were also up and down regulated, gene PA4108, a gene encoding c-di-GMP phosphodiesterase, was down regulated by a Log_2

fold change of 1.37 (Table E-1). The repression of PA4108 suggests that the intracellular level of c-di-GMP would be higher in the SDS treated PAO1 biofilms. Intriguingly, *siaA*, which induces the autoaggregation of *P. aeruginosa* cells in response to SDS stress (Klebensberger *et al.*, 2009) was up regulated (Log_2 fold change of 1.12, Table E-1). Surface components such as adhesins and lipopolysaccharides were also up regulated (Table 7-4). However, *mucA*, which is involved in the biosynthesis of alginate, an extracellular polysaccharide, was down regulated (Table 7-4) by a Log_2 fold change of 1.24. Lastly, genes coding for various secretion systems (e.g. type II and IV secretion) and virulence factors (e.g. hemolysin and pyocin) were also down regulated (Table 7-4).

Table 7-4. List of PAO1 genes that were differentially expressed in SDS treated biofilms

[1]General functions/ pathways	Gene names	[2]Types of regulation
Metabolism		
1- and 2-Methylnaphthalene degradation	PA0508	+
Acetyltransferase activity	*riml*	+
Arginine and proline metabolism	*proC*	+
Aromatic compound catabolism	*fahA*, *maiA*	+
Ascorbate and aldarate metabolism	*pdxB*	+
Cellular amino acid metabolism	*hisG*	+
Cellular energy metabolism	*acsA*, PA5445	+
Cobalamin biosynthesis	*cbiD*, *cobL*	+
Cofactor biosynthesis	PA3169, *thiD*, *thiE*	+
Glycolate oxidase activity	PA4772	+
Lysine biosynthesis	*dapA*	+
Maleylacetoacetate isomerase activity	PA2821	+
Molybdopterin biosynthesis	*moaC*, *moaD*	+
Oxidoreductase activity	*ahpF*, PA0364, PA0366, PA3559	+
Pyrimidine metabolism	*trxB2*	+
Arylesterase activity	*cpo*	-
Butanoate metabolism	*phaC2*	-
Cellular amino acid biosynthesis	PA3459, *ldh*	-
Cellular energy metabolism	*coIII*, *coxA*, *coxB*, *kup*, *lecB*, PA0918, PA2265, PA2550, PA2815,	-

	PA3429, PA3430, PA3460, PA3723, PA5020	
Chitin degradation	*chiC*	-
Denitrification	*norB*, *norC*	-
Fatty acid biosynthesis	PA2142	-
Formaldehyde metabolism	PA2158	-
Glycerolipid metabolism	*glpK*	-
Glycine betaine catabolism	*gbdR*	-
Glycine cleavage	*gcvP1*	-
Glyoxylate and dicarboxylate metabolism	*acnA*, *glcD*, *glcE*, *glcF*	-
Menaquinone biosynthesis	PA3924	-
Nitrogen metabolism	*napA*, *napB*, *napC*, *napD*, *napF*	-
Oxidoreductase activity	PA3795	-
Proteolysis	PA0452, *clpA*, *pfpl*	-
Pyrroloquinoline quinone biosynthesis	*pqqB*, *pqqC*, *pqqD*, *pqqE*	-
Pyruvate metabolism	*ackA*, *gloA3*, *poxB*, PA3415, PA3416, PA3417	-
Starch and sucrose metabolism	*glgB*, *glgP*, PA2152, PA2160, PA2162, PA2164, PA2165	-
Valine, leucine and isoleucine degradation	*bkdA1*, *bkdA2*	-
Biosynthesis of siderophores		
Pyochelin synthesis	*pchE*, *pchF*, *pchG*	+
DNA replication and repair		
Homologous recombination	*ssb*, PA3961	+
Apoptosis	PA1327	-
Motility and attachment		
Flagella assembly	*flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgI*, PA1441,	+
Pilin biosynthesis	*pilE*, *pilY1*, *pilY2*	+
Chemotaxis	PA2920, PA4633, PA2573, PA4915, *aer2*, PA1930, PA0177, PA0178, PA0179, *cttP*	-
Fimbriae	*cupE1*, *cupE5*, *cupE6*	-
Negative regulation of flagella assembly	*amrZ*	-
Protein expression		

Protein folding	*hscA*	+
Ribosome	*rplI*, *rplP*, *rpmC*, PA5369.1	+
RNA helicase	*hepA*	+
Ribosome modulation factor	*rmf*	-
Translational elongation	*fusA2*	-
Regulatory and signaling pathways		
cAMP sythesis	*cyaB*	+
Transcriptional regulation	PA3094, PA3260	+
Two-component system	*amgS*, *fleR*, *phoP*, *phoQ*	+
cAMP binding protein	*cbpA*	-
C-di-GMP phosphodiesterase activity	PA4108	-
Non-coding RNA	*phrS*	-
Transciptional regulation	PA2100, PA2846, PA2896, PA3122, *psrA*	-
Two-component system	*ntrB*, *pprA*, *pprB*, *rsmA*, PA2177, PA2571, PA2572	-
Stress response		
Oxidative stress	*katB*	+
SDS stress	*siaA*	+
Cold response	*cspD*	-
Osmotic response	*osmE*	-
Oxidative stress	*katE*	-
Surface component, secretion system and transport		
ABC transporter	PA1809, PA4222, PA4223	+
Adhesin biosynthesis	*cdrA*	+
Lipopolysaccharide biosynthesis	*waaA*, *arnA*, *arnB*, *arnC*, *arnD*, *arnE*, *arnF*, *arnT*	+
Porin	PA3038	+
Sodium/proton anitporter	PA5529	+
ABC transporter	PA1875, PA1876, PA1877, PA3252, PA3253, PA3573, PA4913, PA5096, *modA*	-
Alginate biosynthesis	*mucA*	-
Bacterial secretion system	PA1666, PA1668, PA1669, *stp1*, PA5210,	-

Copper transport outer membrane porin	*oprC*	-
Iron transporter	PA4880,	-
Lipotoxin	*lptF*	-
Lysozyme inhibitor	*mliC*	-
Type II secretion system	*lasB*, PA2939, *tadA*, *tadB*, *tadZ*	-
Type IV secretion system	*stk1*, *tssC1*, PA2366, PA1031, PA1657, PA1658, PA1667	-
Xanthine transmembrane transporter	PA2938	-
Xenobiotic transporter	PA5030	-
Virulence		
Hemolysin	*rahU*	-
Pyocin	*pyoS5*	-

[1] The table shows only genes that were differentially expressed (Log_2 fold change > 1, *P*-value < 0.05). For the list of genes and their Log_2 fold changes, refer to Table E-1.
[2]+: up regulation; -: down regulation

7.3.2 Genes that were differentially expressed in SDS treated Pf-5 biofilms

An average of 42,293,969 reads with a 126 fold coverage of the coding regions were obtained for each sample. Unlike PAO1, relatively fewer genes were differentially expressed in SDS treated Pf-5 biofilms. In total, only 40 and 43 genes were up and down regulated respectively (Table 7-3). Of these 83 genes, 19 encode hypothetical proteins of unknown functions. Similar to PAO1, genes involved in cellular energy metabolism, e.g. *acsA_1* (acetyl-CoA synthetase) and *aidB* (acyl-CoA dehydrogenase) were down regulated (Table 7-5) by a Log_2 fold change of 1.16 and 1.25 respectively (Table E-2). In contrast, genes involved in amino acid biosynthesis, e.g. *sdaB* (L-serine ammonia-lyase) and DNA replication and repair, such as *lhr* (ATP-dependent helicase) and *polB* (DNA polymerase II), were up regulated by a Log_2 fold change of 1.62, 1.45 and 1.22 respectively (Table E-2). Genes involved in flagella assembly, e.g. *flgB*, *flgC* and *flgD*, were also up regulated by a Log_2 fold change of 1.05 to 1.59 (Table E-2) while genes responsible for chemotaxis, e.g. *pctA_1*, PFL_2182 and PFL_4203, were down regulated (Table 7-5) by a Log_2 fold change of 1.00 to 1.44 (Table E-2), further suggesting that flagella may play a role other than motility during response to SDS. Like PAO1, protein expression was also up regulated, but only one gene associated with protein expression

was differentially expressed in SDS treated Pf-5 biofilms (Table 7-5) compared to six in SDS treated PAO1 biofilms (Table 7-4). In contrast to SDS treated PAO1 biofilms, the biosynthesis of extracellular polysaccharide was down regulated (Table 7-5) in SDS treated Pf-5 biofilms. In addition, genes involved in the biosynthesis of siderophores, e.g. *pvdD*, *pvdI* and *pvdL*, were also down regulated (Table 7-5) by a Log_2 fold change of 1.02 to 1.31 (Table E-2) instead of up regulated as shown in SDS treated PAO1 biofilms (Table 7-4). Furthermore, no SDS stress response gene was differentially expressed in SDS treated Pf-5 biofilms. Instead, genes (*hmp* and PFL_3309) involved in response to nitric oxide were up regulated by a Log_2 fold change of 1.46 to 1.87. Lastly, bacterial secretion systems (e.g. type IV secretion system) and virulence factors (e.g. pyocin) were up regulated (Table 7-5) instead of down regulated, as observed in SDS treated PAO1 biofilms (Table 7-4).

Table 7-5. List of Pf-5 genes that were differentially expressed in SDS treated biofilms

[1]General functions/ pathways	Gene names	[2]Types of regulation
Metabolism		
Ammonia monooxygenase activity	PFL_1436	+
Aspartate ammonia-lyase activity	*aspA*	+
Biosynthesis of amino acids	*sdaB*	+
Glutamate synthase activity	*gltD*	+
Molybdopterin biosynthesis	*moaB_1*	+
Nitrogen metabolism	*cah_3*	+
Oxidoreductase activity	*calB*, PFL_5867	+
Riboflavin biosynthesis	PFL_1034	+
Acyclic terpene utilization	*atuA*	-
Biotin biosynthesis	*tam*	-
Cellular energy metabolism	*acsA_1*, *aidB*, *gcdH*, *liuA*, PFL_0338	-
Fatty acid metabolism	*phlC*	-
Glycolysis and gluconeogenesis	PFL_3668	-
Oxalate catabolism	PFL_0118	-
Oxidoreductase activity	PFL_3558, PFL_5808, *prnD*	-
Phosphate metabolism	PFL_1644	-

Polyketide biosynthesis	*ofaA*, *ofaB*	-
Proteolysis	*aprA*, *aprX*	-
Biosynthesis of siderophores		
Pyoverdine synthesis	*pvdD*, *pvdI*, *pvdL*	-
DNA replication and repair		
Homologous recombination	*lhr*, *polB*	+
DNA modification	PFL_1978	-
Motility and attachment		
Flagella assembly	*flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgJ*	+
Chemotaxis	*pctA_1*, PFL_2182, PFL_4203	-
Flagella protein	PFL_1633	-
Protein expression		
Peptidyl-tRNA hydrolase activity	*pth*	+
Regulatory and signaling pathways		
Two-component system	PFL_4996	+
Diguanylate cyclase activity	PFL_5686	-
Transciptional regulation	PFL_3261	-
Stress response		
Nitric oxide detoxification	*hmp*	+
Response to nitric oxide	PFL_3309	+
Surface component, secretion system and transport		
ABC transporter	PFL_3515, PFL_3573	+
Amino acid transporter	*cycA*, *sdaC*	+
Resistance-nodulation-cell division efflux transporter	*emhB*, PFL_1028, PFL_1157, PFL_1158	+
Type IV secretion system	*tonB3*	+
ABC transporter	PFL_2149	-
Extracellular polysaccharide biosynthesis	*pgaB*	-
Iron aquisition	*hasAp*	-

Multidrug resistance efflux pump	PFL_0258	-
Permease	*gabP*	-
Virulence		
Pyocin	PFL_1227, PFL_1223	+

[1] The table shows only genes that were differentially expressed (Log_2 fold change > 1, *P*-value < 0.05). For the list of genes and their Log_2 fold changes, refer to Table E-2.
[2] +: up regulation; -: down regulation

7.3.3 Genes that were differentially expressed in SDS treated KP-1 biofilms

An average of 45,742,902 reads with a 73 fold coverage of the coding regions were obtained for each sample and a total of 118 and 140 genes were up and down regulated respectively in the SDS treated KP-1 biofilms (Table 7-3). Of these 258 differentially expressed genes, 58 encode hypothetical proteins of unknown functions. Generally, genes that are involved in carbohydrate metabolism such as starch and sucrose metabolism (*ostA*, *ostB*, KP1_5462), fructose and mannose metabolism (*fbaB* and *manC*) as well as carbon metabolism (*gpmA*, *fumB*, *serA*, KP1_2565, KP1_4700) were down regulated (Table 7-6) by a Log_2 fold change of 1.01 to 1.91 (Table E-3). In addition, genes that are responsible for thiamine metabolism such as *thiC*, *thiD* and *thiE* were also down regulated (Table 7-6) by a Log_2 fold change of 1.69 to 2.48 (Table E-3) in the SDS treated KP-1 biofilms. In contrast, genes that are involved in nitrogen metabolism were up regulated by a Log_2 fold change of 1.02 to 1.65 (Table E-3). In addition, genes (*fdoG*, *fdoH*, *fdoI* and *fdhF*) that are responsible for glyoxylate and dicarboxylate metabolism were up regulated (Table 7-6) by a Log_2 fold change of 1.01 to 1.32 (Table E-3). Other metabolic genes that were up regulated include those that are involved in pyrimidine metabolism, e.g. *deoA*, *cdd* and *udp* as well as those that are involved in sulfur metabolism, e.g. *dmsA*, *dmsB*, *dmsC* and *aegA* (Table 7-6). Additionally, *entA*, *entB*, *entC*, *entE* and *entF* (biosynthesis of siderophores) were down regulated (Table 7-6) by a Log_2 fold change of 2.09 to 2.33 (Table E-3). An array of regulatory and signaling pathways were also up and down regulated in SDS treated KP-1 biofilms (Table 7-6) while a few stress response proteins such as superoxide dismutase (*sodB*), phage shock protein A (*pspA*), putative universal stress protein G (KP1_2611) and Cpx envelope stress response proteins (*cpxP* and *degP*) were up regulated (Table 7-6) by a Log_2 fold change of 1.04 to 2.30 (Table E-3). In

addition, genes involved in protein transport (*tatA*, KP1_0713, KP1_0714 and KP1_0946), extracellular polysaccharide (KP1_5310) and lipoprotein (*ybjP*) syntheses were also up regulated (Table 7-6) by a Log_2 fold change of 1.12 to 1.91 (Table E-3). Furthermore, ABC transporters (encoded by *mppA* and *oppB*) involved in oligopeptide transport were also induced by a Log_2 fold change of 3.52 and 1.24 respectively (Table E-3). In contrast, a total of 30 genes involved in the transport of siderophores and iron were down regulated (Table 7-6) by a Log_2 fold change of 1.36 to 4.23 (Table E-3).

7.3.4 Transcriptomes from the mixed species biofilms

The results from the mono species biofilms provide an understanding of the stress responses that occurred during SDS treatment and can be compared against the stress responses exhibited by the mixed species biofilms. RNA sequencing of the mixed species biofilms yielded an average of 43,141,246 reads for each sample. The majority of the reads, 61%, were uniquely mapped to KP-1 while 0.06 and 0.01% of the reads were uniquely mapped to Pf-5 and PAO1 respectively (Figure 7-1). These relative proportions are in agreement with the overall biomass contributions of the three species to the biofilm biovolume. Additionally, it was observed that the rest of the reads from the mixed species biofilms could be mapped to more than one species, probably due to the presence of genes that are commonly conserved among all three species, such as those involved in central metabolism and those associated with transcription and translation. As shown in Figure 7-1, 5.4, 3.8 and 2.2% of the reads was mapped to both PAO1 and Pf-5, PAO1 and KP-1 as well as Pf-5 and KP-1 respectively. In addition, a significant number of the reads, 26.7%, were mapped to all three species. Therefore, more computational analysis is needed to correctly assign these transcripts before any conclusion can be drawn from the transcriptomes of the mixed species biofilms.

Table 7-6. List of KP-1 genes that were differentially expressed in SDS treated biofilms

[1]General function/ pathways	Gene name	[2]Type of regulation
Metabolism		
Anaerobic respiration	*frdA*, *frdB*, *frdC*, *frdD*	+
Cellular amino acid biosynthesis	*tdcB*	+
Cellular amino acid metabolism	*ynfE*	+
Cellular energy metabolism	*cybC*, *yjiM*	+
Formate dehyrogenase activity	*fdhE*	+
Glycolysis and gluconeogenesis	*ascB*	+
Glyoxylate and dicarboxylate metabolism	*fdoG*, *fdoH*, *fdoI*, *fdhF*	+
Nitrogen metabolism	*narG*, *narH*, *narI*, *narJ*, *nuoC*, *nuoE*, *nuoF*, , *nuoI*, *nuoJ*, *nuoK*, *nuoL*, *ydbK*	+
Porphyrin and chlorophyll metabolism	*finA*, *cysG*, *hemG*	+
Protein catabolic process	*clpS*	+
Pyrimidine metabolism	*deoA*, *cdd*, *udp*, KP1_1582	+
Pyruvate metabolism	*pckA*	+
Sulfur metabolism	*dmsA*, *dmsB*, *dmsC*, *aegA*	+
Terpenoid backbone biosynthesis	*ispA*	+
Transferase activity	KP1_1405, *wbbO*	+
Valine, leucine and isoleucine degradation	KP1_0552	+
Vitamin B6 metabolism	*pdxA*	+
Aminobenzoyl-glutamate utilization	*abgA*, *abgB*	-
Biotin metabolism	*bioA*	-
Carbon metabolism	*gpmA*, *fumB*, *serA*, KP1_2565, KP1_4700	-
Cellular amino acid metabolism	*ygjG*	-
Cysteine and methionine metabolism	KP1_1280	-
D-glutamine and D-glutamate metabolism	KP1_2495	-
Fructose and mannose metabolism	*fbaB*, *manC*	-
Glycogen biosynthesis	*glgS*	-
Iron/sulfur metabolism	*sufA*	-
Nucleotidyltransferase activity	*citX*	-
Oxidoreductase activity	*nrdH*, *nrdI*, *yncE*, KP1_3653	-
Pantothenate and CoA biosynthesis	KP1_4354	-

Phosphotransferase system	KP1_5463	-
Purine metabolism	*cysC*, *cysN*, *nrdE*, *nrdF*, *ade*	-
Starch and sucrose metabolism	*ostA*, *ostB*, KP1_5462	-
Thiamine metabolism	*thiC*, *thiD*, *thiE*, *thiF*, *thiG*, *thiH*, *thiM*, *thiS*, KP1_2847, *sufS*	-
Tryptophan metabolism	KP1_3993	-
Biosynthesis of siderophores		
Siderophore biosynthesis	*entA*, *entB*, *entC*, *entE*, *entF*	-
Protein expression		
Protein folding	*groES*	+
Ribosome	*rrfG*	-
Regulatory and signaling pathways		
Transcriptional regulation	*cytR*, *emrR*, *iclR*, *nhaR*, *tdcA*, *lrp*, *ybeF*, *yfeC*, KP1_0886, KP1_1405, KP1_1581, KP1_1583	+
Transcriptional regulation	KP1_1573, KP1_3190, KP1_4265, *cbl*	-
Stress response		
Cpx envelope stress response	*cpxP*, *degP*	+
Nitrotoluene degradation	*nfsA*	+
Oxidative stress	*ahpF*, *sodB*	+
Phage shock protein	*pspA*	+
Universal stress protein	KP1_2611	+
Nitric oxide detoxification	*hmpA*	-
Response to tellurium ion	*tehB*	-
Surface component, secretion system and transport		
ABC transporter	*atpI*, *dppC*, *mppA*, *oppB*, *yhhJ*, *yhiH*, KP1_0700	+
Efflux pump	*ybiF*	+
Electrochemical potential-driven transporter	*ydgE*, *ydgF*	+
Extracellular polysaccharide synthesis	KP1_5310	+
Iron aquisition	*ftnB*	+

Lipoprotein synthesis	*ybjP*	+
Major Facilitator Superfamily transporter	KP1_2665	+
Nucleoside/sodium symporter	KP1_0794	+
Protein transporter	*tatA*, KP1_0713, KP1_0714, KP1_0946	+
Transmembrane transporter	*dcuB*, *narK*, *perM*, *spy*, *yedE*	+
ABC transporter	KP1_1570, KP1_1571, KP1_2849, KP1_2850, KP1_2851, KP1_3864, *fliY*	-
Iron transporter	KP1_3191, KP1_3192, KP1_3194, KP1_3195, *sitA*, *sitB*, *sitC*, *sitD*	-
Iron/siderophore transporter	*fecB*, *fecD*, *fecE*, *fepA*, *fepB*, *fepC*, *fepD*, *fepG*, *fes*, *hmuT*, *hmuU*, *hmuV*, *iroN*, *tonB*, *ybdA*, *ybiL*, KP1_1466, KP1_2101, KP1_3196, KP1_3427, KP1_4356, KP1_4358	-
Iron/sulfur binding cluster	*bfd*, *hcp*, *fhuF*, *sufB*, *sufC*, *sufD*	-
Nucleoside/sodium symporter	*nupC*	-
Protein transporter	*exbB*, *exbD*	-
Sodium/dicarboxylate symporter	*ydjN*	-
Sulfate/thiosulfate transporter	*cysA*, *cysP*, *cysT*, *cysW*	-
Transmembrane transporter	*cirA*, *eitD*, *ompF*, *mntH*, *yeiU*, *yicE*, *tehA*, KP1_2563	-

[1] The table shows only genes that were differentially expressed (Log_2 fold change > 1, *P*-value < 0.05). For the list of genes and their Log_2 fold changes, refer to Table E-3.
[2] +: up regulation; -: down regulation

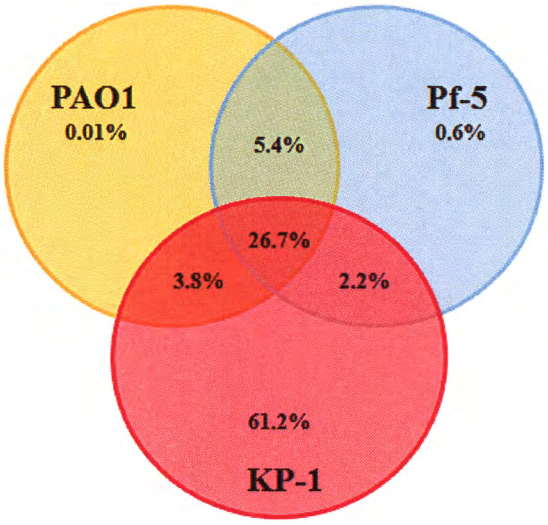

Figure 7-1. Transcripts mapped to more than one species. The venn diagram shows the percentages of reads that mapped to more than one species. The percentage shown is an average of six samples (three biological replicates of SDS untreated mixed species biofilms and three biological replicates of SDS treated mixed species biofilms).

7.4 Discussion

Next-generation sequencing and metatranscriptomic analysis were employed in this study to investigate the differential gene expression profiles during exposure to SDS. SDS exposure resulted in the differential expression of 323 and 259 genes for PAO1 and KP-1 mono species biofilms respectively, while only 83 genes were differentially expressed in SDS treated Pf-5 mono species biofilms. These results are in agreement with the observations that PAO1 and KP-1 biofilms showed greater resistance to SDS than the Pf-5 biofilms. In SDS treated PAO1 and Pf-5 biofilms, genes that are generally associated with flagella assembly were up regulated, but genes that are responsible for chemotaxis were down regulated. Hence, flagella may have a different role as part of the SDS response, rather than their usual function in motility. Indeed, flagella, but not motility, have been reported to be essential in protecting *P. aeruginosa* cells from 0.25% SDS, where flagella were found to be critical in the expression of lipopolysaccharides and the maintenance of outer membrane stability, both of which were important in preventing the lysis of *P. aeruginosa* cells by SDS (Zhang *et al.*, 2007). Notably, genes coding for numerous proteins involved in the biosynthesis of lipopolysaccharides were up regulated in PAO1

biofilms, but not in Pf-5 biofilms. In addition, adhesins encoded by *cdrA* were also up regulated in PAO1 biofilms and has been demonstrated to reinforce the biofilm extracellular matrix (Borlee *et al.*, 2010), thus stabilizing PAO1 biofilms during SDS treatment. While these surface structures would stabilize PAO1 biofilms during SDS stress, it is not clear how they could contribute to the cross protection of Pf-5 in the mixed species biofilms, unless these structures also bind and enmesh Pf-5 cells within the mixed species biofilms. Furthermore, *siaA* was also up regulated and promoted the autoaggregation of PAO1 cells as a response to SDS (Klebensberger *et al.*, 2009). These mechanisms may explain the increased SDS resilience that PAO1 biofilms displayed over Pf-5 biofilms. Surprisingly, enzymes that break down SDS, e.g. SDS hydrolase, were not found to be differentially expressed in SDS treated PAO1 biofilms.

In SDS treated KP-1 mono species biofilms, various stress response proteins such as the *sodB* encoded superoxide dismutase, the *pspA* encoded phage shock protein A, the KP1_2611 encoded putative universal stress protein and the *cpxP*, *degP* encoded Cpx envelope stress response proteins were up regulated. Moreover, the chaperonin GroES was also up regulated, suggesting that the cells were actively responding and rescuing themselves from lysis by SDS. In particular, the induction of Cpx envelope stress response proteins has previously been demonstrated to protect the bacterial envelope from a variety of stresses (Danese and Silhavy, 1998). In contrast, a large number of genes responsible for the synthesis and transport of siderophores and irons were down regulated in SDS treated KP-1 mono species biofilms. While this implies that a reduction in either iron transport or metabolism is important for the SDS response, further experiments are required to determine whether the uptake of iron renders KP-1 cells more susceptible to SDS lysis or that treatment with SDS decreases the need for iron uptake by KP-1 cells. In addition, genes that are involved in glyoxylate and dicarboxylate metabolism were up regulated, whereas genes involved in thiamine metabolism were down regulated in SDS treated KP-1 mono species biofilms. While glyoxylate and dicarboxylate metabolism is involved in the biosynthesis of carbohydrates from fatty acids (de Figueiredo *et al.*, 2008; Kornberg and Madsen, 1957), thiamine metabolism and its product, thiamine pyrophosphate, was reported to be an essential cofactor for various enzymes that

participate in the catabolism of carbohydrates (Settembre *et al.*, 2003). Therefore, the level of carbohydrates should be high in SDS treated KP-1 cells. Furthermore, pyrimidine metabolism was also up regulated in SDS treated KP-1 mono species biofilms. Induced pyrimidine metabolism was shown to be involved in the maintenance of a pyrimidine pool that could be used for DNA related functions such as DNA replication and repair, but was also reported to increase the production of cellulose and curli fibers, both of which were essential components of the extracellular matrix (Garavaglia *et al.*, 2012). As a result, the increased level of carbohydrates and their subsequent incorporation into extracellular polysaccharides may be essential for KP-1 cells to defend against SDS and other surfactants. Lastly, genes involved in the biosynthesis of extracellular polysaccharide (KP1_5310) and lipoprotein (*ybjP*) were induced in SDS treated KP-1 mono species biofilms, further suggesting that the extracellular matrix of SDS treated KP-1 mono species biofilms was enhanced.

Although various genes were determined to be differentially expressed during SDS treatment, the changes in expression could also be indirect consequences of SDS treatment. In metatranscriptomic analysis, some biases are also introduced during sequencing library preparation, cluster amplification and sequencing (Aird *et al.*, 2011). As a result, the expression and function of these genes have to be verified by quantitative reverse transcriptase PCR and site-directed mutagenesis respectively. The comparison between the transcriptomes of the mono species biofilms and the transcriptomes of the mixed species biofilms will be essential to better understand how different species in the mixed species biofilms interact. Preliminary data indicated that transcripts from all three species were present. However, the analyses are hampered by the complexity of the samples. First, 38.1% of the transcripts could be mapped to more than one species, probably due to the presence of highly conserved genes in all three species, such as those involved in central metabolism, transcription and translation. Hence, further computational analysis is required to differentiate these transcripts. Second, the low abundance of PAO1 (less than 1% of the total 16S rRNA, Table C-2) in the mixed species biofilms decreases the sensitivity and detection limit for PAO1 transcripts. More sequencing runs, at a deeper depth of coverage, may be required for the complete

interpretation of the changes in mRNA expression for PAO1. Therefore, due to time constraints, the results from the mixed species biofilms are not presented here. However, the analyses of the mono species biofilms serve as an essential platform for the comparison between community and population based biofilm stress response.

To conclude, the resilience of PAO1 and KP-1 mono species biofilms when exposed to SDS was strongly linked to the increased expression of genes involved in the production and export of EPS. In addition, stress response proteins encoded by *siaA* in PAO1 as well as *cpxP* and *degP* in KP-1, may also play important roles in defending PAO1 and KP-1 cells from lysis by SDS. Pf-5 mono species biofilms, which showed neither up regulation of genes involved in EPS production and export nor relevant stress response proteins, were most susceptible to SDS treatment and showed a much reduced response to SDS exposure than the more resistant PAO1 and KP-1 mono species biofilms (Chapter 5). While it will be rewarding to explore the mixed species transcriptomes to address mixed consortia stress resistance, the data presented here have begun to suggest mechanisms that are used individually by PAO1 and KP-1. These defense mechanisms may ultimately be involved in the mixed community protection.

CHAPTER 8 . CONCLUSIONS

Bacteria predominantly grow as biofilms in their natural habitats and these biofilms are typically comprised of multiple species. Interspecies interactions between different organisms in a mixed species biofilm affect their behaviours, and therefore the development, structure and function of the biofilm community (Elias and Banin, 2012; James *et al.*, 1995; Moons *et al.*, 2009; Rendueles and Ghigo, 2012). More importantly, such interactions and their impacts on the biofilm cannot be predicted from investigating mono species biofilms. Therefore, the studies of interspecies interactions and their impacts on mixed species biofilms become vital for the manipulation of biofilms in all natural settings. In this study, state of the art confocal laser scanning microscopy, image analysis and next-generation sequencing were employed to study a mixed species biofilm model consisting of *Pseudomonas aeruginosa* PAO1, *Pseudomonas protegens* Pf-5 and *Klebsiella pneumoniae* KP-1. These three bacterial species have been shown to coexist in metalworking fluid and the gut of the silkmoth, *Bombyx mori* (Anand *et al.*, 2010; Chazal, 1995). In addition, this mixed species biofilm was shown to be reproducible with respect to specific number of viable cells, structure (Jackson *et al.*, 2001) and biovolume per unit base area (Figure 5-7), and is therefore a good mixed species biofilm model to investigate:

a) The impacts that interspecies interactions have on the development and resilience of a mixed species biofilm;
b) The effects that interspecies diversity and interactions have on intraspecies diversity; and
c) The genes involved in driving biofilm fitness.

Results from Chapter 5 demonstrated that interspecies interactions can affect the development and resilience of the mixed species biofilms significantly. Development of the mixed species biofilms was delayed relative to all comparable mono species biofilms (Figure 5-4). In addition, the biofilm structure of each species in the mixed species biofilms also differed from the structures they exhibited when grown alone (Figure 5-5 and Figure C-1). Moreover, the proportion of each species in the mixed species biofilms

also changed with varying glucose concentrations along the channel of the flow cell (Figure 5-8), suggesting that interactions between these species vary at different nutrient levels, and result in biofilms with distinct structures and compositions. In addition, results from Chapter 5 also showed that *K. pneumoniae* dominated the mixed species biofilm, but the two Pseudomonads were never eliminated from the biofilm. Previous studies have attributed failure of the faster growing *K. pneumoniae* to outcompete *P. aeruginosa* to a slower diffusion of glucose into *K. pneumoniae* microcolonies and faster detachment of *K. pneumoniae* cells from the surfaces of the biofilms (Siebel and Characklis, 1991; Stewart *et al.*, 1997). However, it was shown in this study that the growth of KP-1 was slower than the growth of PAO1 and Pf-5 at lower glucose concentrations (Figure C-3). The slower growth of KP-1 may in part explain the decreased proportion of KP-1 within a mixed species biofilm growing at the outlet end of the flow cell, where the glucose concentration had decreased by 2.5 to 8 fold (Figure C-4) and was in the range associated with slower growth of KP-1 relative to PAO1 and Pf-5 (Figure C-3). These results support the concept that the relative proportions of the bacteria in a mixed species biofilm community are in part a consequence of resource competition.

In addition to changes in biofilm development and structure, the mixed species biofilms were also observed to be more resistant to tobramycin and SDS (Figure 5-9 and Figure 5-10). Although earlier studies by Cowan *et al.* (2000) have demonstrated that insensitive members could surround and physically shield sensitive members in a mixed species biofilm from antimicrobial compounds, the increased community level resistance observed in this study was not achieved through such physical shielding. For example, the mixed species biofilms were resistant to tobramycin due to the presence of resistant Pf-5 (Figure 5-9). However, the relative abundance of Pf-5 was only 10-15% of the total biomass and was found to be randomly distributed in the mixed species biofilms (Figure 5-5) instead of surrounding the sensitive strains, KP-1 and PAO1, as proposed by Cowan *et al.* (2000). Nevertheless, members within the mixed species biofilms must be closely associated to provide the biofilm community with resilience to tobramycin and SDS (Figure 5-11). This was supported by the planktonic experiments where both PAO1 and KP-1 in the mixed planktonic cultures were eradicated by treatment with tobramycin

(Figure C-7), further suggesting that a structured environment and interspecies interactions are essential for the resilience of mixed species community to stress. The community level biofilm resistance was also not a result of selection for the resistant species as the proportion of each member was similar in the control and treated mixed species biofilms (Figure 5-9 and Figure 5-10). Instead, the community level resistance observed in this study is proposed to be achieved through the sharing of public goods such as enzymes and EPS. In fact, studies have shown that enzymes such as SDS hydrolase and catalase were responsible for protecting the *P. aeruginosa* biofilms from SDS and hydrogen peroxide respectively (Hagelueken *et al.*, 2006; Stewart *et al.*, 2000) while another study by Chiang *et al.* (2013) has demonstrated that eDNA could shield *P. aeruginosa* biofilms from aminoglycosides. However, metatranscriptomic analyses of SDS treated PAO1 mono species biofilms indicated that gene encoding SDS hydrolase was not induced. Hence, SDS hydrolase may not play a significant role in SDS stress protection under the conditions used in this study. Nevertheless, the metatranscriptomic analyses do support the proposition that the resilience of a mixed species biofilm to SDS is due to sharing of public goods such as the EPS and surface adhesins (discussed below). Furthermore, the community level resistance observed in this study was achieved regardless of the relative abundance of the resistant species in the mixed species biofilm. Thus, this is the first study to demonstrate that the community level resistance of a mixed species biofilm has no correlation to the abundance of the resistant species in the mixed species biofilm. As a result, it will be particularly exciting to compare the transcriptomes of SDS treated mono species and mixed species biofilms to determine the mechanism involved in this community level resistance to SDS.

Interspecies interactions not only influenced the development and resilience of a mixed species biofilm, but also had an impact on the formation of morphotypic variants, and thus intraspecies diversity. Earlier studies have shown that the formation of morphotypic variants increased the diversity of mono species biofilms and their resistance to antibiotics, hydrogen peroxide and predation (Boles *et al.*, 2004; Drenkard and Ausubel, 2002; Koh *et al.*, 2012). However, there are no reports on the effects that interspecies interactions can have on variant formation, although evolutionary and ecological theories have proposed

the possible relationships between interspecies and intraspecies diversity (Harper, 1977; Van Valen, 1965; Vellend and Geber, 2005). Here, results from Chapter 6 showed that an increase in species richness (interspecies diversity) within the mixed species biofilms decreased the frequency and diversity of morphotypic variants (intraspecies diversity) formed by each member (Figure 6-5). Notably, the decrease in frequency and diversity of the morphotypic variants formed in Pf-5 biofilms could also be brought about by the addition of cell free effluents obtained from KP-1 and PAO1 biofilms (Figure 6-6 and Figure 6-7). This indicates that extracellular molecules that are present in the effluent can regulate the formation of morphotypic variants by Pf-5. In fact, studies by Garbeva *et al.* (2011) have shown that bacteria can sense and recognize neighboring cells, but the mechanisms of such recognition and the signals involved were not identified. While PAO1 is known to produce and respond to AHL QS signal molecules, neither Pf-5 nor KP-1 were observed to produce AHLs in this study (data not shown). Nonetheless, it has been reported that some bacteria, such as *E. coli*, which do not produce AHLs can respond to these signals via orphan signal receptors (Sitnikov *et al.*, 1996) and it remains possible that Pf-5 similarly responds to AHLs produced by PAO1. Therefore, the identities of these extracellular molecules will be of utmost interest for unraveling the role of interspecies cell-cell signaling in the control of self-generated genetic diversity. Further investigation employing analytical tools such as high-performance liquid chromatography, mass spectrometry and add back experiments could be used to identify these extracellular molecules.

Collectively, the observations presented above have highlighted the effects of interspecies interactions on biofilm development, resilience and intraspecies diversity. However, little is known about the molecular mechanisms that drive these observations. Therefore, metatranscriptomic analyses were performed and the results in Chapter 7 have delivered some understanding on the genes that may be involved in SDS resistance. The resilience of PAO1 and KP-1 biofilms to SDS is probably mediated by increasing the production and export of EPS. The biofilm matrix or EPS, which consists mainly of eDNAs, lipids, proteins and polysaccharides, is the "glue" that keeps bacterial cells in a biofilm. It is also responsible for the mechanical strength of a biofilm and its defense against external

stresses e.g. antibiotics and surfactants (Arciola *et al.*, 2005; Flemming and Wingender, 2010). For instance, this study has shown that genes involved in the biosynthesis of lipopolysaccharides and stress response proteins were up regulated in SDS treated PAO1 and KP-1 mono species biofilms (Table 7-4 and Table 7-6). Lipopolysaccharides are negatively charged and can thereby repel negatively charged SDS. In addition, different stress response proteins can also protect PAO1 and KP-1 cells from lysis by SDS. For example, the up regulation of *siaA* in SDS treated PAO1 mono species biofilms would lead to the autoaggregation of PAO1 cells. Such autoaggregation has been reported to be a defense mechanism employed by PAO1 when exposed to SDS stress (Klebensberger *et al.*, 2009). In addition, Cpx envelope stress response proteins were induced in SDS treated KP-1 mono species biofilms, where Cpx envelope stress response proteins were shown to prevent cell lysis by maintaining the stability of the cell envelope (Danese and Silhavy, 1998). Intriguingly, the transcriptomes of SDS treated Pf-5 mono species biofilms indicated that fewer genes were observed to be differentially expressed relative to those observed for PAO1 and KP-1 mono species biofilms. Given that PAO1 and KP-1 mono species biofilms were resistant to SDS stress, perhaps the lack of response from Pf-5 is a reflection of its inability to resist SDS stress. The genes that were observed to be induced in PAO1 and KP-1 mono species biofilms would serve as a basis for the metatranscriptomic analyses of SDS treated mixed species biofilms, where the importance of these genes in the SDS response of mixed species biofilms will be determined. Such analyses are in progress and preliminary data suggest that the assignment of transcripts to specific function and species is challenging due to the presence of highly conserved genes among all the three species. This issue can be resolved by comparing the conserved genes to identify SNPs that are specific to each species, using genomes of the three species sequenced in this study.

The results from this study are consistent with others that have shown that mixed species biofilms are better adapted than mono species biofilms to survive in extreme environments with limited resources, antibiotics, xenobiotics, oxidative stresses as well as invasion by other bacterial species and higher organisms (Breugelmans *et al.*, 2008; Burmolle *et al.*, 2006; Christensen *et al.*, 2002). Moreover, this study has also

demonstrated that the increased resilience of a mixed species biofilm was likely to be due to the sharing of defenses among individuals in the community rather than selection for the more resistant species (Figure 5-9 and Figure 5-10). Combining these results together, it seems that conclusions made from mono species biofilm studies ought to be revisited as the effects that interspecies interactions have on various aspects of a mixed biofilm community are substantial, and should not be excluded in the quest to understand and control biofilms in natural, medical and industrial settings. As a result of the enhanced mixed species biofilm resistance, the effective concentration of an antibiotic that is derived from test performed on mono species biofilm, may not be effective against *in vivo* biofilms comprised of multiple species. For example, the biofilm inhibitory concentration (geometric mean) of tobramycin for mono species *P. aeruginosa* biofilms was reported to be 7.1 $\mu g\ mL^{-1}$ (Fernandez-Olmos *et al.*, 2012). However, the results from Chapter 5 have shown that 20 $\mu g\ mL^{-1}$ tobramycin could reduce the biovolume of *P. aeruginosa* biofilms by 33.6%, but did not significantly affect the mixed species biofilms comprised of *P. aeruginosa*, *P. protegens* and *K. pneumoniae* (Figure 5-9). Similarly, 0.1% w/v SDS was effective in eradicating 80% of the biomass of *C. albicans* K1 mono species biofilms (Nett *et al.*, 2008), but this concentration may not be effective when a resistant species such as *K. pneumoniae* is present in the biofilms (Figure 5-10).

Subsequently, the increase in interspecies diversity have also been demonstrated to reduce intraspecies diversity in biofilms. Genetic diversity at the intraspecies level and species diversity within a community are unlikely to function independently in a community (Vellend and Geber, 2005; Whitham *et al.*, 2006). As the new field of community genetics focuses on both the consequences of genetic diversity at the community level and the relationships between these two levels of biodiversity (Agrawal, 2003; Hersch-Green *et al.*, 2011; Rowntree *et al.*, 2011), it is clear that intraspecies genetic variation has profound and widespread effects on both community and population performances (Rowntree *et al.*, 2011; Whitham *et al.*, 2006). However, the relative importance of interspecies diversity versus intraspecies diversity is unclear. The results in Chapter 6 have suggested that interspecies diversity could be more important than intraspecies diversity in maintaining the performances of the mixed species biofilms.

CONCLUSIONS

Since the studies of ecological theories using higher organisms can be time consuming and laborious as higher organisms have long life spans and reproductive cycles, a bacterial community model that can be easily replicated with good reproducibility and the capacity for genetic manipulation may be more practical and powerful for testing ecological theories. In contrast to animals and plants used in traditional ecological experiments, bacteria have relatively short life cycles and are small in size so that they can be grown in large numbers in the laboratory. However, the use of microbial systems in ecological studies is limited in part due to the historical division between the field of microbiology and ecology (Jessup *et al.*, 2004), although there is an increasing effort to bridge the two fields. In fact, some recent studies have begun to test ecological theories on microbial systems. For example, Kerr *et al.* (2002) have tested the ecological theory of coexistence using a microbial system comprised of three competing *E. coli* strains and showed that coexistence could only be preserved in a structured environment (agar plate), but not in a non-structured environment (chemostat). Coincidentally, the results presented in this thesis on community level resilience was also achieved in a structured biofilm, but not in a non-structured planktonic culture. In addition, a microbial system comprised of bacteria, algae and protists has also been used to test and show that biodiversity is an insurance against the fluctuation in performance of an ecosystem (Naeem and Li, 1997). The results from Chapter 6 of this study have also shown that increased interspecies diversity could lead to a decrease in intraspecies diversity as proposed by Van Valen (1965). Certainly, there are still several issues to be resolved with regards to the use of microbial systems for ecological studies. For example, the blurred distinction between bacterial genotypes and species is a challenge for ecological studies that involve evolution (Vellend and Geber, 2005). In addition, certain ecological questions related to genetic drift and extinction require a small number of individuals, which can be monitored over time. Bacteria being small in size will be difficult to manipulate and track specifically in such experiments (Jessup *et al.*, 2004). Furthermore, studies on age and behavioural ecology may also be difficult as bacteria are clonal, unicellular and deficient in morphological differentiation (Adrews, 1991). However, as technologies such as microfluidics, image-based cell sorting and sequencing improve, it will ultimately be possible to perform single cell 'omics' to track mutation and changes in gene expression

over time. Therefore, some of the limitations mentioned for bacterial systems are likely to be overcome in the near future. As a result, based on the spatial and temporal benefits of using microbial systems, their ease of replication, along with the wealth of genetic information that is available through the explosion in bacterial genome sequencing, may make microbial models the systems of choice to test ecological theories in the future.

In summary, this study has demonstrated the importance of interspecies interactions in mixed species biofilms, where their development, structure and resilience were altered relative to mono species biofilm development. This may partly explain why natural biofilms do not respond in the same way as mono species biofilms of laboratory adapted strains. This may be expected since mono species biofilms also behave differently from planktonic cultures (Dotsch *et al.*, 2012; Mah and O'Toole, 2001; Sauer *et al.*, 2002). Hence, antibiotic treatments and cleaning regimes may need to be revisited with added knowledge from mixed species biofilm research. Further, interspecies interactions also have an impact on intraspecies diversity as these interactions decreased the frequency and diversity of morphotypic variants generated by all members within the mixed species biofilms. The reduction in individual morphotypic variant frequency and diversity in the presence of increased interspecies diversity is one of the few emperical experiments that provided evidence for the proposition that interspecies and intraspecies diversity is "substitutable". It is also clear that a mixed species biofilm can be useful, genetically tractable, experimental model to assess ecological theories to address how communities function.

APPENDIX A

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APPENDIX A

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APPENDIX A

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Figure A-1. Alignment of KP-1 16S rRNA sequence to the 16S rRNA sequence of NTUH-K2044. E-value = 0.0. Identities: 1366/1368 (99%). Gaps: 0/1368 (0%).

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APPENDIX A

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Query  1023     ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCC  1082
                |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  4801427  ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCC  4801486

Query  1083     GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA  1142
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4801487  GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA  4801546

Query  1143     TCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGA  1202
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4801547  TCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGA  4801606
```

APPENDIX A

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Query  1203     CCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACT  1262
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4801607  CCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACT  4801666

Query  1263     CGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTT  1322
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4801667  CGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTT  4801726

Query  1323     CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAA  1370
                ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4801727  CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAA  4801774
```

Figure A-2. Alignment of KP-1 16S rRNA sequence to the 16S rRNA sequence of MGH78578. E-value = 0.0. Identities: 1365/1368 (99%). Gaps: 0/1368 (0%).

Table A-1. List of tRNAs predicted for KP-1 genome

Contig no.	tRNA no.	Begin	End	tRNA type	Anti-codon
supercontig4	1	34969	35045	Val	GAC
supercontig4	2	35050	35126	Val	GAC
supercontig4	3	35131	35207	Val	GAC
supercontig4	4	238623	238707	Tyr	GTA
supercontig4	5	238923	239007	Tyr	GTA
supercontig5	1	139833	139908	Asn	GTT
supercontig5	2	145552	145627	Asn	GTT
supercontig5	3	156307	156382	Asn	GTT
supercontig5	4	370528	370604	Pro	GGG
supercontig5	5	487370	487444	Arg	CCT
supercontig5	6	510658	510733	Val	TAC
supercontig5	7	510780	510855	Val	TAC
supercontig5	8	510902	510977	Val	TAC
supercontig5	9	510982	511057	Lys	TTT
supercontig5	10	507959	507884	Ala	GGC
supercontig5	11	507842	507767	Ala	GGC
supercontig5	12	154549	154474	Asn	GTT
supercontig5	13	138841	138752	Ser	CGA
supercontig5	14	117646	117571	Gly	GCC
supercontig5	15	117530	117457	Cys	GCA
supercontig5	16	117443	117357	Leu	TAA

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supercontig7	1	250548	250624	Met	CAT
supercontig7	2	250658	250734	Met	CAT
supercontig7	3	250767	250843	Met	CAT
supercontig7	4	389236	389163	Gly	CCC
supercontig7	5	100281	100189	Ser	GCT
supercontig7	6	100184	100108	Arg	ACG
supercontig7	7	99957	99881	Arg	ACG
supercontig7	8	99819	99743	Arg	ACG
supercontig7	9	99514	99438	Arg	ACG
supercontig8	1	47179	47254	Phe	GAA
supercontig8	2	119958	120033	Met	CAT
supercontig9	1	121184	121109	Thr	GGT
supercontig9	2	36765	36679	Leu	GAG
supercontig9	3	32748	32672	Met	CAT
supercontig11	1	342119	342213	SeC	TCA
supercontig11	2	204205	204129	Pro	CGG
supercontig14	1	52	128	Asp	GTC
supercontig14	2	182	257	Trp	CCA
supercontig14	3	38290	38366	Arg	CCG
supercontig14	4	38421	38496	His	GTG
supercontig14	5	38520	38606	Leu	CAG
supercontig14	6	38649	38725	Pro	TGG
supercontig18	1	54455	54530	Gly	GCC
supercontig18	2	54599	54674	Gly	GCC
supercontig18	3	54747	54822	Gly	GCC
supercontig18	4	193299	193383	Leu	CAA
supercontig18	5	12417	12342	Phe	GAA
supercontig19	1	95500	95414	Leu	CAG
supercontig19	2	95385	95299	Leu	CAG
supercontig19	3	95267	95181	Leu	CAG
supercontig21	1	205	281	Asp	GTC
supercontig21	2	9652	9728	Asp	GTC
supercontig21	3	276756	276832	Arg	TCT
supercontig21	4	291368	291443	Thr	TGT
supercontig21	5	624364	624439	Lys	TTT
supercontig21	6	624565	624640	Val	TAC
supercontig21	7	624643	624718	Lys	TTT

APPENDIX A

supercontig21	8	624771	624846	Val	TAC
supercontig21	9	624850	624925	Lys	TTT
supercontig21	10	565743	565667	Met	CAT
supercontig21	11	565658	565574	Leu	TAG
supercontig21	12	565550	565476	Gln	TTG
supercontig21	13	565439	565365	Gln	TTG
supercontig21	14	565341	565265	Met	CAT
supercontig21	15	565217	565143	Gln	CTG
supercontig21	16	565067	564993	Gln	CTG
supercontig21	17	61742	61667	Thr	CGT
supercontig22	1	102	177	Lys	TTT
supercontig22	2	311	386	Lys	TTT
supercontig22	3	297861	297948	Ser	TGA
supercontig22	4	354175	354088	Ser	GGA
supercontig22	5	350754	350667	Ser	GGA

APPENDIX B

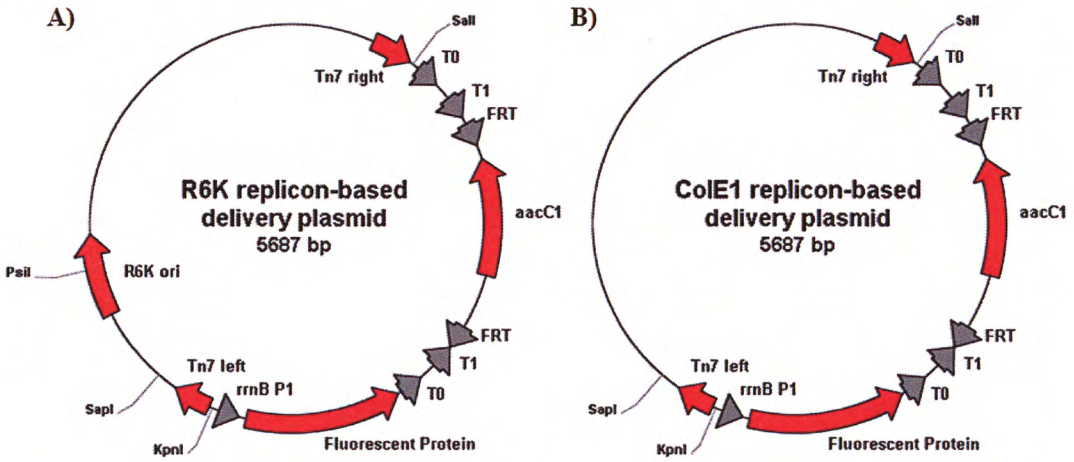

Figure B-1. Delivery plasmid maps. A) Plasmid map of R6K replicon-based delivery plasmids. The R6K *ori* yields a 580 bp fragment when digested with *Psi*I and *Sap*I. B) Plasmid map of ColE1 replicon-based delivery plasmid. The absence of the R6K *ori* would result in linearization of the plasmid when digested with *Psi*I and *Sap*I.

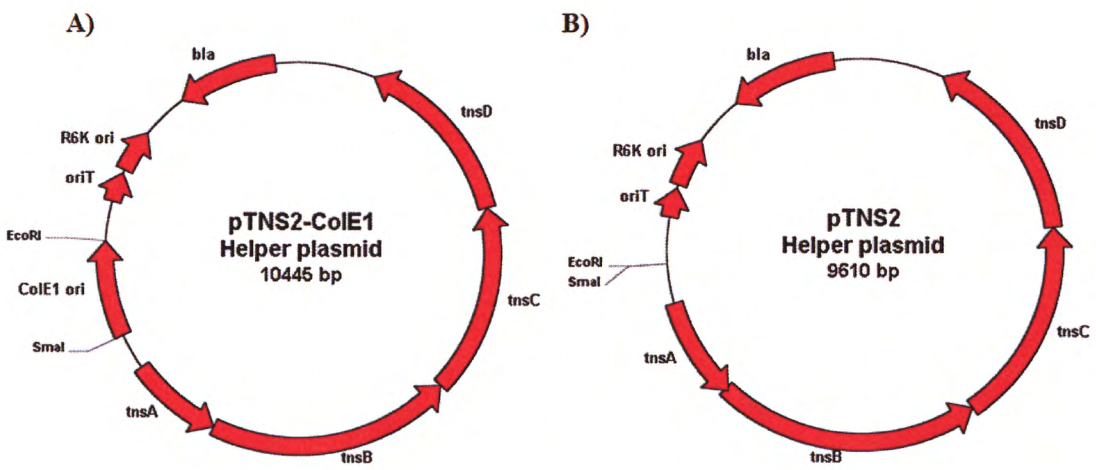

Figure B-2. Helper plasmid maps. A) Plasmid map of pTNS2-ColE1. The ColE1 *ori* is an approximately 840 bp fragment when digested with *EcoR*I and *Sma*I. B) Plasmid map of pTNS2. Digestion of pTNS2 with *EcoR*I and *Sma*I would result in a very small fragment of approximately 13 bp.

APPENDIX B

A)

5'AGTTTAGATCTATTTTGTTCAGTTTAAGACTTTATTGTCCGCCCACAGGCTTC
TCGGGCACGCGGCCGGCGTTACTCGACGGTGACCGACTTGGCCAGGTTGCGC
GGCTGGTCGACGTCGGTGCCCTTGAGCACGGCGACATGGTACGAGAGCAATT
GCAACGGGATGGTGTAGAGGATCGGCGAGAGCACG3'

B)

5'AGTTTAGATCTATTTTGTTCAGTTTAAGACTTTATTGTCCGCCCACACACCTG
GGCGGTCACTGTGGATAACTCACTCCACGGTCACCGACTTGGCGAGGTTGCGC
GGCTGGTCGACGTCGGTGCCCTTGAGCACGGCCACGTAGTAGGACAGCAGCT
GCAGCGGGATGGTGTAGAGGATCGGCGAGAGGAT3'

C)

5'TTTAGATCTATTTTGTTCAGTTTAAGACTTTATTGTCCGCCCACAGCAGCCCT
TTGCAGGATAAGATGCTTTACTCAACCGTTACCGATTTCGCCAGGTTACGCGG
CTGGTCAACGTCAGTGCCTTTGATCAGCGCGACGTGATAAGCCAACAGCTGCA
GCGGAACCGTATAGAAGATAGGGGCGATGGTCTCTTCCACATGCGGCATCTC
AATGATGTGCATGTTATCGCTACCGTTGAAGCCGGCTTCACCGTCGGCGAAGA
CGTATAGTTCACCGCCGCGGGCACGAACCTCTTCGATGTTGGATTTCAGTTTTT
CCAGCAGTTC3'

Legend:
TCA: coding region of *glmS* gene
AAC: intergenic region
ACA: right end of Tn7

Figure B-3. Sequences of PCR products analyzed for the exact site of Tn7 insertion. Sequences of PCR products amplified for A) tagged PAO1; B) tagged Pf-5 and C) tagged KP-1. Legend shows the corresponding components for different segments of the sequences.

APPENDIX B

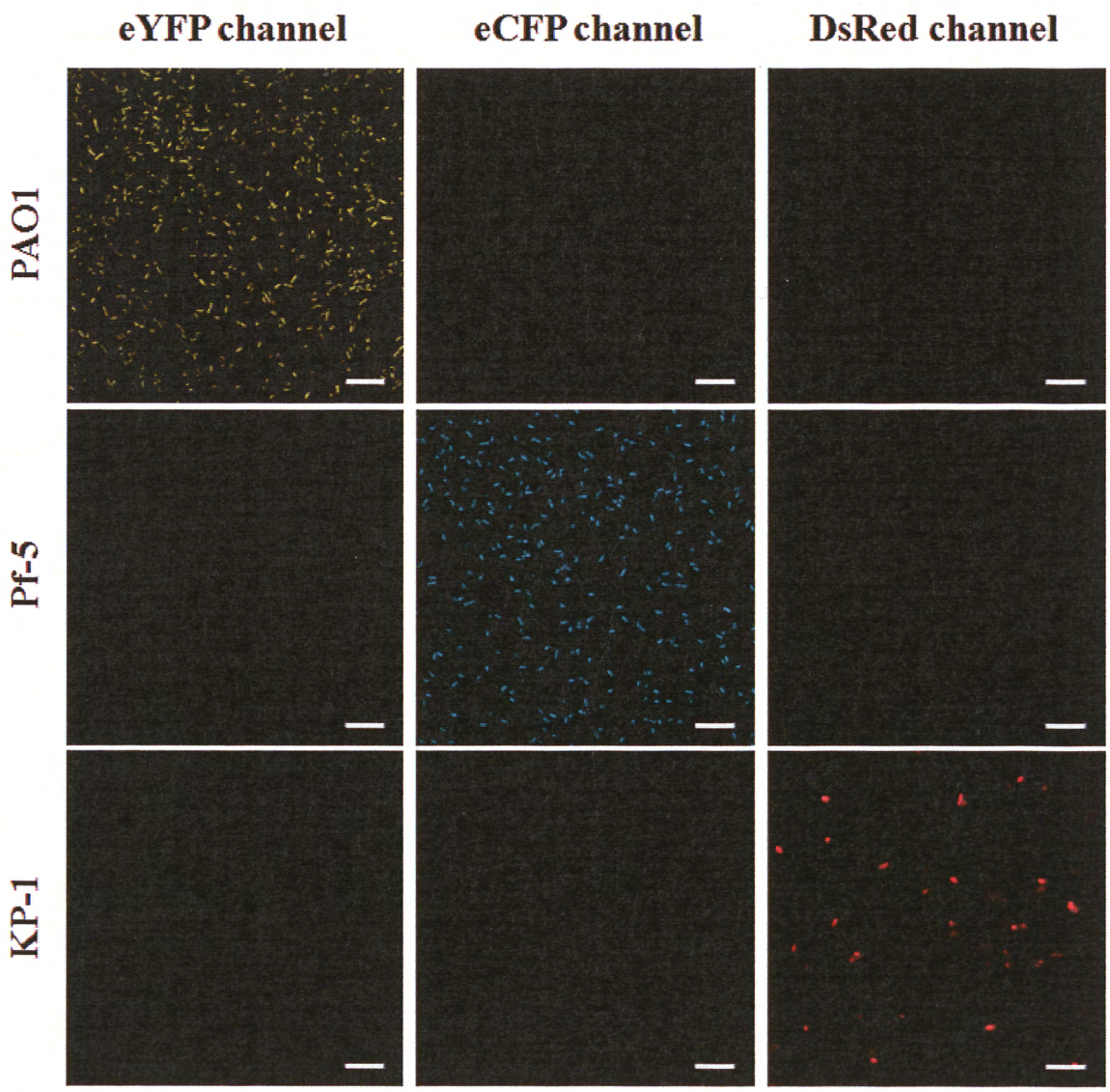

Figure B-4. Absence of fluorescent bleed-through. Confocal images of PAO1, Pf-5 and KP-1 taken with the 3 different fluorescent channels. Magnification: 100 x. Scale bar: 10 µm.

APPENDIX B

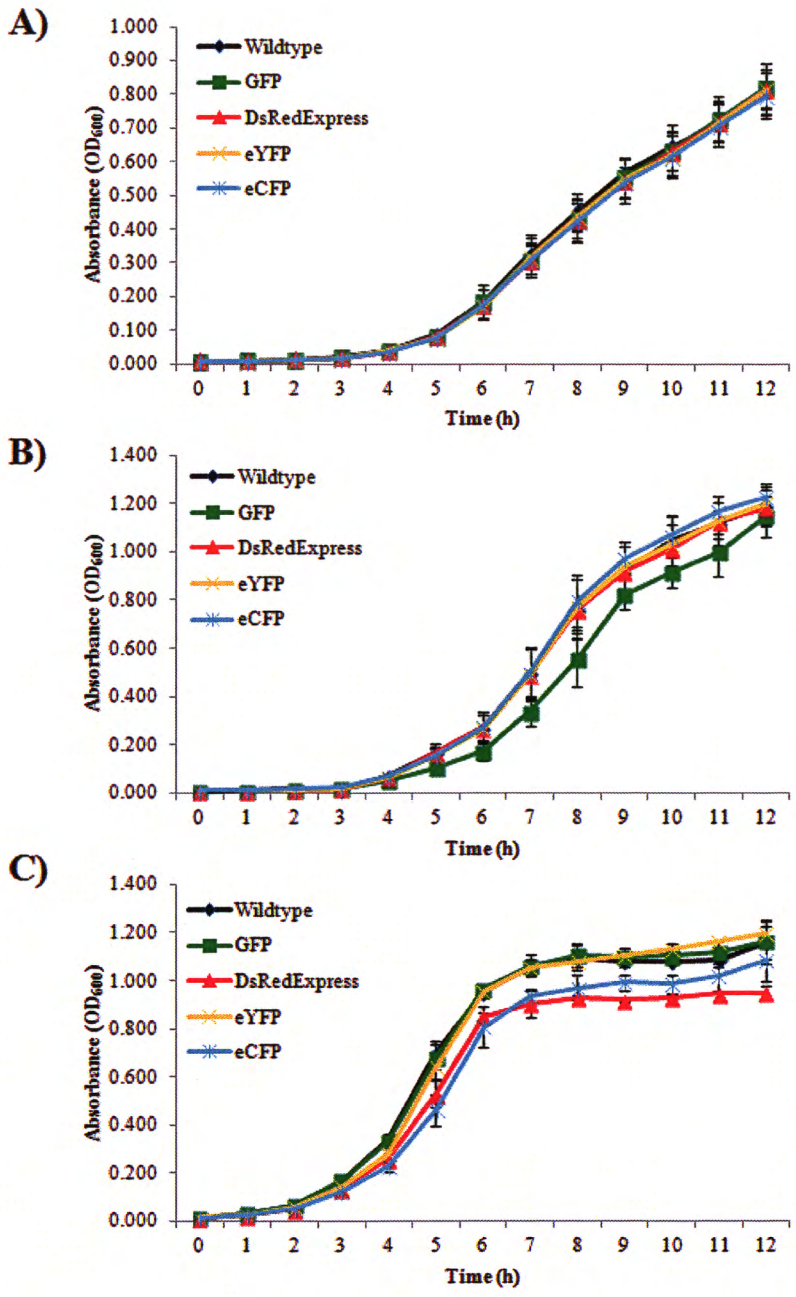

Figure B-5. Growth profiles of tagged and untagged bacteria. A) Optical density measured over a period of 12 h for tagged and untagged A) PAO1, B) Pf-5 and C) KP-1. Error bars represent standard deviations (n = 3).

APPENDIX B

attn7 recognition sequence	CAACCGTAACCGATTTTGCCAGGTTACG
KP-1 sequence	CAACCGTTACCGATTTCGCCAGGTTACG

Figure B-6. BLASTN analysis between the sequences of the PCR product amplified from tagged KP-1 and the *attn7* recognition site from Waddell and Craig (1989). Two mismatches (highlighted in yellow) were identified.

APPENDIX C

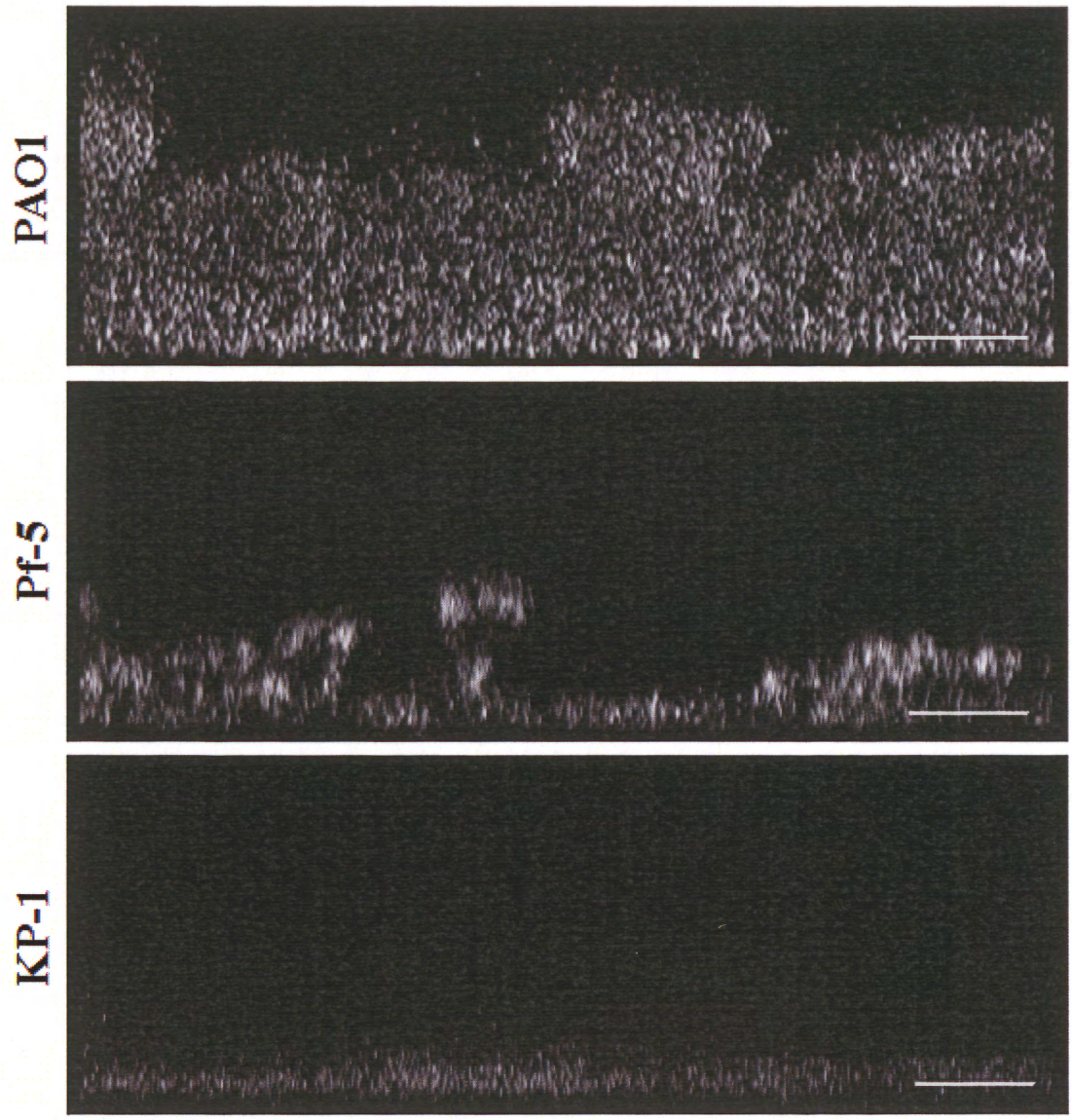

Figure C-1. Structures observed for mature biofilms. Mushroom-like microcolony was observed for mature PAO1 biofilm. Tower-like microcolony was observed for mature Pf-5 biofilm. Mature KP-1 biofilm was flat with no distinct structure. Scale bar: 50 µm.

APPENDIX C

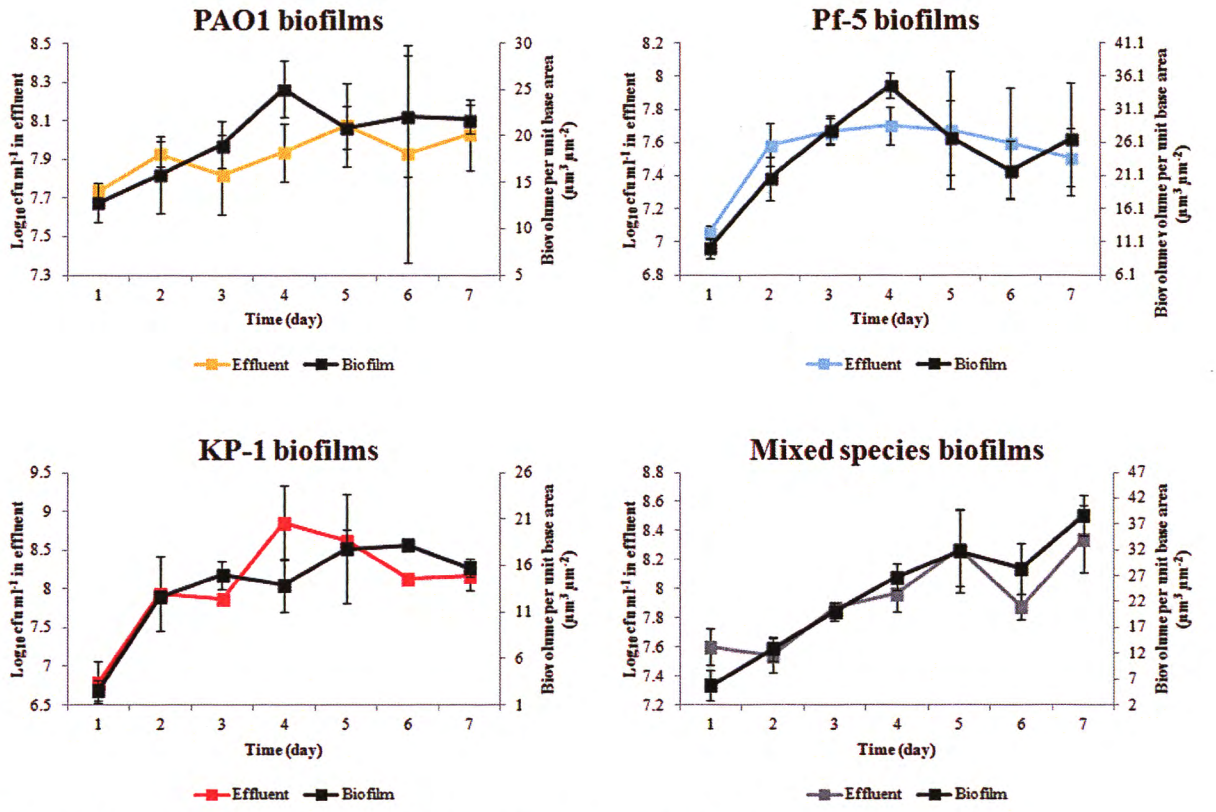

Figure C-2. Comparisons between colony forming units in the effluents and biovolume per unit base area. Colony forming units in the effluents of mono and mixed species biofilms were compared to their respective biovolume per unit base area. Error bars represent standard deviations (n = 3).

APPENDIX C

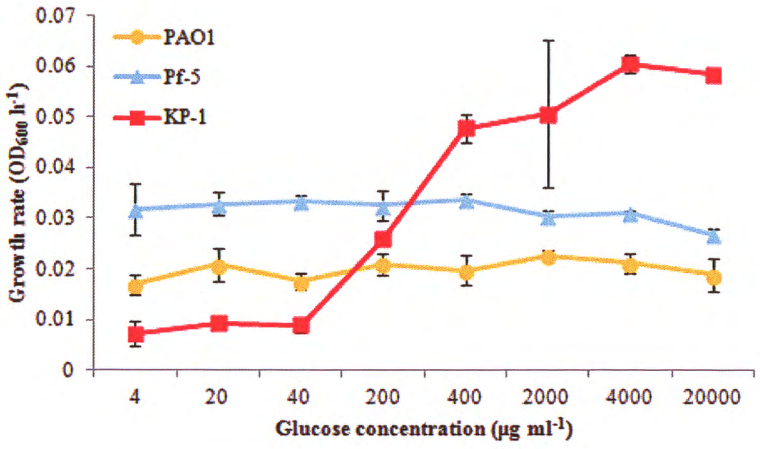

Figure C-3. Growth rates at varying glucose concentrations. Growth rates of PAO1, Pf-5 and KP-1 at varying glucose concentrations. Error bars represent standard deviations (n = 3).

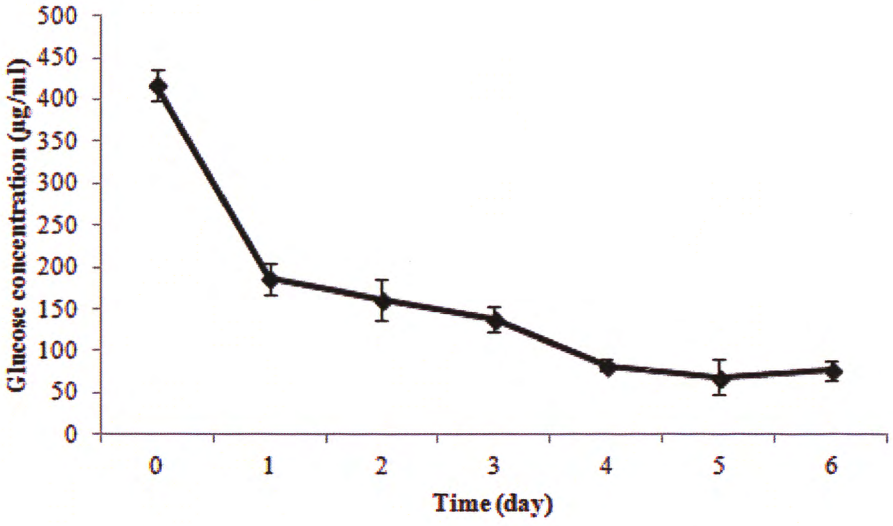

Figure C-4. Glucose concentration at the outlet of a flow cell. Glucose concentration at the outlet of a flow cells decreased over 7 d of biofilm growth. Error bars represent standard deviations (n = 3).

APPENDIX C

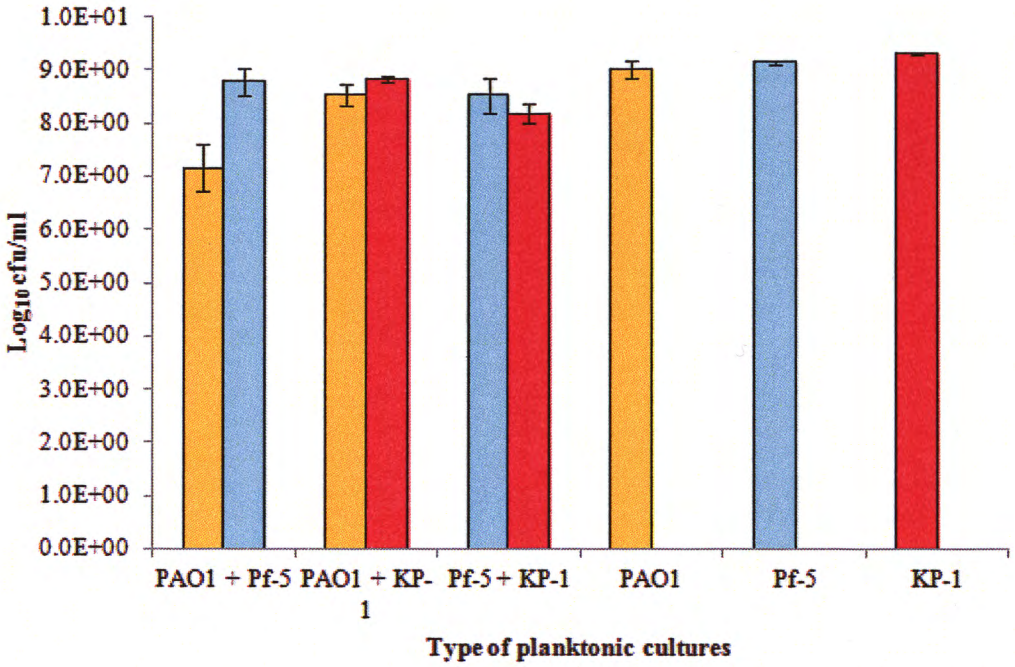

Figure C-5. Mono and dual species planktonic cultures. Colony forming units of each species in mono and dual species planktonic cultures. Error bars represent standard deviations (n = 2).

APPENDIX C

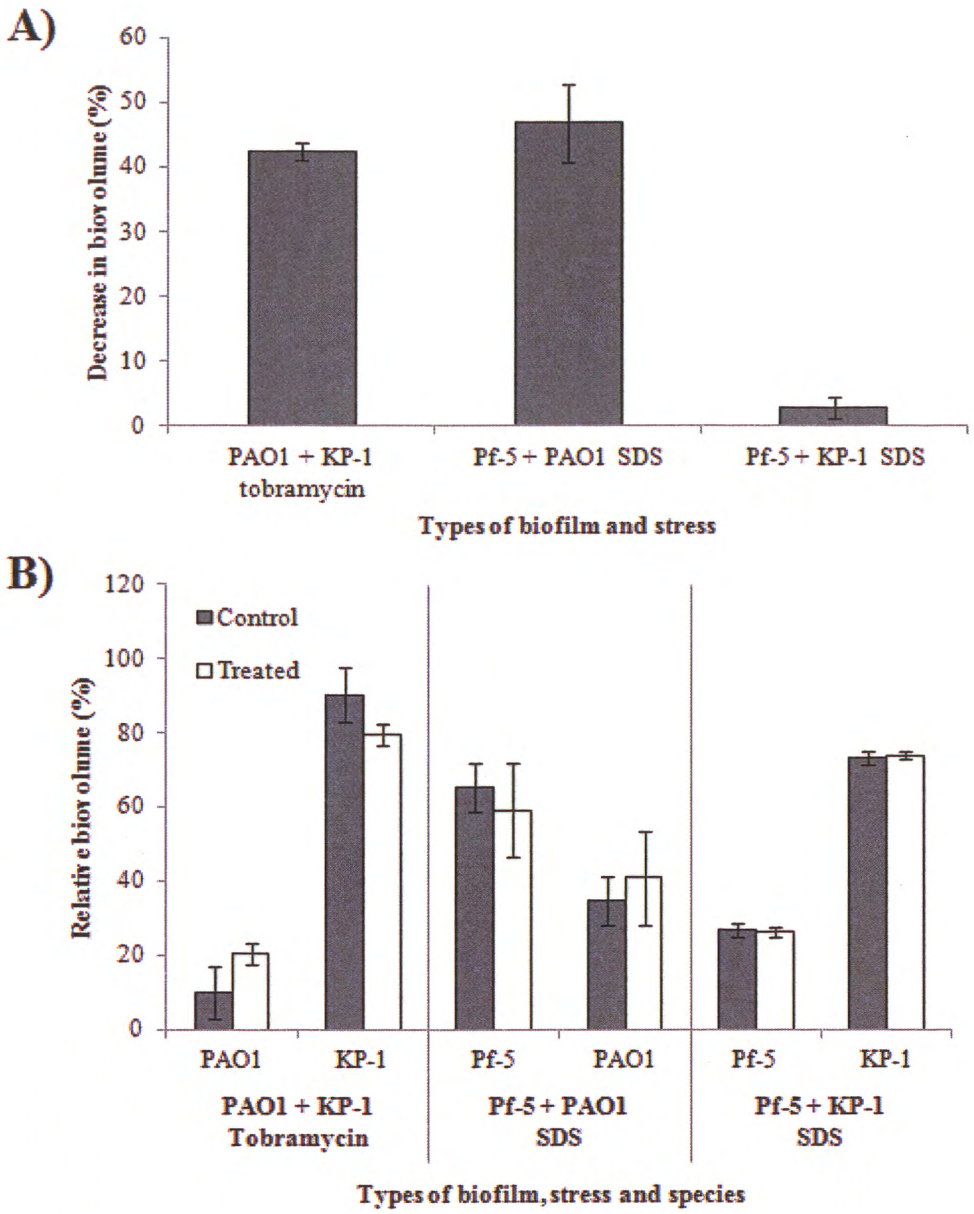

Figure C-6. Dual species biofilms treated with tobramycin and SDS. A) Percentage decrease in biovolume for dual species biofilms after treatment with either 10 μg mL^{-1} tobramycin or 0.1% w/v SDS. Error bars represent standard deviations (n = 3). B) Relative biovolume of each member within the control and treated dual species biofilms. Error bars represent standard deviations (n = 3).

APPENDIX C

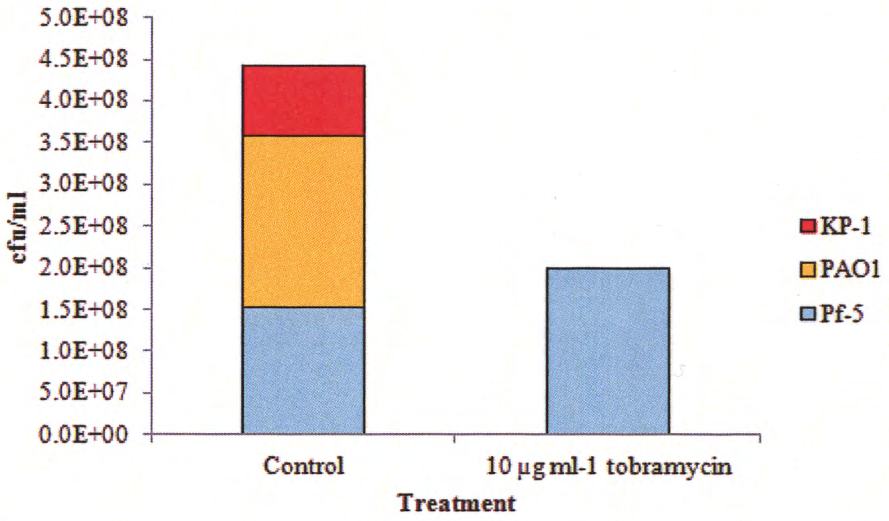

Figure C-7. Tobramycin treated mixed planktonic cultures. Colony forming units per milliliter (cfu mL^{-1}) for control and 10 µg mL^{-1} tobramycin treated mixed planktonic cultures.

Table C-1. List of species-specific tag sequence

Species	16S rRNA region	
	V4	**V6**
KP-1	TCTGTCAAGTCGGATGTGAAATCCCCGGGCTCA TCTGTCAAGTCGGATGTGAAATCCCCGGGCTTA	GTCTCACAGTTCCCGAAGGCACCAATCCATCTC GTCTCACAGTTCCCGAAGGCACCAAAGCATCTC
PAO1	TTCAGCAAGTTGGATGTGAAATCCCCGGGCTCA	GTGTCTGAGTTCCCGAAGGCACCAATCCATCTC
Pf-5	TTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCA	GTCTCAATGTTCCCGAAGGCACCAATCTATCTC

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Table C-2. Percentage of KP-1, PAO1 and Pf-5 in the mixed species biofilms

	Species		
Age of biofilm	**KP-1**	**PAO1**	**Pf-5**
Day 4/Replicate 1	95.7237	0.0890	4.1873
Day 4/Replicate 2	95.1642	0.1524	4.6834
Day 4/Replicate 3	93.6699	0.0491	6.2810
Day 5/Replicate 1	89.9652	0.0282	10.0066
Day 5/Replicate 2	92.4255	0.1568	7.4177
Day 5/Replicate 3	87.7307	0.0506	12.2188
Day 6/Replicate 1	79.7816	0.0571	20.1613
Day 6/Replicate 2	92.6467	0.1170	7.2363
Day 6/Replicate 3	88.4488	0.0364	11.5147

Table C-3. One-way ANOVA comparisons between the percentages of KP-1, PAO1 and Pf-5 obtained from image analysis by IMARIS and 16S rRNA sequencing

Comparison between	**Degrees of freedom**	***P*-value**
Day 4 mixed species biofilm		
KP-1	1, 4	0.076
PAO1	1, 4	0.174
Pf-5	1, 4	0.069
Day 5 mixed species biofilm		
KP-1	1, 4	0.186
PAO1	1, 4	0.171
Pf-5	1, 4	0.201
Day 6 mixed species biofilm		
KP-1	1, 4	0.705
PAO1	1, 4	0.027
Pf-5	1, 4	0.785

APPENDIX D

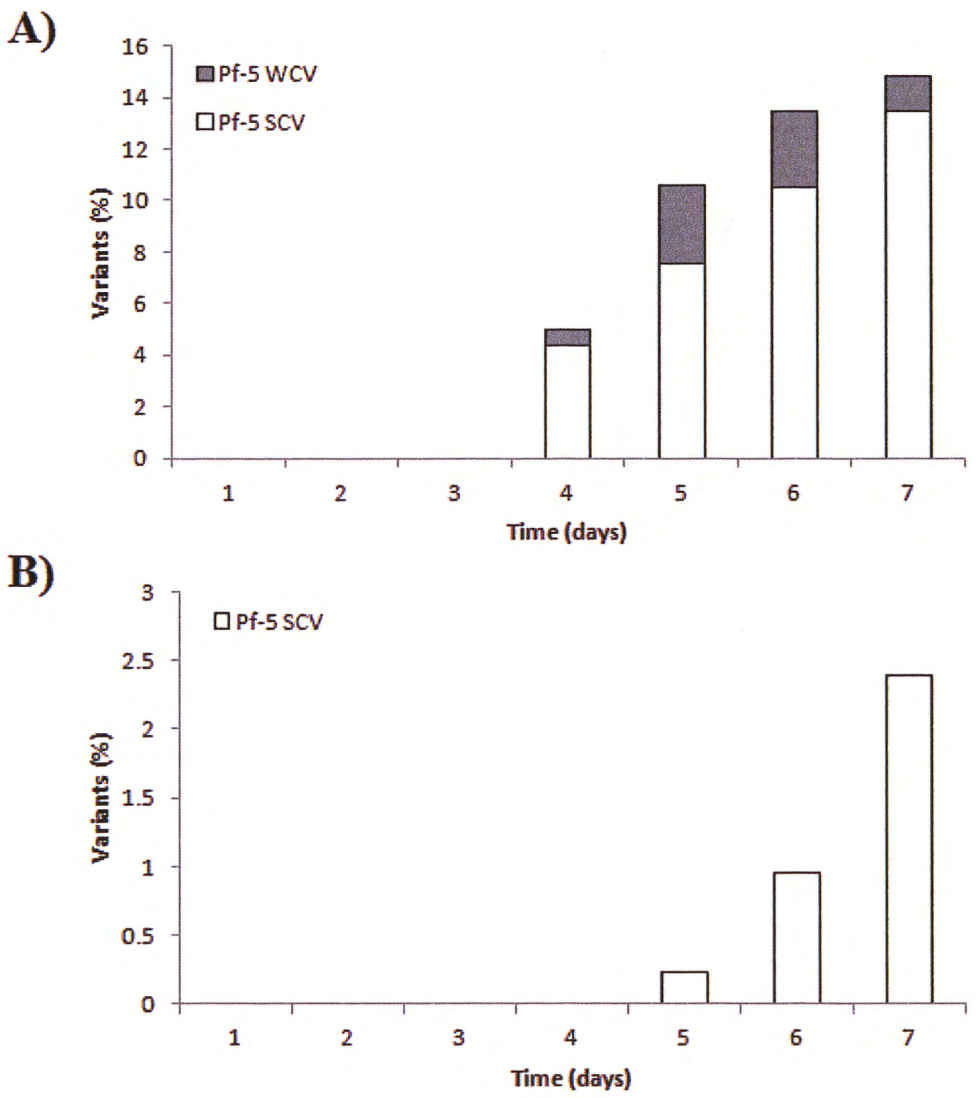

Figure D-1. Biofilm development and variant formation by Pf-5 RCV and Pf-5 WCV. A) Frequencies and proportions of Pf-5 WCV and Pf-5 SCV in the effluent of Pf-5 RCV biofilm. B) Frequency of Pf-5 SCV in the effluent of Pf-5 WCV biofilm.

APPENDIX D

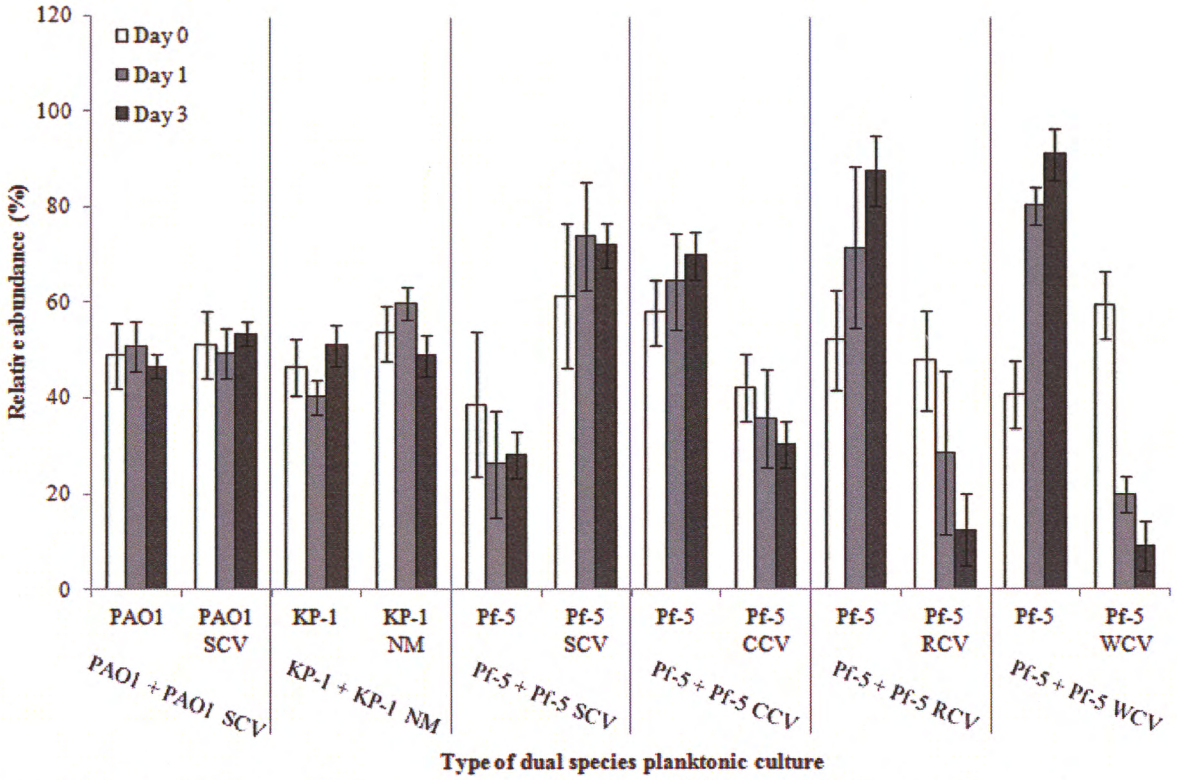

Figure D-2. Competitive fitness of morphotypic variants in planktonic cultures. A) Relative abundance (%) of morphotypic variants and their respective parent strains in planktonic co-culture. Error bars represent standard deviations (n = 3).

APPENDIX D

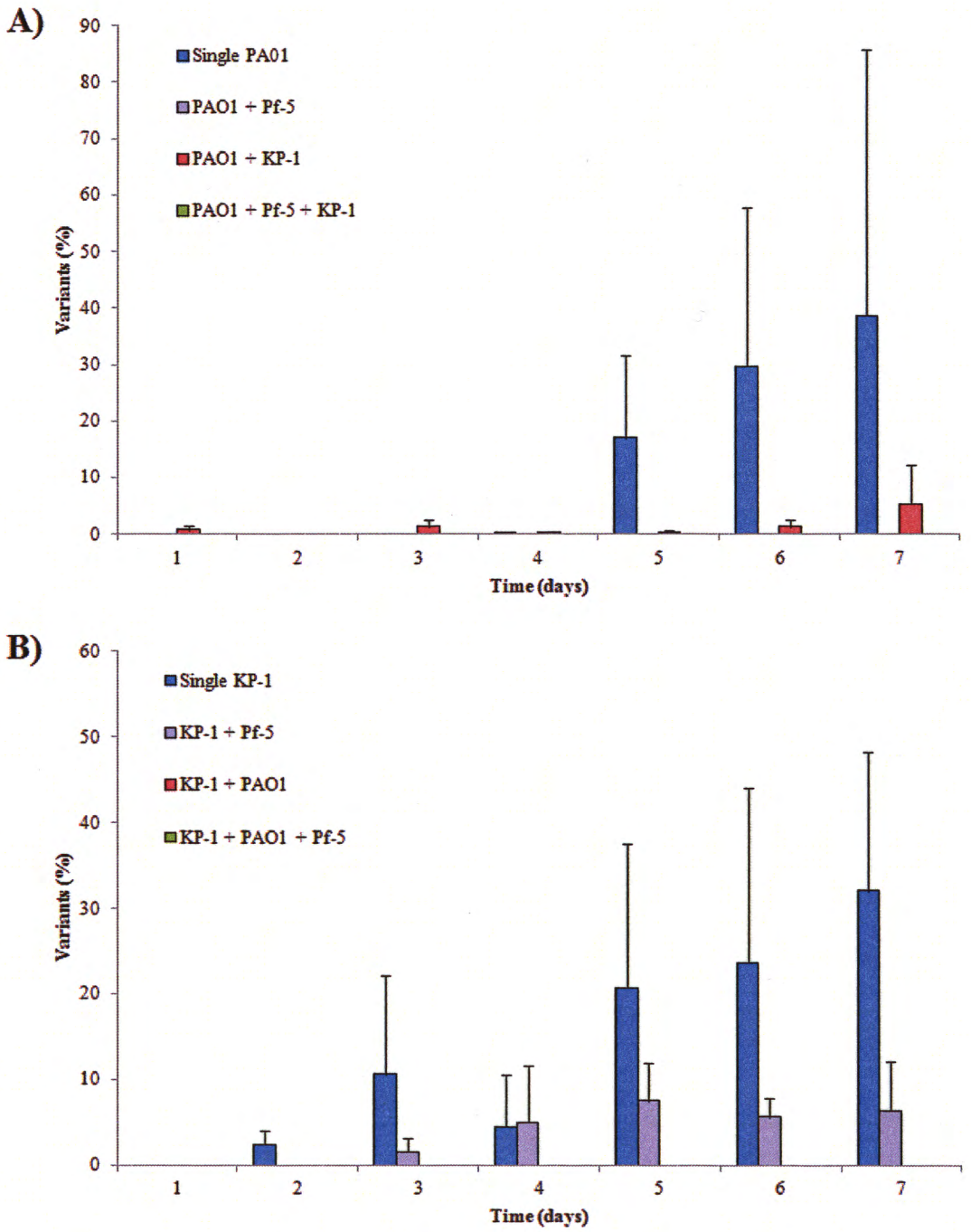

Figure D-3. Frequencies of PAO1 SCV and KP-1 NM. A) Frequencies of PAO1 SCV in the effluents of mono, dual and triple species biofilms. Error bars represent standard deviations (n = 3). B) Frequencies of KP-1 NM in the effluents of mono, dual and triple species biofilms. Error bars represent standard deviations (n = 3).

APPENDIX D

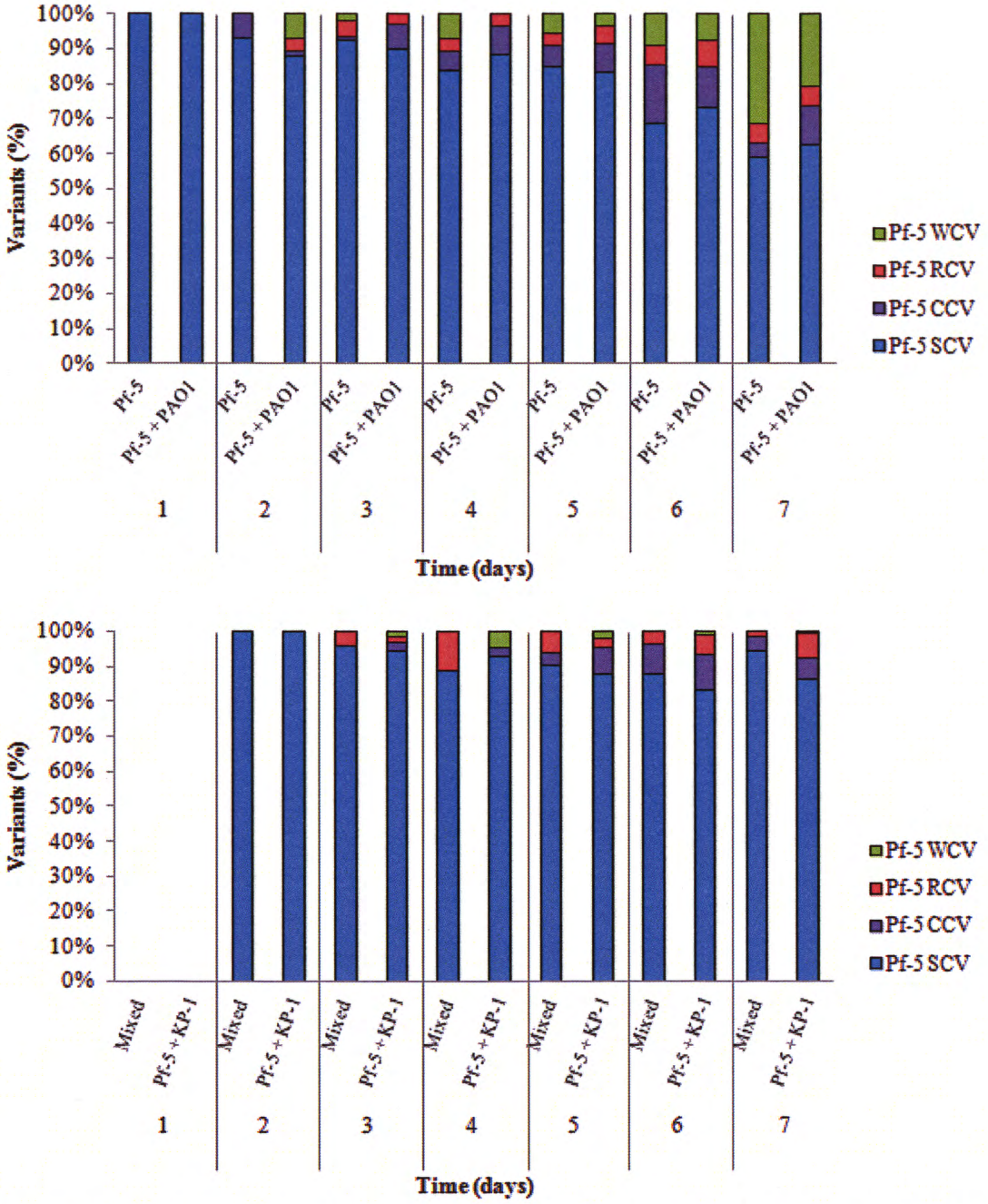

Figure D-4. The diversity and frequency of Pf-5 morphotypic variants in mono, dual and triple species biofilms. The diversity and frequency of Pf-5 morphotypic variants in Pf-5 as well as Pf-5 and PAO1 dual species biofilms were similar.

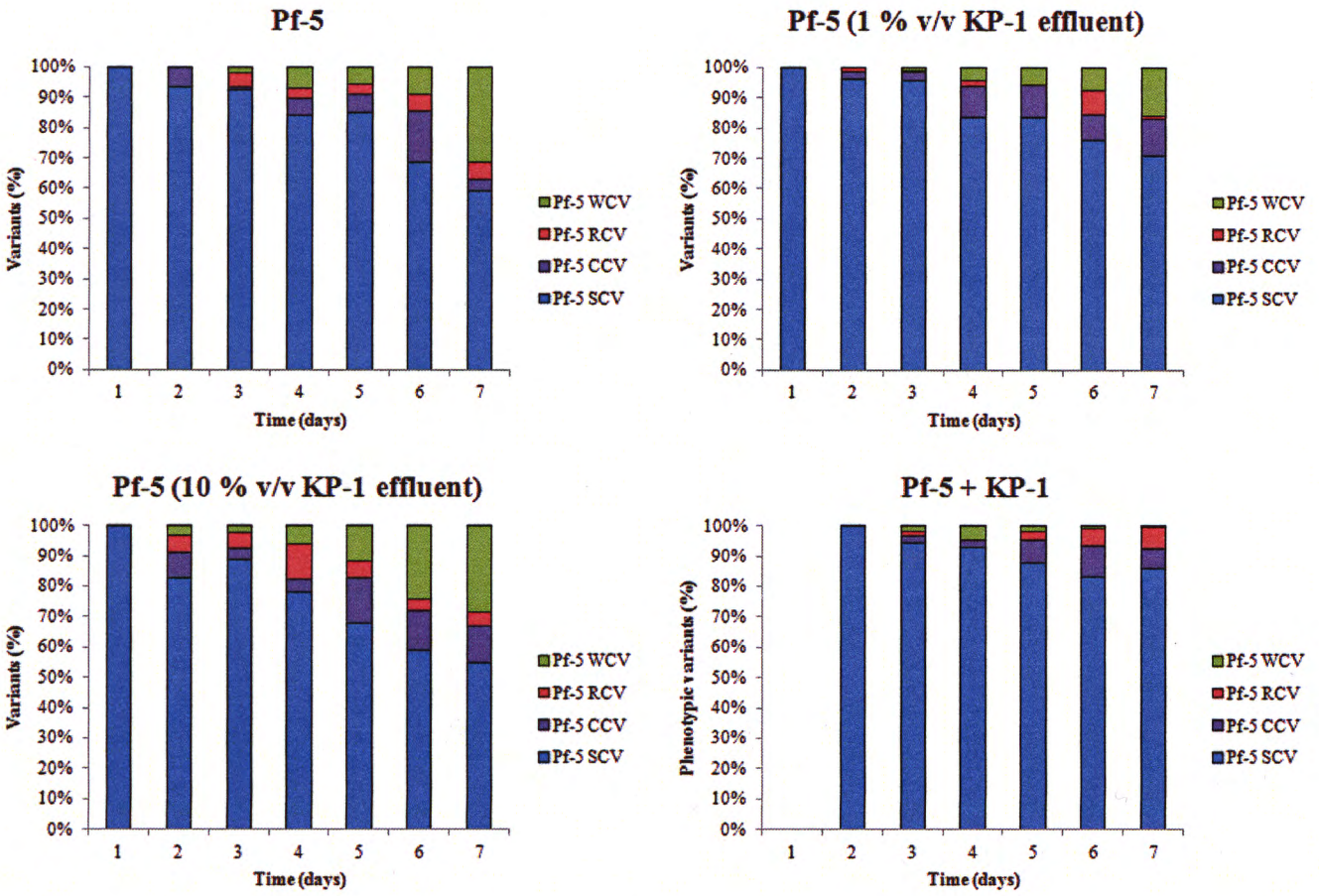

Figure D-5. Effluent of KP-1 biofilm has minimal effect on the diversity of Pf-5 morphotypic variants. The morphotypic variants of Pf-5 from Pf-5 mono species biofilms grown with and without KP-1 biofilm effluent had similar diversity, but were different from the diversity of Pf-5 morphotypic variants formed for Pf-5 and KP-1 dual species biofilms.

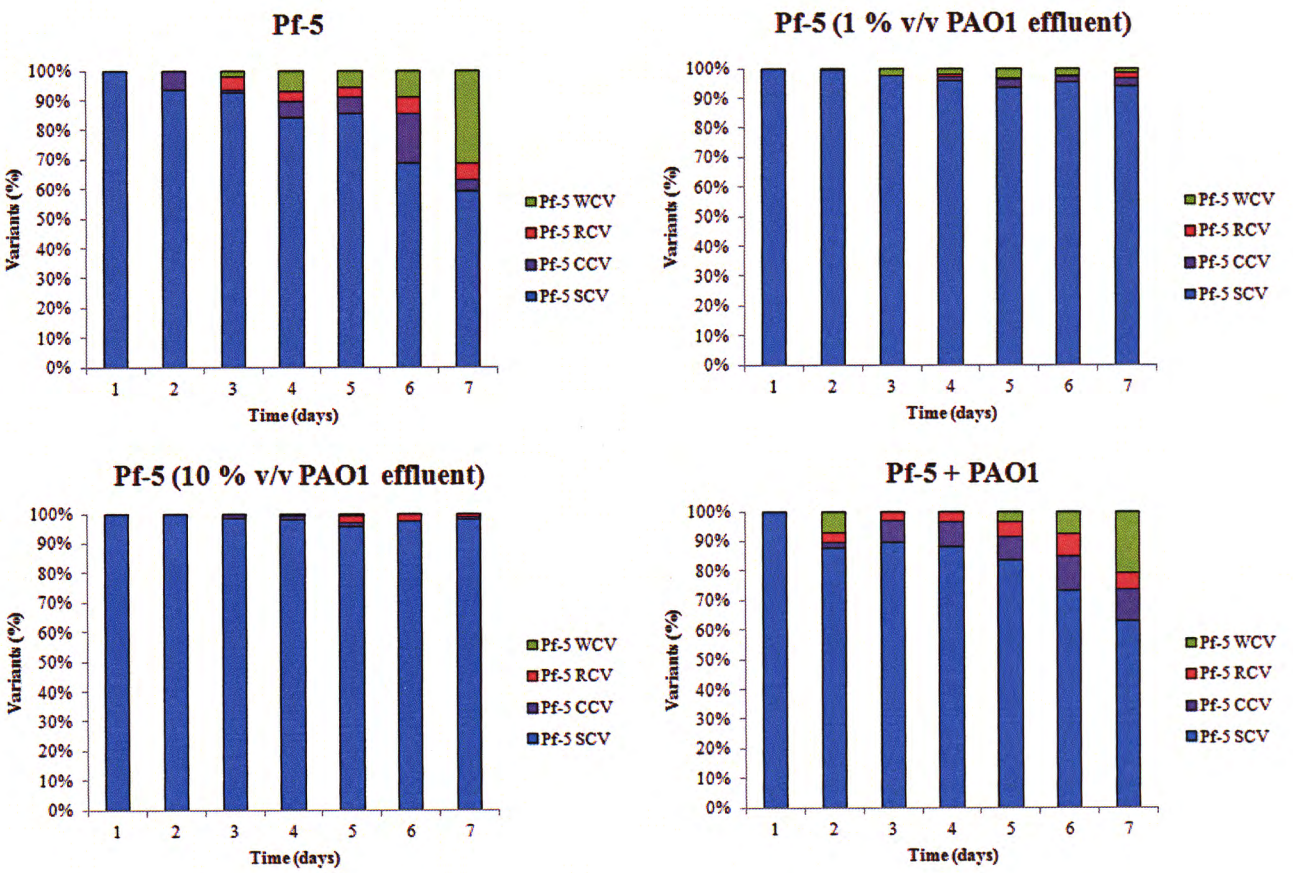

Figure D-6. Effluent of PAO1 biofilm decreased the diversity of Pf-5 morphotypic variants. The diversity of Pf-5 morphotypic variants from Pf-5 mono species biofilms grown with PAO1 biofilm effluent were lowered significantly. They were also different from the diversity of Pf-5 morphotypic variants formed for Pf-5 mono species biofilms grown without PAO1 biofilm effluent as well as Pf-5 and PAO1 dual species biofilms.

APPENDIX E

Table E-1. Alphabetical list of differentially expressed genes and fold change in SDS treated PAO1 biofilms

Gene name	[1,2] Log_2 fold change
ackA	-1.08
acnA	-1.58
acsA	2.30
aer2	-1.77
ahpF	1.37
amgS	1.20
amrZ	-1.11
arnA	1.49
arnB	1.49
arnC	1.49
arnD	1.49
arnE	1.49
arnF	1.49
arnT	1.49
bkdA1	-1.72
bkdA2	-1.72
cbiD	1.49
cbpA	-1.13
cdrA	2.06
chiC	-1.29
clpA	-1.23
cobL	1.49
coIII	-1.26
coxA	-1.26
coxB	-1.26
cpo	-1.62
cspD	-1.70
cttP	-1.08
cupE1	-1.97
cupE5	-1.40
cupE6	-1.40

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cyaB	1.15
dapA	1.12
fahA	2.24
fleR	1.02
flgB	1.11
flgC	1.27
flgD	1.51
flgE	1.19
flgF	1.24
flgI	1.58
fusA2	-1.08
gbdR	-1.18
gcvP1	-1.42
glcD	-1.78
glcE	-1.78
glcF	-2.10
glgB	-1.05
glgP	-1.55
gloA3	-1.06
glpK	-1.08
hepA	1.01
hisG	1.04
hscA	1.06
katB	1.64
katE	-1.46
kup	-1.18
lasB	-1.07
ldh	-1.38
lecB	-1.93
lptF	-1.50
maiA	2.24
mliC	-1.09
moaC	1.44
moaD	1.44
modA	-2.34
mucA	-1.24
napA	-1.73

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napB	-2.39
napC	-1.52
napD	-1.73
napF	-1.73
norB	-1.49
norC	-1.49
ntrB	-1.32
oprC	-1.22
osmE	-1.62
PA0007	-1.07
PA0063	1.78
PA0107	-1.26
PA0122	-1.67
PA0177	-2.01
PA0178	-2.01
PA0179	-2.18
PA0256	-1.16
PA0308	1.57
PA0364	3.12
PA0365	1.78
PA0366	1.69
PA0451	-1.95
PA0452	-1.95
PA0453	-1.78
PA0484	-1.86
PA0508	1.08
PA0545	-1.11
PA0575	-1.02
PA0585	-1.96
PA0586	-1.33
PA0587	-1.33
PA0588	-1.64
PA0713	-1.54
PA0732	-2.05
PA0737	-1.42
PA0758	1.52
PA0788	-2.02

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PA0918	-2.06
PA0959	-1.33
PA1041	-1.42
PA1053	1.12
PA1242	-1.77
PA1323	-1.55
PA1324	-1.38
PA1327	-1.23
PA1414	-1.01
PA1415	-1.50
PA1441	1.43
PA1639	1.49
PA1657	-1.12
PA1658	-1.15
PA1666	-1.07
PA1667	-1.07
PA1668	-1.25
PA1669	-1.08
PA1730	-1.77
PA1731	-1.31
PA1732	-1.31
PA1733	-1.51
PA1784	-1.89
PA1809	1.31
PA1874	-1.60
PA1875	-1.60
PA1876	-1.60
PA1877	-1.60
PA1887	-1.70
PA1888	-1.70
PA1914	-1.71
PA1930	-2.23
PA1969	infinite
PA2072	-2.04
PA2100	-1.41
PA2141	-2.04
PA2142	-2.04

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PA2150	-1.88
PA2151	-1.42
PA2152	-1.05
PA2158	-1.40
PA2160	-1.24
PA2161	-1.58
PA2162	-1.58
PA2163	-1.58
PA2164	-1.58
PA2165	-1.58
PA2167	-1.78
PA2168	-1.78
PA2171	-1.65
PA2172	-1.61
PA2176	-1.74
PA2177	-1.18
PA2179	-1.67
PA2180	-1.67
PA2190	-2.06
PA2265	-1.18
PA2366	-1.03
PA2372	-1.75
PA2381	-1.40
PA2456	-1.02
PA2504	-1.59
PA2550	-2.46
PA2562	-1.29
PA2566	-1.48
PA2571	-1.51
PA2572	-1.65
PA2573	-1.50
PA2618	-1.47
PA2621	-1.02
PA2705	-1.03
PA2708	-2.16
PA2751	-1.53
PA2754	-1.44

PA2780	-1.18
PA2781	-1.18
PA2815	-1.31
PA2821	1.04
PA2846	-1.78
PA2858	1.37
PA2864	-1.96
PA2895	-1.58
PA2896	-1.58
PA2909	1.49
PA2920	-1.02
PA2938	-1.50
PA2939	-1.55
PA3023	-1.48
PA3038	1.12
PA3040	-1.95
PA3041	-1.57
PA3042	-1.71
PA3093	1.03
PA3094	1.03
PA3122	-1.25
PA3169	1.33
PA3224	-1.69
PA3250	-1.51
PA3251	-1.15
PA3252	-1.15
PA3253	-1.15
PA3260	2.48
PA3261	-1.02
PA3282	-1.09
PA3283	-1.09
PA3311	-1.14
PA3362	-2.82
PA3415	-1.47
PA3416	-1.63
PA3417	-1.63
PA3429	-1.58

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PA3430	-1.58
PA3459	-1.03
PA3460	-1.25
PA3461	-1.25
PA3559	1.49
PA3573	-1.14
PA3611	-1.08
PA3688	-1.46
PA3691	-1.44
PA3723	-1.37
PA3748	1.03
PA3752	-1.38
PA3753	-1.38
PA3795	-1.39
PA3922	-1.71
PA3923	-1.77
PA3924	-2.32
PA3945	-1.19
PA3961	1.50
PA4015	-1.24
PA4017	-1.12
PA4063	-1.09
PA4108	-1.37
PA4222	1.04
PA4223	1.04
PA4311	-1.37
PA4312	-1.37
PA4313	-1.37
PA4364	-1.18
PA4523	-1.39
PA4606	-1.08
PA4607	-1.61
PA4610	-1.09
PA4627	1.33
PA4633	-1.08
PA4679	1.15
PA4691	-1.38

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PA4692	-1.38
PA4698	1.25
PA4699	1.25
PA4702	-1.32
PA4739	-1.00
PA4772	1.00
PA4851	1.08
PA4879	-1.53
PA4880	-1.77
PA4913	-1.67
PA4915	-1.54
PA4917	-1.86
PA4929	-1.69
PA5020	-1.42
PA5030	-1.12
PA5096	-1.02
PA5101	-2.04
PA5210	-1.18
PA5212	-1.37
PA5250	1.42
PA5359	-1.15
PA5369.1	infinite
PA5445	1.89
PA5494	-1.35
PA5515	1.21
PA5528	1.30
PA5529	1.22
pchE	1.04
pchF	1.04
pchG	1.04
pdxB	2.20
pfpI	-1.63
phaC2	-1.33
phoP	1.07
phoQ	1.07
phrS	-1.49
pilE	1.03

pilY1	1.11
pilY2	1.03
poxB	-1.80
pprA	-2.04
pprB	-1.75
pqqB	-1.25
pqqC	-1.83
pqqD	-1.83
pqqE	-1.83
proC	1.01
pslN	-1.74
psrA	-1.05
pyoS5	-1.10
rcpC	-2.36
rimI	1.15
rmf	-1.69
rplI	1.24
rplP	1.14
rpmC	1.14
rsmA	-1.12
siaA	1.12
ssb	1.01
stk1	-1.08
stp1	-1.08
tadA	-1.60
tadB	-1.60
tadG	-1.58
tadZ	-1.60
thiD	1.06
thiE	1.38
trxB2	2.17
tssC1	-1.22
waaA	1.24

[1]Log_2 fold change = Log_2 (FPKM value of SDS treated biofilm/FPKM value of SDS untreated biofilm)
[2]infinite: gene detected only in SDS treated biofilms but not in SDS untreated biofilms

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Table E-2. Alphabetical list of differentially expressed genes and fold change in SDS treated Pf-5 biofilms

Gene name	[1,2] Log_2 fold change
acsA_1	-1.16
aidB	-1.25
aprA	-1.41
aprX	-1.48
aspA	1.70
atuA	-1.52
cah_3	1.04
calB	2.64
cycA	1.41
emhB	1.36
flgB	1.59
flgC	1.22
flgD	1.05
flgE	1.15
flgF	1.19
flgJ	1.12
gabP	-1.25
gcdH	-1.08
gltD	1.26
hasAp	-1.95
hmp	1.46
lhr	1.45
liuA	-1.10
moaB_1	1.78
ofaA	-1.31
ofaB	-1.59
pctA_1	-1.01
PFL_0118	-1.40
PFL_0258	-1.82
PFL_0338	-1.28
PFL_1028	2.77
PFL_1034	1.96
PFL_1035	2.64

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PFL_1157	1.01
PFL_1158	1.08
PFL_1214	1.57
PFL_1223	1.06
PFL_1227	1.16
PFL_1421	4.60
PFL_1431	-3.02
PFL_1436	1.64
PFL_1633	-1.14
PFL_1644	-1.10
PFL_1711	-1.24
PFL_1978	-3.45
PFL_2100	-1.16
PFL_2149	-1.54
PFL_2182	-1.44
PFL_2367	1.28
PFL_2455	1.29
PFL_2485	-1.48
PFL_3099	-4.65
PFL_3261	-1.27
PFL_3309	1.87
PFL_3515	1.01
PFL_3558	-1.07
PFL_3573	1.40
PFL_3668	-1.34
PFL_4203	-1.00
PFL_4432	-1.23
PFL_4824	1.01
PFL_4996	2.51
PFL_4998	infinite
PFL_5116	-1.15
PFL_5548	-1.19
PFL_5686	-1.34
PFL_5808	-1.59
PFL_5866	3.14
PFL_5867	2.33
PFL_6235	-1.35

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PFL_6282	-1.19
pgaB	-1.23
phlC	-1.18
polB	1.22
prnD	-1.18
pth	1.09
pvdD	-1.15
pvdI	-1.02
pvdL	-1.31
sdaB	1.62
sdaC	1.21
tam	-1.03
tonB3	1.01

[1]Log_2 fold change = Log_2 (FPKM value of SDS treated biofilm/FPKM value of SDS untreated biofilm)
[2]infinite: gene detected only in SDS treated biofilms but not in SDS untreated biofilms

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Table E-3. Alphabetical list of differentially expressed genes and fold change in SDS treated KP-1 biofilms

Gene name	[1,2] Log_2 fold change
abgA	-3.2626
abgB	-3.2626
ade	-3.04418
aegA	1.21201
ahpF	1.22592
ascB	1.83187
atpI	1.27493
bfd	-2.68046
bioA	-1.15026
cbl	-2.16526
cdd	1.40244
cirA	-3.10468
citX	-1.05094
clpS	1.27874
cpxP	1.63781
cybC	1.63479
cysA	-1.7408
cysC	-1.80509
cysG	1.39026
cysN	-1.80509
cysP	-1.7408
cysT	-1.7408
cysW	-1.7408
cytR	1.04888
dcuB	1.31247
degP	1.41361
deoA	1.42753
dmsA	2.23131
dmsB	1.52706
dmsC	1.97784
dppC	1.54949
eitD	-1.38479
emrR	1.09454

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entA	-2.10451
entB	-2.10451
entC	-2.33463
entE	-2.08517
entF	-2.17824
exbB	-1.86258
exbD	-1.2675
fbaB	-1.01332
fdhE	1.18014
fdhF	1.00984
fdoG	1.31945
fdoH	1.18014
fdoI	1.18014
fecB	-1.38479
fecD	-1.38479
fecE	-1.38479
fepA	-2.30116
fepB	-2.09042
fepC	-1.35829
fepD	-1.35829
fepG	-1.35829
fes	-2.1254
fhuF	-2.17273
fliY	-1.38134
frdA	1.15475
frdB	1.15475
frdC	1.09203
frdD	1.09203
ftnA	1.85318
ftnB	1.47145
fumB	-1.35411
glgS	-1.07375
gpmA	-1.06956
groES	1.00324
hcp	-1.4822
hemG	1.16233
hmpA	-1.37962

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hmuT	-3.73272
hmuU	-3.73272
hmuV	-3.73272
iclR	1.17293
iroE	-2.47126
iroN	-2.15611
ispA	1.0607
KP1_0018	1.21746
KP1_0271	1.72623
KP1_0328	-1.67989
KP1_0466	-1.95022
KP1_0509	-1.17432
KP1_0552	1.70373
KP1_0655	1.16763
KP1_0656	1.16763
KP1_0700	1.62052
KP1_0713	1.14142
KP1_0714	1.14142
KP1_0715	1.90167
KP1_0716	1.90167
KP1_0794	1.38989
KP1_0886	1.25286
KP1_0946	1.90518
KP1_0947	2.32159
KP1_1190	1.45516
KP1_1280	-1.37567
KP1_1318	1.06192
KP1_1405	1.0944
KP1_1436	3.88016
KP1_1465	-2.63522
KP1_1466	-2.34889
KP1_1562	-2.05972
KP1_1570	-1.27737
KP1_1571	-1.27737
KP1_1573	-1.76266
KP1_1574	-1.76266
KP1_1581	1.20133

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KP1_1582	1.20133
KP1_1583	1.20133
KP1_1692	1.06251
KP1_1844	1.23853
KP1_2035	-2.75395
KP1_2036	-2.33817
KP1_2037	-2.44924
KP1_2068	1.09279
KP1_2100	1.47046
KP1_2101	-2.60552
KP1_2216	-1.03229
KP1_2355	3.52033
KP1_2452	1.09214
KP1_2461	-1.73776
KP1_2462	-1.73776
KP1_2495	-1.02972
KP1_2513	1.23441
KP1_2563	-1.88712
KP1_2565	-1.83004
KP1_2585	-3.01618
KP1_2611	1.73398
KP1_2665	1.29828
KP1_2691	1.12134
KP1_2847	-1.93106
KP1_2849	-1.93106
KP1_2850	-1.93106
KP1_2851	-1.33119
KP1_2980	1.25757
KP1_3190	-1.95318
KP1_3191	-1.95318
KP1_3192	-1.95318
KP1_3194	-1.95318
KP1_3195	-1.95318
KP1_3196	-3.69569
KP1_3256	-1.05543
KP1_3267	-1.81475
KP1_3289	-1.44496

KP1_3339	-1.51844
KP1_3358	-1.16319
KP1_3427	-2.71701
KP1_3461	1.04339
KP1_3479	-1.09523
KP1_3490	2.26631
KP1_3536	1.03745
KP1_3653	-2.48339
KP1_3688	1.14898
KP1_3689	1.14898
KP1_3690	1.14898
KP1_3837	-5.53718
KP1_3864	-1.62076
KP1_3993	-1.26865
KP1_4265	-1.69618
KP1_4266	-1.33815
KP1_4354	-1.87697
KP1_4356	-4.22605
KP1_4358	-3.73272
KP1_4467	1.34524
KP1_4596	1.15733
KP1_4681	1.51592
KP1_4700	-1.90789
KP1_4706	-1.86258
KP1_4810	1.61637
KP1_4948	-2.1356
KP1_5113	-1.41225
KP1_5282	1.14923
KP1_5292	1.5437
KP1_5310	1.64723
KP1_5462	-1.67486
KP1_5463	-1.67486
lrp	1.0136
manC	-1.03964
mntH	-2.39399
mppA	3.52033
narG	1.64637

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narH	1.64637
narI	1.64637
narJ	1.64637
narK	1.22087
nfsA	1.23853
nhaR	1.23153
nrdE	-2.71225
nrdF	-2.71225
nrdH	-2.71225
nrdI	-2.71225
nuoC	1.02109
nuoE	1.2987
nuoF	1.2987
nuoI	1.1669
nuoJ	1.13786
nuoK	1.13786
nuoL	1.13786
nupC	-1.13912
nupC	-1.13912
ompF	-2.09817
oppB	1.23566
otsA	-1.25461
otsB	-1.25461
pckA	1.12337
pdxA	1.10973
perM	1.21099
pspA	1.03898
rrfG	-1.06023
serA	-1.90309
sitA	-3.12197
sitB	-3.12197
sitC	-3.12197
sitD	-3.12197
sodB	2.29828
spy	2.02571
sufA	-1.80986
sufB	-2.17981

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sufC	-2.22309
sufD	-2.22309
sufS	-2.22309
tatA	1.12389
tdcA	1.06255
tdcB	1.34101
tehA	-1.64874
tehB	-1.64874
thiC	-2.48283
thiD	-1.68926
thiE	-2.48283
thiF	-2.48283
thiG	-2.31082
thiH	-2.31082
thiM	-1.68926
thiS	-2.48283
tonB	-1.57603
udp	1.20865
wbbO	1.01925
ybdA	-1.44643
ybeF	1.58017
ybiF	1.49149
ybiL	-2.36917
ybjP	1.22804
yciW	-2.07143
ydbK	1.13547
ydgD	-3.01618
ydgE	2.07211
ydgF	2.07211
ydjN	-1.52463
yedE	1.23441
yeiU	-5.53718
yfeC	2.0196
ygjG	-1.39224
yhfC	-1.33819
yhhJ	1.62052
yhiH	1.62052

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yicE	-1.49578
yjiM	1.43531
yncE	-2.05861
ynfE	1.90809

[1]Log_2 fold change = Log_2 (FPKM value of SDS treated biofilm/FPKM value of SDS untreated biofilm)
[2]infinite: gene detected only in SDS treated biofilms but not in SDS untreated biofilms

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