The Effect of Biofilm and the Role of *rpoS* on the Survival of a *p*-Nitrophenol-Degrading *Pseudomonas putida*

Author: Miranda Maki

M.Sc. Candidate Biology

August 31st, 2008

Supervisor: Dr. K.T. Leung

Committee members: Dr. H. Schraft and Dr. D. Law

Your file Votre référence
ISBN: 978-0-494-43426-0
Our file Notre référence
ISBN: 978-0-494-43426-0

Table of Contents

List of Figures

Chapter 1: Literature Review

Chapter 2: The Survival and *p*-Nitrophenol Degradation of Planktonic and Biofilm *Pseudomonas putida* Cells

Chapter 3: The Role of *rpoS* on the Survival of a *p*-Nitropheol Degrading *Pseudomonas putida* Strain in Planktonic and Biofilm Phases

List of Tables

Chapter 3: The Role of *rpoS* on the Survival of a *p*-Nitropheol Degrading *Pseudomonas putida* Strain in Planktonic and Biofilm Phases

Abstract

Pseudomonas putida is a Gram negative bacterium that can be found naturally in the environment as planktonic free-living cells, or in concert with other bacterial species in biofilms. In this study, the *P. putida* strain has the ability to degrade *p*-nitrophenol (PNP), a moderately toxic organic pollutant found world-wide. Both the survivability and PNP degradative abilities of planktonic and biofilm *P. putida* cells were compared in buffer and river water samples containing varying concentrations of PNP. The *P. putida* biofilm cells showed higher survival rate in both the buffer and river water samples compared to the planktonic *P. putida* cells. In the buffer experiment, *P. putida* planktonic cell density decreased from 8.25 to 5.7 and 5.0 log (CFU/mL) after 17 days treatment with 3000 and 4000 μM PNP. However, *P. putida* biofilm cells in the buffer system had a decrease in cell density from 10.2 to 8.7 and 9.0 log (CFU/g glass wool) after 17 days treatment with 3000 and 4000 μM PNP, respectively. The survival fraction revealed that biofilm cells had a 100-fold greater survival compared to planktonic cells at 3000 μM and 4000 μM PNP by day 17. In the river water system at 3000 and 4000 μM PNP, the planktonic cell density decreased from 9.4 to 3.0 log (MPN/mL) and biofilm cell density decreased from 10.3 to 9.5 and 10 log (MPN/g glass wool), respectively, after 17 days treatment. Survival fractions of the bacteria in the river water system revealed that biofilm cells survived 350- and 450-fold greater than planktonic cells at 3000 and 4000 μM PNP, respectively. Monitoring PNP degradation in the buffer system, revealed that the *P. putida* planktonic cells degraded 1500 μM PNP completely, degraded 2600 μM PNP by 50% and 3000 μM PNP by 40% and did not degrade 4000 μM PNP within 18 days. The biofilm cells in the buffer completely degraded 1500 and 2600 μM PNP,

degraded 3000 μM PNP by 55%, and did not degrade 4000 μM PNP, in 18 days. In the river water system, the *P. putida* planktonic cells did not degrade any PNP. By contrast, the *P. putida* biofilm cells in the river water were found to completely degrade 1500 μM PNP in 9 days. It was observed that high concentrations of PNP inflicted injury to cells located on the surface of biofilms while cells in the interior of biofilms remained viable. The *P. putida* biofilms were able to survive in and degrade PNP better than planktonic cells, and these findings indicate potential improvement of bioremediation using biofilm cells.

The expression of the stationary phase sigma factor gene (*rpoS*) and biofilm formation are important bacterial stress-survival strategies. To further explore the relationship between these two factors, the survival and competitiveness of the PNP-degrading *P. putida* strain as well as its *rpoS*$^-$ mutant were examined in both planktonic and biofilm phases. To distinguish wild-type (WT) and knock-out (KO) strains in mixed samples, they were labeled with a red fluorescent protein-gentamycin resistant and a green fluorescent protein-gentamycin resistant gene cassette via a Tn7 transposon system, respectively. In single cultures, the KO-*gfp* biofilm featured a greater than 4-fold increase in biovolume than the WT-*rfp*. However, mixed biofilm biovolume revealed that KO-*gfp* was reduced by 75.6% compared to the single culture KO-*gfp* biofilm biovolume, suggesting that the WT-*rfp* suppressed KO-*gfp* biofilm formation. Competitiveness studies showed that carbon-starved planktonic WT-*rfp* single cultures achieved a 3.5-fold greater survival rate in 0.85% saline than their KO-*gfp* counterparts, and out-competed them by more than 13-fold in mixed culture samples. Conversely, there were no significant survival differences between the KO-*gfp* and WT-*rfp* strains in

biofilm samples. Finally, differences in cellular cohesiveness were evident, after 60 min washing with 0.2% SDS resulted in 32 and 89% cells detached from the KO and WT samples, respectively. These results indicate that although the KO produced more biofilm than the WT, it may in fact not confer an advantage in survival because the KO biofilm has a lower percentage of surviving cells and cannot readily detach to colonize new habitats. Overall, these findings provide new perspectives towards a more complete understanding of the role of *rpoS* in biofilm formation.

Acknowledgements

I would like to thank my supervisor, Dr. Kam Leung, for providing me the opportunity to complete my Master's thesis in his Applied Environmental Microbiology laboratory and for all his support and mentorship. I would also like to thank my committee members, Dr. Heidi Schraft and Dr. David Law, for donating their time and energy throughout my thesis work. I would also like to express my appreciation to the staff and students of the Applied Environmental Microbiology laboratory, especially Shawn Minor for his work during his internship. Special thanks to Dr. Søren Molin for the donation of the mini-Tn7–*rfp* and –*gfp* plasmids, without which this work could not have been possible. Finally, thank you to Mr. Alan Mackenzie for the instruction and access to the equipment in the Lakehead University Instrumentation laboratory. This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC).

Chapter 1: Literature Review

1.0. *Pseudomonas putida*

Pseudomonas putida is a Gram negative, rod-shaped bacterium. It is characterized as non-spore forming and motile with one or more polar flagellum (or flagella). It is known to be capable of forming a biofilm. *P. putida* also undergoes aerobic metabolism and has the capability of growing on several different organic substrates (Holt *et al.*, 1994). In doing so, it has been observed that *P. putida* strains can degrade a wide variety of organic pollutants such as toluene, phenol and organomercurial products (Horn *et al.*, 1994; Annadurai *et al.*, 2002; Dinkla and Janssen, 2003).

P. putida can also be found ubiquitously within the environment and is non-pathogenic to humans. Thus, it has been proposed as a safe and useful bioremediation agent (Raghavan and Vivekenandan, 1999). It is currently being used to bioremediate soil, because a *P. putida* rhizobacterial strain demonstrated increased metal-binding for cadium in plant roots. It has been suggested that if this heavy-metal binding *P. putida* also possessed organic-degradation capabilities, it could be used to remediate mixed organic-heavy metal polluted soils (Wu *et al.*, 2006). Due to its broad degradation capabilities and environmentally-friendly nature, *P. putida* has a potential to be developed into an effective bioremediation agent.

1.1. *p*-Nitrophenol (PNP)

Organic pollutants such as nitrophenols, benzoate, naphthalene-sulfonic acids and steroids are found worldwide as soil and aquatic environmental contaminants, which are often anthropogenically-derived by-products (Tremp *et al.*, 1993; Cinar and Grady,

2001). *p*-Nitrophenol (PNP) is one such organic pollutant. It is a toxic and possibly mutagenic by-product found in agricultural soils, industrial waste sites and rainwater runoff. PNP can contaminate aquatic environments such as rivers, lakes and streams and groundwater. The U.S. EPA considers *p*-nitrophenol and other nitrophenolic compounds to be major pollutants because they can be found in a wide variety of environments (US Environmental Protection Agency, 1976).

PNP can be found in agricultural soils because it is a hydrolytic product of parathion, a chemical which is often used in pesticides (Munnecke and Hsieh, 1976). PNP is also a precursor to pharmaceuticals such as acetaminophen and 4-aminosalicylic acid and is often found in industrial waste sites world-wide (Boehncke *et al.*, 2000). It is also able to contaminate rainwater because it can be found in diesel fuel and gasoline exhaust (Tremp *et al.*, 1993). Many derivatives found in diesel exhaust have been studied to investigate their toxicity and in doing so PNP has been found to have vasodilatory activity in mice (Kamata *et al.*, 2004). It was also shown *in-vitro* that PNP has estrogenic and anti-androgenic activities according to a recombinant yeast screening assay (Taneda *et al.*, 2004). Most recently, Li *et al.* (2006) demonstrated, using mice, the toxicity of PNP, where it was shown to have estrogenic and anti-androgenic activities *in-vivo*.

Some organic pollutants and organic solvents are known to be toxic to microorganisms. They have been found to segregate in the membrane of cells, making them more fluid and permeable (Heipieper *et al.*, 1991; Heipieper and de Bont, 1994). Despite this toxicity, some microorganisms are able to adapt to high concentrations of such pollutants and ultimately metabolize them.

The oxidative release of nitrite from *p*-nitrophenol in a soil pseudomonad was first identified by Simpson and Evans (1953). Since this time, several bacteria species have been isolated and identified as degraders of PNP. These include such species as: *Nocardia* (Hanne *et al.*, 1993), *Arthrobacter* (Bhushan *et al.*, 2000; Jain *et al.*, 1994), *Sphingomonas* (Leung *et al.*, 1997), *Flavobacterium* (Raymond and Alexander, 1971), *Burkholderia* (Bhushan *et al.*, 2000; Prakash *et al.*, 1996), *Bacillus* (Kadiyala *et al.*, 1998), *Pseudomonas* (Munnecke and Hsieh, 1976), *Ralstonia* (Bhushan *et al.*, 2000) and *Moraxella* (Spain and Gibson, 1991).

The PNP degradation pathway of the *Moraxella* sp. strain (re-classified as *Pseudomonas putida* by Tallon and Leung (2005)) isolated from a sludge sample by Spain and Gibson (1991) is shown in Figure 1. Initially, the nitro group is removed from PNP by a PNP-monoxygenase which results in the formation of *p*-benzoquinone. The *p*-benzoquinone is then converted to hydroquinone as an intermediate before ring cleavage and complete degradation of PNP (Spain and Gibson, 1991).

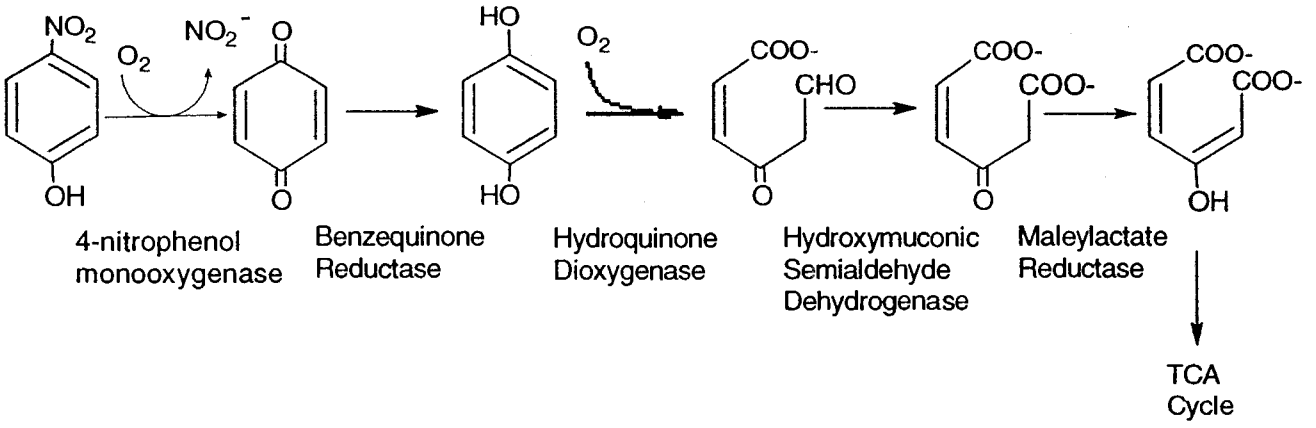


Figure 1. Proposed pathway of PNP degradation by *Moraxella* sp. (Spain and Gibson, 1991).

The genes responsible for the biodegradation of PNP have been shown to be carried on a plasmid in several species, such as *P. putida*, *P. cepacia* and *Arthrobacter protophormiae* (Chauhan *et al.*, 2000; Kulkarni and Chaudhari, 2006; Prakash *et al.*, 1996). Recently, it has been found that the rate of PNP degradation in *P. putida* increases with alkalinity, completely degrading 300 ppm in 16.5 hours (Kulkarni and Chaudhari, 2006). Increasing PNP-degradation rate is important for rapid bioremediation of PNP pollution in the environment. Other studies have shown that a *Moraxella* strain can be genetically modified to degrade *p*-nitrophenol and some organophosphorus pesticides at the same time, which proves also to be valuable for bioremediation (Lei *et al.*, 2005; Shimazu *et al.*, 2001).

1.2. Toxicity and Tolerance of Organic Pollutants

Some bacteria naturally possess the ability to degrade toxic organic pollutants. However, high pollutant concentrations can cause physiological changes and detrimental effects on bacterial cells. Recent studies have examined the effects of various toxic organic compounds on planktonic cells. Organic contaminants such as solvents have been found to affect membrane fluidity and permeability of *P. putida* and *Escherichia coli* because the organic pollutants partition preferentially into their cell membranes. This causes a collapse in the potassium gradient of bacterial cells, which has been evaluated by quantification of cellular potassium content (Heipieper *et al.*, 1991). It has been shown in *E. coli* that the addition of glucose helps restore the potassium gradient in the presence of sub-bactericidal concentrations of phenol. At bacteriostatic and bactericidal levels of phenol, small cellular metabolites have been shown to be lost from cells. One such metabolite is ATP (Heipieper *et al.*, 1991; Heipieper and de Bont, 1994).

Furthermore, because several organic pollutants have been found to disrupt the lipid bilayer, it is the concentration of the pollutant that is lethal to a cell and not the actual structure of the organic compound itself. The toxicity of a compound has been found to correlate directly with the logarithm of its partitioning coefficient (Isken and de Bont, 1998). Thus the toxicity of organic compounds to microorganisms can thus be easily determined.

Additionally, mechanisms for bacterial tolerance to toxic organic compounds have also been examined. Inoue and Horikoshi (1989) were the first to identify a *P. putida* strain that could actively grow in 50% (v/v) toluene. Since this discovery, studies have focused on investigating how microorganisms tolerate such organic pollutants.

In a pollutant-degrading *P. putida* strain, it has been observed that there is a reduction in cell size if grown in the presence of phenol and chlorophenols. Also noted was a change in shape from rod to spherical cells. These observations were correlated with an overall drop in dry weight but an increase in cell number. Cells growing in glucose and 500 ppm phenol had a 0.788 g/L change in dry weight after complete degradation (Fakhruddin and Quilty, 2006). Qualitative changes in the cell membrane, such as a notable reduction in cell shape and size, are thought to be an important strategy for pollutant-degrading microorganisms to tolerate high concentrations of organic pollutants (Sardessai and Bhosle, 2002).

In addition to such visible changes in cell structure, there are several less visible adaptations Gram negative bacteria undergo in response to toxic organic pollutants. The first is a change in the unsaturated fatty acids of the lipid bilayer. This has been readily observed in *P. putida*, where several studies have shown that there is a change from *cis-*

to *trans*- unsaturated fatty acids with modifications in the lipopolysaccharides. The increased isomerization from *cis*- to *trans*- unsaturated fatty acids allows the cell membrane to become more rigid and thus less fluid (Holtwick *et al.*, 1997; Junker and Ramos, 1999; Pinkart *et al.*, 1996; Weber *et al.*, 1994).

Another mechanism that has also been observed frequently in *Pseudomonas* sp. is the activation of efflux pumps in response to various concentrations of toxic organic pollutants. One example is the multi-drug efflux pumps of *P. aeruginosa*, which are known to be involved in intrinsic and acquired multiple antibiotic resistance of this species. Li et al. (1998) generated various efflux pump mutants in *P. aeruginosa.* After exposure to *n*-hexane and *p*-xylene, they determined that the expression of the MexAB-OprM efflux pump could be correlated directly with increased tolerance to these organic pollutants. Efflux pumps have also been identified in *P. putida*, which have been found to remove toxic organic compounds from the cell membrane, and contribute to its high tolerance of organic compounds (Kieboom *et al.*, 1998; Ramos *et al.*, 1998).

Other cellular mechanisms contributing to the toxic organic compound-tolerance of bacteria include the observed decrease in cell surface hydrophobicity, as well as changes in proportions or chemical composition of lipids and proteins (Kobayashi *et al.*, 1999; Ramos *et al.*, 1997). In *E. coli*, the alkylhydroperoxide reductase operon *ahpCF* was found to be involved in tolerance to tetralin and cyclohexane. The expression of these genes allowed for *E. coli* to possess decreased cell surface hydrophobicity (Ferrante *et al.*, 1995). At this time, several genes or proteins, such as the TonB system and *flip* of *Pseudomonas putida* DOT-T1E, have been identified as being involved in the tolerance to organic solvents in a wide variety of species (Goday *et al.*, 2001; Segura *et al.*, 2001).

Such genes have been characterized as being involved in the wide variety of tolerance mechanisms previously mentioned.

1.3. Biofilms

1.3.1. Biofilm physiology

In many natural environments, microorganisms are often found living in the form of complex multicellular biofilm structures adhering to surfaces. The advantage of biofilm formation is that it stabilizes the metabolism of resident bacteria living in continuously changing environments (Hall-Stoodley and Stoodley, 2005). Studies have shown that biofilms have significant relevance to medical, industrial and environmental settings because of increased survival of bacteria within biofilms when treated with such compounds as antimicrobials and antibiotics (Brown and Gilbert, 1993; Mah and O'Toole, 2001; Stewart and Costerton, 2001). In one study, bacteria isolated from multiple catheters showed that regardless of the bacterial species, once bacteria developed a biofilm structure, the minimal antibiotic dose to exterminate biofilm cells was 100-fold higher than the highest patient dose possible (Bartoszewicz *et al.*, 2007). As a result bacterial biofilms are a concern due to their increased resistance to antibiotics and antimicrobials and, more studies have been initiated to understand the physiology and genetic regulations of biofilm bacteria (Brown and Gilbert, 1993; Mah and O'Toole, 2001; Stewart and Costerton, 2001).

There are several factors thought to contribute to the increased survival of biofilm cells and, in particular, their increased resistance to antimicrobials. The four major hypotheses for the increased resistance of biofilm cells to antibiotics and antimicrobials

include physiological heterogeneity within the biofilm community, expression of adaptive stress responses, poor penetration of the biocides through the biofilm, and the presence of persister cells or high variance in the phenotypes of some cells (Brown *et al.*, 1988; Davies, 2003; Lewis, 2001; Stewart, 2002).

Nutrient limitation and substrate utilization are two factors that affect the physiology of biofilm cells. The depletion of substrates produces regions of inactive growth in the interior of the biofilm; these areas are termed physiological heterogeneity. Nutrient limitation causes these cells to be stressed and therefore much hardier than active growing cells. Physiological heterogeneity was examined in a study done on a toluene-degrading *P. putida*; a *gfp*-reporter gene was used to monitor cellular growth activity of *P. putida* biofilm cells when grown in benzyl alcohol-supplemented media. The rRNA levels were monitored and different levels of growth activity were observed within the biofilms over time (Sternberg *et al.*, 1999). Similar results were seen with the use of acridine orange, a nucleic acid stain. Biofilm cells stained green or red depending on the levels of DNA and RNA, respectively, within a cell. This allowed for visualization and quantification of variations in growth rate within *Klebsiella pneumoniae* biofilms (Wentland *et al.*, 1996).

Oxygen limitation increases biofilm survival through the expression of adaptive stress responses. Borriello *et al.* (2004) noted that 48 h biofilms of *Pseudomonas aeruginosa* were 210 μm thick, and much more resistant to tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, and tetracycline exposure for 12 h. Upon analysis of oxygen penetration, it was observed that oxygen only penetrated 50 μm into the biofilm and protein synthesis occurred at a narrow (30 μm) band located at the air-

interface of the biofilms. This suggests that most of the cells in these 48 h-old biofilms are in an oxygen-depleted stationary phase state (Borriello *et al.*, 2004). Because the cells are in a stationary phase state, this means that several stress resistance genes are turned on allowing these bacteria to be more resistant to toxic compounds and this has been noted in a range of bacteria species (Walters *et al.*, 2003; Xu *et al.*, 1998).

An explanation of the antibiotic/antimicrobial resistance of biofilm cells is thought to be low penetration of the biocides. It has been suggested that antimicrobials cannot reach all of the cells within the biofilm, thereby allowing a large number of cells to survive within the biofilm. However, this has proven not to be the case in some studies. In both *Staphylococcus epidermidis* and *P. aeruginosa,* the antibiotics rifampin and ciprofloxacin, and tobramycin, respectively, were found to penetrate the biofilms completely without killing all of the bacteria (Walters *et al.*, 2003; Zheng and Stewart, 2002).

In addition, Rani *et al.* (2005) used fluorescent tracers to determine the rate of diffusion of molecules equivalent in size to average antibiotics and biocides. In doing so, they revealed that Rhodamine B and fluoroscein had diffusion rates through cell clusters (200 - 600 μm thick) of 3.7×10^{-7} and 1.6×10^{-6} cm^2 s^{-1}, respectively. These studies suggested that slow penetration does not play a role in biofilm survival to such toxic compounds as antibiotics and biocides.

The resilience of biofilms may also be caused by the development of persister cells. This is a hypothesis that within biofilms, a sub-population may transform into a unique phenotype that allows them to become more resistant (Mah and O'Toole, 2001; Stewart, 2000). The reason for such a hypothesis comes from the fact that several studies

have shown a rapid killing of most biofilm cells by various antibiotics, however, there remains a sub-population of cells within the biofilm that exhibit stationary phase characteristics and are found to be viable. This has even been observed in considerably thinner biofilms, suggesting that persister cells are developed throughout the growth of biofilms (Anderl *et al.*, 2003; Brooun *et al.*, 2000; Goto *et al.*, 1999).

1.3.2. Biofilm development

It has been determined that the formation of a bacterial biofilm follows a series of developmental stages, including attachment, formation of microcolonies, proliferation and production of exopolymeric substances (EPS), maturation of the biofilm and finally detachment or dispersal of bacterial cells from the biofilm structure (Dalton and March, 1998; Stoodley *et al.*, 2000).

The formation bacterial biofilms begins with the interaction of planktonic cells with a surface. *P. aeruginosa* PA14 mutants defective in surface attachment have lent insights into the genes involved biofilm initiation. It has been found that the *flgK* affects cell-surface interaction suggesting that flagella and motility are required to make initial contact with a surface. In addition, the type IV pili were shown to be required for the formation of microcolonies on an abiotic surface via twitching motility (O'Toole and Kolter, 1998a). Similarly, both flagella and pili have also been shown to be involved in substrate contact and attachment of *Pseudomonas fluorescens* (O'Toole and Kolter, 1998b). In *P. aeruginosa,* the *pel* genes, which are known to encode proteins involved in polysaccharide production, also play a role in the early attachment of biofilm cells (Vasseur *et al.*, 2005).

After the attachment and formation of microcolonies, proliferation and the production of exopolysaccharide (EPS) occur. Quorum-sensing (QS) is referred to as cell-to-cell communication and is responsible for the initiation of proliferation of bacterial microcolonies to mature biofilms (Fuqua *et al.*, 1994; Salmond *et al.*, 1995). Passador *et al.* (1993) identified two acyl-homoserine lactone (AHL) quorum–sensing signal systems in *P. aeruginosa*, the LasR-LasI and RhlR-RhlI regulatory circuits. The LasR-LasI and RhlR-RhlI global gene regulators were found to be responsible for the production of and response to *N*-(3-oxododecanoyl)-AHL and *N*-butryl-AHL, respectively. Using a halogenated furanone, Hentzer *et al.* (2002) prohibited the production of AHL signals in *P. aeruginosa* causing the shutdown of QS and suppressing biofilm formation. It has also been shown that *P. aeruginosa lasI* mutants were deficient in AHL production compared to the wild-type (Davies *et al.*, 1998).

Coinciding with the proliferation of biofilms, is the production of extracellular polymeric substances (EPS), which are important to biofilm structure, growth and bacterial adhesion to substrata (Rickard *et al.*, 2003). EPS is composed of a complex mixture of polysaccharides, nucleic acids and/or proteins (Flemming *et al.*, 2000; Sutherland, 2001). However, the function and composition of the EPS varies depending on the microbial species (Sutherland, 2001). In *P. aeruginosa*, the production of EPS has been well studied. It is known to contribute to the mushroom-mound structure of mature *P. aeruginosa* biofilms (Hentzer *et al.*, 2002). In addition, EPS is thought to play a role in protecting biofilm cells from the environment. EPS has been shown to shelter biofilm cells from a variety of environmental stresses including changes in pH, osmotic shock,

diffusion of toxic compounds such as antimicrobials; desiccation, and UV radiation (Flemming, 1993; Ophir and Gutnick, 1994; Gilbert *et al.*, 1997; Elasir and Miller, 1999).

To date, most biofilm studies have focussed on the attachment stage and quorum sensing mechanism. Consequently, little is known about the detachment mechanism of biofilm bacteria. Detachment is one of the crucial steps of biofilm development, enabling bacteria to colonize new surfaces as nutrients are exhausted by a mature biofilm (Allison and Sutherland, 1984; Bester *et al.*, 2005; Gjermansen *et al.*, 2005). In support of this hypothesis, Hunt *et al.* (2004) showed that nutrient starvation contributes to cell detachment in biofilms using computer modeling. However, a study with *P. aeruginosa* has shown that an increase in nutrients may contribute to biofilm dispersal (Sauer *et al.*, 2004). Bester *et al.* (2005) also demonstrated that detachment occurs in continuously nutrient-fed high-density *Pseudomonas* biofilms. This infers that detachment occurs throughout the growth of biofilms and can take place when nutrients are present.

Currently, several proteins have been identified as being involved in biofilm dispersal in different *Pseudomonas* species. Many of these proteins have been linked with environmental signals and cellular levels of cyclic-di-GMP (c-di-GMP). In *P. aeruginosa*, a transducer protein BdlA (biofilm dispersion locus), has been linked with environmental-que-induced biofilm cell dispersion (Morgan *et al.*, 2006). While another study in *P. putida* has shown the genes PP0164 and PP0165 are involved in biofilm dispersal. The PP0165 protein is linked to c-di-GMP signaling and the PP0164 protein acts as a transducer of the signal (Gjermansen *et al.*, 2005). These studies provide environmental evidence that changes can contribute to a biofilm's ability to disperse.

Further examination into biofilm detachment has allowed researchers to find that biofilm dispersal may not only depend on environmental ques but can also be influenced by signal molecules that are produced by cells within the biofilm. Baurrad and colleagues (2006) were the first to link nitric oxide (NO) to the dispersal of *P. aeruginosa* biofilms. Nitric oxide has been identified as a product of anaerobic metabolism; and although toxic at high levels, low levels have been shown to disperse *P. aeruginosa* biofilms. This suggests that toxic metabolites produced in mature biofilms may act as dispersal signals in low doses.

As well, Morgan *et al.* (2006) were able to show that the biofilm dispersal in *Shewanella oneidensis* could be controlled by intercellular concentrations of cyclic-di-GMP. This was an important study because they were able to bypass the previously identified environmental que of molecular oxygen depletion for biofilm dispersal (Thormann *et al.*, 2006). This means they were able to show that without the influence of depletion of nutrients, biofilms of *Shewanella oneidensis* could still disperse because of the production of intercellular signal molecules such as cyclic-di-GMP.

Lastly, surfactant production in bacteria has also been linked to biofilm dispersal. Davey *et al.* (2003) demonstrated that the production of the rhamnolipid surfactants in wild-type *P. aeruginosa* results in channels clear of cells around macrocolonies. Whereas, mutant *P. aeruginosa* cells incapable of the production of rhamnolipids, were unable to keep open channels around macrocolonies therefore had thicker more continuous biofilms (Davey *et al.*, 2003).

With all this said, there is still much to learn about the control of biofilm dispersal mechanisms. Detachment of biofilms is a complex process which is suggested to be dependent on several factors and does seem to vary between different microbial species.

1.4. The Role of RpoS in Planktonic Cells

The stationary phase sigma factor (RpoS) in *E. coli*, coded for by the *rpoS* gene, is known to be the central regulator for the expression of more than 50 genes for cells in stationary phase (Hengge-Aronis, 2002a, b; Kolter *et al.*, 1993). The *rpoS* gene was first identified and described by Lange and Hennge-Aronis (1991). They studied the physiological and morphological changes of *E. coli* in stationary phase induced by carbon starvation. They found that *rpoS* could prolong survival of the bacteria during C-starvation stress.

Transcription initiation sites for the promoters of *P. aeruginosa* and *P. putida* have been identified at 366 and 373 bp, respectively, upstream from the translation start. Both strains have promoter sequences typical for σ^{70} at -10 and -35 bp. It has been shown that the *rpoS* promoters of *P. putida* and *P. aeruginosa* are functionally very similar (Fujita *et al.*, 1994; Kojic and Venturi, 2001; Kojic *et al.*, 2002). However, the *rpoS* promoter of *P. putida* and *P. aeruginosa* are not functional in *E. coli* (Kojic and Venturi, 2001). This suggests that *rpoS* between different genera can be quite different. However, regulation of the *rpoS* remains very similar between species of the same genus.

In *P. aeruginosa*, RpoS levels are known to increase upon entering stationary phase. Studies of the mRNAs revealed that an array of mRNAs was highly induced during stationary phase. Fujita *et al.* (1994) observed a 5-fold increase in RpoS mRNA in the *P. aeruginosa* cells when the bacterial culture entered stationary phase.

For planktonic cells such as a PNP-degrading *P. putida* strain (formerly identified as *Moraxella* sp.), it has been shown that carbon starvation will trigger an increase in cellular resistance to oxidation, high temperature shock, osmotic stresses and many other environmental stresses. The carbon-starved cells were about 2 log more resistant to osmotic (2.7 M NaCl) and high temperature (43.5°C) stresses in comparison to non-starved log phase *P. putida* cells (Moore *et al.*, 2005).

Specifically knocking out *rpoS* has been shown to affect the stress response as well as the expression of virulence factors in *P. aeruginosa.* Notably, it was found that the *rpoS*⁻ mutant produced 50% less exotoxin A than the wild-type. As well, incubation of the mutant and parent strains at 50°C for 8 mins revealed that the wild-type could survive 50-times greater than the *rpoS*⁻ mutant (Suh *et al.*, 1999).

The stress response has also been recently investigated in *Pseudomonas fluorescens* Pf-5, by the creation of an *rpoS*⁻ mutant from the wild-type. Cells starved in aerated PBS for a period of a month, showed that the wild-type *P. fluorescens* would decrease only 0.5 logs in cell density, in comparison to a 1-1.5 log decrease for the *rpoS*⁻ mutant. Similarly, they were able to show that *rpoS* allowed for a survival advantage when exposed to UV irradiation, low temperature stress and desiccation (Stockwell and Loper, 2005). Ramos-Gonzalez and Molin (1998) knocked out the *rpoS* of a *Pseudomonas putida* strain and also showed that the *rpoS*⁻ mutant was more susceptible to 18% v/v ethanol, 200 μM H_2O_2 and 2.4 M NaCl.

Although much is known about the role of *rpoS* for the survival of planktonic cells, information related to the functions of *rpoS* on biofilm cells is limited.

1.5. Biofilms and RpoS

RpoS has been extensively studied and characterized in planktonic/free-living bacteria cells. However, little is known about the role and regulation of RpoS in biofilms and thus remains the subject of some controversy.

Recently, some studies have shown that *rpoS* plays a role in biofilms. They describe qualitative differences in the biofilm growth of wild-type bacteria versus their *rpoS* knockout strains. One particular study done on an *Escherichia coli* strain, has shown that without the *rpoS* gene there is a considerable decrease in biofilm growth compared to the wild-type. Grown on glass coverslips and measured in CFU per square centimeter, the *rpoS* knockout was reduced by 50% ($P < 0.05$) compared to the wild-type (Adams and McLean, 1999). Supporting this study, it has also been shown in *E. coli* that *rpoS* mutant strains are incapable of attaching to surfaces. Both of these studies suggest that there is a relationship between RpoS and biofilm formation in *E. coli* (Prigent-Combaret *et al.*, 2001; Schembri *et al.*, 2003).

However, contrary to the findings of Adams and McLean (1999), Corona-Izquierdo and Membrillo-Hernández (2002) observed an increase in biofilm formation for their *E. coli* K12 *rpoS*$^-$ mutant strain. After 48 h of growth the *rpoS*$^-$ mutant strain formed 3-5-fold greater biofilm than the wild-type. They also suggested that the *rpoS* mutant strain was secreting an extracellular factor that caused a similar increase in biofilm production to the wild-type strain; however the extracellular factor was not identified.

Also, an *rpoS*$^-$ knockout mutant was produced from a *P. aeruginosa* strain by Heydorn *et al.* (2002). They found that the *rpoS*$^-$ *P. aeruginosa* mutant produced notably

thicker biofilms in comparison to the wild-type. As well, in a PNP-degrading *P. putida* strain (formerly classified as *Moraxella* sp.), it was found that the *rpoS*⁻ mutant strain produced thicker biofilms than the wild-type when grown on metal coupons (Nandakumar *et al.*, 2006).

All of these observations, whether an *rpoS* knockout strain produces more or less biofilm than the parent strain, show that there is a relationship between RpoS and biofilm formation. These observations indicate the need for further investigation into the role of *rpoS* in relation to biofilm growth.

It is first important to note, how biofilm growth is regulated. Initiation and proliferation of biofilm cells requires cell-cell communication, referred to as quorum-sensing. This allows bacteria to live within a community to regulate growth, structure and architecture. It also allows communities of cells to regulate physiological processes such as bioluminescence, competence development, conjugal plasmid transfer and antibiotic or metabolite synthesis (Fuqua *et al.*, 1994; Salmond *et al.*, 1995; Losick and Kaiser, 1997; Davies *et al.*, 1998).

Two acyl-homoserine lactone (acyl-HSL) quorum–sensing signal systems have been identified in *P. aeruginosa* (Passador *et al.*, 1993). These two systems are the LasR-LasI and RhlR-RhlI autoinducer-dependent regulatory circuits, referred to as global gene regulators. These two systems are responsible for the production of and response to *N*-(3-oxododecanoyl)-AHL and *N*-butryl-AHL, respectively. There has been a lot of work done identifying these global regulatory systems in other Gram negative bacteria such as the homologous LuxI and LuxR systems found in a *P. putida* rhizobacterium (Haas and Keel, 2003).

To date, studies have clearly shown that *rpoS* and quorum-sensing are linked, providing evidence for the hypothesis that *rpoS* is in fact involved in biofilm formation. Originally, it was thought in *P. aeruginosa*, that expression of the *rpoS* was regulated by the LasR/I and RhlI quorum-sensing systems (Latifi *et al.*, 1996). However, more recently it has been discovered that *rpoS* negatively regulates the expression of *rhlI*, in *P. aeruginosa* (Schuster *et al.*, 2004).

Explicitly, this was first described by Whitley *et al.* (2000) in *P. aeruginosa* that *rpoS* down-regulated *rhlI* expression. Similarly, RpoS and N-acyl homoserine lactone (AHL)–dependent quorum sensing have been found to regulate each other in a rhizosphere *P. putida* WCS358 (Bertani and Venturi, 2004). In *P. aeruginosa*, it has been established that the two signal systems (AHL and RpoS) regulate virulence factors and biofilm formation (Schuster *et al.*, 2003; Smith and Iglewski, 2003).

Finally, *rpoS* has been shown to be linked to biofilm detachment. Nielsen *et al.* (2006) observed in an *rpoS*$^-$ knock-out *Vibrio cholerae* mutant, a decreased ratio of fluid: mucus membrane cells. This suggests that the *rpoS*$^-$ mutant biofilms will retain more cells attached to the mucosal epithelium, thus preventing the spread *Vibrio cholerae.* In support of these findings, Müller *et al.* (2007) found that the biofilm of an *rpoS*$^-$ mutant strain of *Vibrio cholerae* retained nearly 100% of its biomass in the biofilm. Both of these studies show a clear relationship between *rpoS* and biofilm detachment which is important for biofilm survival.

The stationary phase sigma factor (RpoS) appears to play a significant role in both the growth and survival of biofilm cells. However, this role has not been well defined.

1.6. Thesis Objectives

To date, biofilms are rigorously studied because biofilms can have relevance to medical, industrial and environmental applications. With regard to the PNP-degrading *Pseudomonas putida* strain, information related to biofilm formation and *rpoS* functions of the bacteria is crucial for enhancing the effectiveness of using this bacterial strain as a bioremediation agent.

The first objective of this study is to determine the survival and PNP-degradative abilities of biofilm versus planktonic PNP-degrading *Pseudomonas putida* strain, in both a buffer and river water system. Second, the toxic effect of PNP on *P. putida* biofilm cells is examined using propidium iodide viability staining procedure. Third, the *p*-nitrophenol-degrading *Pseudomonas putida* strain and its *rpoS*$^-$ mutant are used to examine the role of *rpoS* in biofilm development, specifically their competitiveness in both the planktonic and biofilm phases. Fourth, the biofilm cohesiveness of both the *P. putida* wild-type and the *rpoS*$^-$ mutant are also compared.

References

Adams JL and McLean RJC. 1999. Impact of *rpoS* deletion on *Escherichia* coli biofilms. Appl Environ Microbiol 65:4285-7.

Allison DG and Sutherland IW. 1984. A staining technique for attached bacteria and its correlation to extracellular carbohydrate production. J Microbiol Methods 2:93-9.

Anderl JN, Zahller J, Roe F, Stewart PS. 2003. Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial Agents Chemother 47:1251-6.

Annadurai G, Juang RS, Lee DJ. 2002. Biodegradation and adsorption of phenol using activated carbon immobilized with *Pseudomonas putida*. J Environ Sci Health A Tox Hazard Subst Environ Eng 37:1133-46.

Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. 2006. Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. J Bacteriol 188:7344-53.

Bartoszewicz M, Rygiel A, Przondo-Mordarska A. 2007. Analysis of the effect of selected antiseptics and antibiotics on the survival of planktonic cells and biofilm cells. Int J Antimicrob Agents 29:S140.

Bertani I and Venturi V. 2004. Regulation of the N-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. Appl Environ Microbiol 70:5493-502.

Bester E, Wolfaardt G, Joubert L, Garny K, Saftic S. 2005. Planktonic-cell yield of a Pseudomonad biofilm. Appl Environ Microbiol 71:7792-8.

Bhushan B, Chauhan A, Samanta SK, Jain RK. 2000. Kinetics of biodegradation of *p*-nitrophenol by different bacteria. Biochem Biophys Res Commun 274:626-30.

Boehncke A, Koennecker G, Mangelsdorf I, Wibbertmann. 2000. Mononitrophenols, concise international chemical assessment document 20. [http://www.who.int/ipcs/publications/cicad/en/cicad20.pdf]. (21/05/08).

Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrobial Agents Chemother 48:2659-64.

Brooun A, Liu SH, Lewis K. 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents Chemother 44:640-6.

Brown MRW and Gilbert P. 1993. Sensitivity of biofilms to antimicrobial agents. J Appl Bacteriol 74:S87-97.

Brown MRW, Allison DG, Gilbert P. 1988. Resistance of bacterial biofilms to antibiotics - a growth-rate related effect. J Antimicrob Chemother 22:777-80.

Chauhan A, Chakraborti AK, Jain RK. 2000. Plasmid-encoded degradation of *p*-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*. Biochem Biophys Res Commun 270:733-40.

Cinar O and Grady CPL. 2001. Aerobic and anoxic biodegradation of benzoate: stability of biodegradative capability under endogenous conditions. Water Res 35:1015-21.

Corona-Izquierdo FP and Membrillo-Hernandez J. 2002. A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. FEMS Microbiol Lett 211:105-10.

Dalton HM, March PE. 1998. Molecular genetics of bacterial attachment and biofouling. Curr Opin Microbiol 9:252-5.

Davey ME, Caiazza NC, O'Toole GA. 2003. Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1. J Bacteriol 185:1027-36.

Davies D. 2003. Understanding biofilm resistance to antibacterial agents. Nature Reviews Drug Discovery 2:114-22.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-8.

Dinkla IJT and Janssen DB. 2003. Simultaneous growth on citrate reduces the effects of iron limitation during toluene degradation in *Pseudomonas*. Microb Ecol 45:97-107.

Elasir MO, and Miller RV. 1999. Study of the response of a biofilm bacterial community to UV radiation. Appl Environ Microbiol 65:2025-31.

Fakhruddin ANM and Quilty B. 2006. The response of *Pseudomonas putida* CP1 cells to nutritional, chemical and environmental stresses. World J Microbiol Biotechnol 22:507-14.

Ferrante AA, Augliera J, Lewis K, Klibanov AM. 1995. Cloning of an organic solvent-resistance gene in *Escherichia* coli: The unexpected role of alkylhydroperoxide reductase. Proc Natl Acad Sci USA 92:7617-21.

Flemming H-C. 1993. Biofilms and environmental protection. Water Sci Technol 27:1-10.

Flemming, H.-C.,Wingender J, Mayer C, Korstgens V and Borchard W. 2000. Cohesivenessin biolfim matrix polymers. In: Allison, D., P. Gilbert, H.M. Lappin-Scott, and M. Wilson,editor. Community Structure and Cooperation in Biofilms. Cambridge: Cambridge University Press. pp. 87-105.

Fujita M, Tanaka K, Takahashi H, Amemura A. 1994. Transcription of the principal sigma-factor genes, *rpod* and *rpos*, in *Pseudomonas aeruginosa* is controlled according to the growth-phase. Mol Microbiol 13:1071-7.

Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria - the *luxr-luxi* family of cell density-responsive transcriptional regulators. J Bacteriol 176:269-75.

Gilbert P, Das J, Foley I. 1997. Biofilms susceptibility to antimicrobials. Adv Dent Res 11:160-7.

Gjermansen M, Ragas P, Sternberg C, Molin S, Tolker-Nielsen T. 2005. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. Environ Microbiol 7:894-906.

Goday P, Ramos-Gonzalez MI, Ramos JL. 2001. Involvement of the TonB system in tolerance to solvents and drugs in *Pseudomonas putida* DOT-T1E. Bacteriol 183:5285-92.

Goto T, Nakame Y, Nishida M, Ohi Y. 1999. *In vitro* bactericidal activities of beta-lactamases, amikacin, and fluoroquinolones against *Pseudomonas aeruginosa* biofilm in artificial urine. Urol 53:1058-62.

Haas D and Keel C. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41:117-153.

Hall-Stoodley L and Stoodley P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol 13:300-1.

Hanne LF, Kirk LL, Appel SM, Narayan AD, Bains KK. 1993. Degradation and induction specificity in *Actinomycetes* that degrade *p*-nitrophenol. Appl Environ Microbiol 59:3505-8.

Heipieper HJ and de Bont JA. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl Environ Microbiol 60:4440-4.

Heipieper HJ, Keweloh H, Rehm HJ. 1991. Influence of phenols on growth and membrane-permeability of free and immobilized *Escherichia coli*. Appl Environ Microbiol 57:1213-7.

Hengge-Aronis R. 2002a. Recent insights into the general stress response regulatory network in *Escherichia coli*. J Mol Microbiol Biotechnol 4:341-436.

Hentzer M, *et al.* 2002. Inhibition of quorum-sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology 148:87-102.

Heydorn A, Ersboll B, Kato J, Hentzer M, Parsek MR, Tolker-Nielsen T, Givskov M, Molin S. 2002. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: Impact of mutations in genes involved in twitching motility, cell-to-cell signaling, and stationary-phase sigma factor expression. Appl Environ Microbiol 68:2008-17.

Holt JG, Krieg NR, Sneath PHA., Staley JT, Williams ST. 1994. Berygey's Manual of Determinative Bacteriology 9th Ed. Williams and Wilkens Publishing Ltd. Baltimore, USA. p.155.

Holtwick R, Meinhardt F, Keweloh H. 1997. Cis-trans isomerization of unsaturated fatty acids: Cloning and sequencing of the *cti* gene from *Pseudomonas putida* P8. Appl Environ Microbiol 63:4292-7.

Horn JM, Brunke M, Deckwer WD, Timmis KN. 1994. *Pseudomonas putida* strains which constitutively overexpress mercury resistance for biodetoxification of organomercurial pollutants. Appl Environ Microbiol 60:357-62.

Hunt SM, Werner EM, Huang BC, Hamilton MA, Stewart PS. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol 70:7418-25.

Inoue A and Horikoshi K. 1989. A *Pseudomonas* thrives in high-concentrations of toluene. Nature 338:264-6.

Isken S and de Bont JAM. 1998. Bacteria tolerant to organic solvents. Extremophiles 2:229-38.

Jain RK, Dreisbach JH, Spain JC. 1994. Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. Appl Environ Microbiol 60:3030-2.

Junker F and Ramos JL. 1999. Involvement of the cis-trans isomerase *cti* in the solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181:5693-700.

Kadiyala V, Smets BF, Chandran K, Spain JC. 1998. High affinity *p*-nitrophenol oxidation by *Bacillus sphaericus* JS905. FEMS Microbiol Lett 166:115-20.

Kamata K, Hayashi H, Taneda S, Suzuki A, Toda N, Sakush-Ima A, Seki K, Mori Y. 2004. Isolation and identification of nitrophenols in diesel exhaust particles (DEP) that exhibit vasodilating activity using bioassay-directed fractionation. Toxicol Appl Pharmacol 197:237.

Kieboom J, Dennis JJ, Zylstra GJ, de Bont JAM. 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J Bacteriol 180:6769-72.

Kobayashi H, Takami H, Hirayama H, Kobata K, Usami R, Horikoshi K. 1999. Outer membrane changes in a toluene-sensitive mutant of toluene-tolerant *Pseudomonas putida* IH-2000. J Bacteriol 181:4493-8.

Kojic M and Venturi V. 2001. Regulation of *rpoS* gene expression in *Pseudomonas*: Involvement of a TetR family regulator. J Bacteriol 183:3712-20.

Kojic M, Aguilar C, Venturi V. 2002. TetR family member PsrA directly binds the *Pseudomonas rpoS* and *psrA* promoters. J Bacteriol 184:2324-30.

Kolter R, Siegele DA, Tormo A. 1993. The stationary phase of the bacterial life cycle. Annu Rev Microbiol 47:855-74.

Kulkarni M and Chaudhari A. 2006. Biodegradation of *p*-nitrophenol by *P. putida*. Bioresour Technol 97:982-8.

Lange R and Hengge-aronis R. 1991. Identification of a central regulator of stationary-phase gene-expression in *Escherichia coli*. Mol Microbiol 5:49-59.

Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. 1996. A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol 21:1137-46.

Lei Y, Mulchandani A, Chen W. 2005. Improved degradation of organophosphorus nerve agents and *p*-nitrophenol by *Pseudomonas putida* JS444 with surface-expressed organophosphorus hydrolase. Biotechnol Prog 21:678-81.

Leung KT, Tresse O, Errampalli D, Lee H, Trevors JT. 1997. Mineralization of *p*-nitrophenol by pentachlorophenol-degrading *Sphingomonas* spp. FEMS Microbiol Lett 155:107-14.

Lewis K. 2001. Riddle of biofilm resistance. Antimicrobial Agents Chemother 45:999-1007.

Li CM, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. 2006. Estrogenic and anti-androgenic activities of 4-nitrophenol in diesel exhaust particles. Toxicol Appl Pharmacol 217:1-6.

Li XZ, Zhang L, Poole K. 1998. Role of the multidrug efflux systems of *Pseudomonas aeruginosa* in organic solvent tolerance. J Bacteriol 180:2987-91.

Losick R and Kaiser D. 1997. Why and how bacteria communicate. Sci Am 276:68-73.

Mah TFC and O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34-9.

Moore M, Trevors J, Lee H, Leung KT. 2005. Stress-survival responses of a carbon-starved *p*-nitrophenol-mineralizing *Moraxella* strain in river water. Can J Microbiol 51:223-9.

Morgan R, Kohn S, Hwang SH, Hassett DJ, Sauer K. 2006. Bd1A, a chemotaxis regulator essential for biofilm dispersion in *Pseudomonas aeruginosa.* J Bacteriol 188:7335-43.

Müller J, Miller M, Nielsen A, Schoolnik G, Spormann A. 2007. *vspA*- and *luxO*-dependent biofilms of *Vibrio cholerae.* FEMS Microbiol Lett 275:199-206.

Munnecke DM and Hsieh DP. 1976. Pathways of microbial metabolism of parathion. Appl Environ Microbiol 31:63-9.

Nandakumar K, Keeler W, Schraft H, Leung KT. 2006. Visible laser and UV-A radiation impact on a PNP degrading *Moraxella* strain and its *rpoS* mutant. Biotechnol Bioeng 94:793-802.

Nielsen A *et al.*. 2006. RpoS Controls the *Vibrio cholerae* Mucosal Escape Response. PloS Pathog 2:934-48.

Ophir T, and Gutnick DL. 1994. A role for exopolysaccharide in the protection of microorganisms from desiccation. Appl Environ Microbiol 60:740-5.

O'Toole GA and Kolter R. 1998a. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30:295-304.

O'Toole GA and Kolter R. 1998b. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. Mol Microbiol 28:449-61.

Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260:1127-30.

Pinkart HC, Wolfram JW, Rogers R, White DC. 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to o-xylene. Appl Environ Microbiol 62:1129-32.

Prakash D, Chauhan A, Jain RK. 1996. Plasmid-encoded degradation of *p*-nitrophenol by *Pseudomonas cepacia.* Biochem Biophys Res Commun 224:375-81.

Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, Dorel C. 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. J Bacteriol 183:7213-23.

Raghavan PUM and Vivekenadan M. 1999. Bioremediation of oil-spilled sites through seeding of naturally adapted *Pseudomonas putida*. Int Biodeterioration Biodegrad 44:29-32.

Ramos JL, Duque E, Godoy P, Segura A. 1998. Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323-9.

Ramos JL, Duque E, RodriguezHerva JJ, Godoy P, Haidour A, Reyes F, FernandezBarrero A. 1997. Mechanisms for solvent tolerance in bacteria. J Biol Chem 272:3887-90.

Ramos-Gonzalez MI and Molin S. 1998. Cloning, sequencing, and phenotypic characterization of the *rpoS* gene from *Pseudomonas putida* KT2440. J Bacteriol 180:3421-31.

Rani SA, Pitts B, Stewart PS. 2005. Rapid diffusion of fluorescent tracers into *Staphylococcus epidermidis* biofilms visualized by time lapse microscopy. Antimicrobial Agents Chemother 49:728-32.

Raymond DGM, Alexander M. 1971. Microbial metabolism and co-metabolism of nitrophenols. Pestic Biochem Physiol 1:123-30.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, and Handley PS. 2003. Bacterial coaggregation: and integral process in the development of multi-species biofilms. Trends Microbiol 11:94-100.

Salmond GPC, Bycroft BW, Stewart GSAB, Williams P. 1995. The bacterial enigma - cracking the code of cell-cell communication. Mol Microbiol 16:615-24.

Sardessai Y and Bhosle S. 2002. Tolerance of bacteria to organic solvents. Res Microbiol 153:263-68.

Sauer K, Cullen MC, Rickard AH, Zeef LAH, Davies DG, Gilbert P. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J Bacteriol 186:7312-26.

Schembri MA, Kjaergaard K, Klemm P. 2003. Global gene expression in *Escherichia coli* biofilms. Mol Microbiol 48:253-67.

Schuster M, Hawkins AC, Harwood CS, Greenberg EP. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. Mol Microbiol 51:973-85.

Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. J Bacteriol 185:2066-79.

Segura A, Duque E, Hurtado A, Ramos JL. 2001. Mutations in genes involved in the flagellar export apparatus of the solvent-tolerant *Pseudomonas putida* DOT-T1E strain impair motility and lead to hypersensitivity to toluene shocks. Bactriol 183:4127-33.

Shimazu M, Mulchandani A, Chen W. 2001. Simultaneous degradation of organophosphorus pesticides and *p*-nitrophenol by a genetically engineered *Moraxella* sp. with surface-expressed organophosphorus hydrolase. Biotechnol Bioeng 76:318-24.

Simpson JR and Evans WC. 1953. The metabolism of nitrophenols by certain bacteria. Biochem J 55:24.

Smith RS and Iglewski BH. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr Opin Microbiol 6:56-60.

Spain JC and Gibson DT. 1991. Pathway for biodegradation of para-nitrophenol in a *Moraxella* sp. Appl Environ Microbiol 57:812-19.

Sternberg C, Christensen BB, Johansen T, Nielsen AT, Andersen JB, Givskov M, Molin S. 1999. Distribution of bacterial growth activity in flow-chamber biofilms. Appl Environ Microbiol 65:4108-17.

Stewart PS. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol 292:107-13.

Stewart PS and Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358:135-8.

Stockwell VO and Loper JE. 2005. The sigma factor RpoS is required for stress tolerance and environmental fitness of *Pseudomonas fluorescens* pf-5. Microbiol 151:3001-9.

Stoodley P, Hall-Stoodley L, Lappin-Scott, HM. 2000. Detachment, surface migration and other dynamic behavior in bacterial biofilms revealed by digital time-lapse imaging. Meth Enzymol 337:306-19.

Suh SJ, Silo-Suh L, Woods DE, Hassett DJ, West SEH, Ohman DE. 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. J Bacteriol 181:3890-7.

Sutherland IW. 2001. The biofilm matrix - an immobilized but dynamic microbial environment. Trends Microbiol 9:222-7.

Tallon P, Leung KT. 2005. The impact of *rpoS* deletion on biofilm formation of a *p*-nitrophenol degrading *Moraxella* strain. Proc Can Soc Microbiol. 55th conference, Halifax, Nova Scotia, Canada p.63.

Taneda S, Mori Y, Kamata K, Hayashi H, Furuta C, Watanabe G, Li C, Takahashi S, Seki K, Sakushima A, *et al.* 2004. Estrogenic and anti-androgenic activity of nitrophenols in diesel exhaust particles (DEP). Biol Pharm Bull 27:835-7.

Thormann KM, Duttler S, Saville RM, Hyodo M, Shukla S, Hayakawa Y, Spormann AM. 2006. Control of formation and cellular detachment from *Shewanella oneidensis* MR-1 biofilms by cyclic di-GMP. J Bacteriol 188:2681-91.

Tremp J, Mattrel P, Fingler S, Giger W. 1993. Phenols and nitrophenols as tropospheric pollutants - emissions from automobile exhausts and phase-transfer in the atmosphere. Water Air Soil Pollut 68:113-23.

US Environmental Protection Agency (1976) Water quality criteria. USEPA, Washington, DC.

Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A. 2005. The *pel* genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology 151:985-97.

Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother 47:317-23.

Weber FJ, Isken S, Debont JAM. 1994. Cis-trans isomerization of fatty acids as a defense mechanism of *Pseudomonas putida* S12 to toxic concentrations of toluene. Microbiol 140:2013-17.

Wentland EJ, Stewart PS, Huang CT, McFeters GA. 1996. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. Biotechnol Prog 12:316-21.

Whiteley M, Parsek MR, Greenberg EP. 2000. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa.* J Bacteriol 182:4356-60.

Wu CH, Wood TK, Mulchandani A, Chen W. 2006. Engineering plant-microbe symbiosis for rhizoremediation of heavy metals. Appl Environ Microbiol 72:1129-34.

Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl Environ Microbiol 64:4035-9.

Zheng ZL and Stewart PS. 2002. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother 46:900-3.

Chapter 2: The Survival and *p*-Nitrophenol Degradation of Planktonic and Biofilm *Pseudomonas putida* Cells

Abstract

Pseudomonas putida is a Gram negative bacterium that can be found naturally in the environment as planktonic free-living cells, or in concert with other bacterial species in biofilms. In this study, the *P. putida* strain has the unique ability to degrade *p*-nitrophenol (PNP), a moderately toxic organic pollutant found world-wide. Both the survivability and PNP degradative abilities of planktonic and biofilm *P. putida* cells were compared in buffer and river water samples containing varying concentrations of PNP. The *P. putida* biofilm cells showed higher survival rate in both the buffer and river water samples compared to the planktonic *P. putida* cells. In the buffer experiment, *P. putida* planktonic cell density decreased from 8.25 to 5.7 and 5.0 log (CFU/mL) after 17 days treatment with 3000 and 4000 μM PNP. However, *P. putida* biofilm cells in the buffer system had a decrease in cell density from 10.2 to 8.7 and 9.0 log (CFU/g glass wool) after 17 days treatment with 3000 and 4000 μM PNP, respectively. The survival fraction revealed that biofilm cells had a 100-fold greater survival compared to planktonic cells at 3000 μM and 4000 μM PNP by day 17. In the river water system at 3000 and 4000 μM PNP, the planktonic cell density decreased from 9.4 to 3.0 log (MPN/mL) and biofilm cell density decreased from 10.3 to 9.5 and 10 log (MPN/g glass wool), respectively, after 17 days treatment. Survival fractions of the bacteria in the river water system revealed that biofilm cells survived 350- and 450-fold greater than planktonic cells at 3000 and 4000 μM PNP, respectively. Monitoring PNP degradation in the buffer system, revealed

that the *P. putida* planktonic cells could degraded 1500 μM PNP completely, degraded 2600 μM PNP by 50% and 3000 μM PNP by 40% and did not degrade 4000 μM PNP within 18 days. The biofilm cells in the buffer completely degraded 1500 and 2600 μM PNP, degraded 3000 μM PNP by 55%, and did not degrade 4000 μM PNP, in 18 days. In the river water system, the *P. putida* planktonic cells did not degrade any PNP. By contrast, the *P. putida* biofilm cells in the river water were found to completely degrade 1500 μM PNP in 9 days. It was observed that high concentrations of PNP inflicted injury to cells located on the surface of biofilms while cells in the interior of biofilms remained viable. The *P. putida* biofilms were able to survive in and degrade PNP better than planktonic cells, and these findings indicate potential improvement of bioremediation using biofilm cells.

2.1. Introduction

Organic pollutants such as nitrophenols, benzoate, naphthalene-sulfonic acids and steroids are found worldwide as soil and water contaminants and they are often anthropogenically-derived by-products (Madyastha and Shanker, 1994; Sterjiades and Plemont, 1989; Wittich *et al.*, 1988). *p*-Nitrophenol (PNP) is toxic, and possibly a mutagen. Originating in agricultural soils, industrial waste sites and rainwater runoff, PNP can eventually contaminate aquatic environments such as rivers, lakes and streams (Tremp *et al.*, 1993). The USEPA considers *p*-nitrophenol and other nitrophenolic compounds to be major pollutants because they are widely distributed (US Environmental Protection Agency, 1976).

PNP is found in agricultural soils because it is a hydrolytic product of parathion, a chemical which is often used in pesticides (Munnecke and Hsieh, 1976). PNP is also a

precursor to pharmaceuticals such as acetaminophen and 4-aminosalicylic acid and is often found in industrial waste sites (Boehncke *et al.*, 2000). It is also able to contaminate rainwater because it can be found in diesel fuel and gasoline exhaust (Tremp *et al.*, 1993). Further study of the toxic effects of PNP from diesel exhaust, have been examined and it was determined to be a vasodilatory and to have estrogenic and anti-androgenic activities on mice (Kamata *et al.*, 2004; Li *et al.*, 2006).

Many phenolic pollutants, such as PNP, are known to be toxic to microorganisms and have been found to segregate in the membrane making it more fluid and permeable. However, some microorganisms are able to adapt to high concentrations of such organic compounds and ultimately metabolize them (Heipieper *et al.*, 1991; Heipieper and DeBont, 1994). In 1953, oxidative release of nitrate from *p*-nitrophenol in a soil pseudomonad was first identified by Simpson and Evans. Since then, several bacterial species have been isolated and identified as degraders of PNP. These include *Nocardia* (Hanne *et al.*, 1993), *Arthrobacter* (Bhushan *et al.*, 2000; Jain *et al.*, 1994), *Sphingomonas* (Leung *et al.*, 1997), *Flavobacterium* (Raymond and Alexander, 1971), *Burkholderia* (Bhushan *et al.*, 2000; Prakash *et al.*, 1996), *Bacillus* (Kadiyala *et al.*, 1998), *Pseudomonas* (Munnecke and Hsieh, 1976), *Ralstonia* (Bhushan *et al.*, 2000) and *Moraxella* (Spain and Gibson, 1991).

Recently, biofilm research has received more attention in both environmental and medical science because most microorganisms exist commonly in a sessile state in the environment (Costerton, 1987). Studies have also shown that biofilms have significant relevance to medical, industrial and environmental settings because of their increased

survival or tolerance to antimicrobials and antibiotics (Brown and Gilbert, 1993; Mah and O'Toole, 2001; Stewart and Costerton, 2001).

Several factors are thought to contribute to the increased survival of biofilm cells. The four major hypotheses for the increased resistance of biofilm cells to antibiotics and antimicrobials include poor penetration of biocides through the biofilm, expression of adaptive stress responses, physiological heterogeneity within the biofilm community, and the presence of persister cells or high variance in the pheonotypes of some cells (Brown *et al.*, 1988; Davies *et al.*, 2003; Lewis *et al.*, 2001; Stewart *et al.*, 2002).

Pseudomonas putida is a Gram negative, rod shaped, soil bacterium. The strain used in this study has the unique ability to degrade *p*-nitrophenol (Spain and Gibson, 1991) and can be a potential bioremediation agent. *P. putida* is also capable of producing biofilms in concert with other bacterial species in river water, but may also be found in river water environments occurring in planktonic form. In a river water environment, there is a limitation of nutrients, competition from other microbial species and cells must adapt to a constantly changing environment as temperatures, pH, and osmolarity fluctuate (Van Veen *et al.*, 1997). Little known about how the concentration of pollutants may affect the bacteria's ability to degrade them. By comparing the survivability and PNP-degradative abilities of these two forms, at various PNP concentrations, one may be able to maximize the efficiency with which *P. putida* can degrade PNP.

Here, the survival and PNP-degradation of planktonic and biofilm phase of a *P. putida* strain are compared in both buffer and river water settings. Also, the toxic effect of PNP on *P. putida* biofilm cells is examined using propidium iodide viability staining procedure.

2.2. Materials and Methods

2.2.1. Bacterial strain

The *P. putida* strain used in this study (formerly identified as a *Moraxella* species) was isolated from an activated sludge sample and has the unique ability to degrade *p*-nitrophenol (PNP) (Spain and Gibson, 1991). The 16S rDNA sequence of this bacterium is more than 99% homology to known *P. putida* 16S rDNA sequences in GenBank (Tallon and Leung, 2005). API 20E test were used (Biomerieux, St. Laurent, Quebec) which resulted in a good-likelihood selection for *P. putida*. Further confirmative biochemical tests verified that the strain was in fact a *P. putida*, included positive test results for catalase, oxidase, fermentation and motility (Holt *et al.*, 1994). The *P. putida* strain is grown optimally in Trypticase Soy Broth (TSB; Becton Dickinson and Co., Sparks, MD) medium at 30°C and was stored in sterile 25% glycerol at -80°C before used. Biofilm cells were grown on the surface of sterile glass wool submerged in half strength TSB.

A *gfp* (green fluorescent protein gene) labeled version of the PNP-degrading *P. putida* strain was constructed by a mini-Tn5-*gfp* transposon system for direct visualization of the bacteria in biofilm conditions (Moore *et al.*, 2005). This *gfp* labeled *P. putida* strain has the same PNP-degrading ability and growth rate as its wild-type parent strain.

2.2.2. Survival of planktonic cells in buffer

The planktonic cells were grown in TSB in a 30°C shaking incubator (200 rpm) to an optical density (OD_{600nm}) of 0.5. The cell suspension (4×150 mL) was harvested by centrifugation at 5000 x g for 15 minutes, washed twice in 150 mL sterile 0.85% saline solution and resuspended in a minimal salts medium (MSM; 1.249 mM KH_2PO_4, 3.73 mM K_2HPO_4, 0.4 mM $MgSO_4.7H_2O$, 0.02 mM $FeSO_4.7H_2O$, 1.4 mM NH_4Cl, pH 7) to an OD_{600nm} of 0.5. Thirty mL of the cell suspension was aliquoted into each of 15 sterile 250 mL centrifuge bottles. Appropriate volumes of a filter sterilized 3 M PNP stock solution were added to the cell suspension samples to achieve a final PNP concentration of 0, 1500, 2600, 3000 or 4000 μM. The planktonic cell suspension samples were placed in a 22°C shaking incubator (200 rpm) and viable plate counts were determined for each sample at day 0, 1, 3, 7 and 17. All cell counts in buffer solution were obtained by plating sample dilutions on TSA plates using the spiral plating method (Gilchrist *et al.*, 1973).

2.2.3. Determining optimum biofilm growth

Before beginning the biofilm buffer experiment, initial biofilm cultures were grown for 24, 48 and 72 hours to determine which cultures had the highest cell counts. Planktonic cells were grown to an OD_{600nm} of 0.5 in TSB in a 30°C shaking incubator (200 rpm). The cell suspension was then centrifuged at 5000 xg for 5 minutes to harvest the planktonic cells. The cells were washed twice in sterile phosphate buffered saline (PBS; NaCl, 137 mM, KCl, 2.7 mM, KH_2PO_4, 1.8 mM, Na_2HPO_4, 10 mM, pH 7.4). The pellet was then suspended in half strength TSB; 100 μL of the cell suspension was added to each of 9 test tubes which contained 8 mL of half strength sterile TSB as well as 0.1g of sterile glass wool. The tubes were placed in a 30°C shaking incubator (70 rpm). After

24 hours three tubes were removed to determine the biofilm cell count. The same was done after 48 and 72 h.

In order to obtain a biofilm cell count, the tubes were first washed three times with sterile PBS. Cell density in each of three washes was determined by plate counting. The cell density in the washes stabilized after three washes. To release the biofilm cells from the glass wool, 8 mL of sterile PBS and 5 g of glass beads (420-600μm) were added to the washed biofilm samples, then vortexed for two min at maximum setting. Following vortexing, the samples were sonicated in a sonication bath at 105 watts (Sonic Dismembrator model 100, Fisher Scientific, Ottawa, Ontario Canada) for 60 s. The biofilm cells, now removed from the glass wool, were plate counted using the same method as previously described for the planktonic cells.

2.2.4. Survival of biofilm cells in buffer

To grow biofilm cultures, planktonic *Pseudomonas putida* cells were first grown to an OD_{600nm} of 0.5 in TSB in a 30°C shaking incubator (200 rpm). The cell suspension (4×2 mL) was then centrifuged at 5000 xg for 5 minutes to harvest the planktonic cells. The cells were washed twice in sterile PBS, and then suspended in sterile half strength TSB.

Sixty-three sterile test tubes containing 8 mL of sterile half strength TSB and 0.1g of glass wool were each inoculated with 100 μL of cell suspension. The test tubes were placed in a 30°C shaking incubator (70 rpm) for 24 hours. After 24 h, the tubes were removed from the incubator and the liquid portion was removed from each sample. The sample tubes were then washed three times with 8mL of sterile PBS.

Three of the tubes were set aside for day 0 biofilm cell count using the method previously described. The remaining 60 tubes were filled with 8 mL of sterile MSM and PNP concentrations equal to those used in the planktonic experiment (12×0 μM, $12 \times$ 1500 μM, 12×2600 μM, 12×3000 μM, 12×4000 μM). Cell counts were determined at each PNP concentration at day 0, 1, 3, 7 and 17 using the biofilm counting method previously described.

2.2.5. Survival of planktonic P. putida cells in river water

The river water samples were obtained from the Kaministiqua River in Thunder Bay, ON, Canada. This site was chosen because it is downstream of a pulp and paper mill and from agricultural sources. The chemical composition of the water sample was analysed by the Lakehead University Centre for Analytical Services. The data, averages of four separate samples collected at the same location and at the same time, are presented as follows in mg/L: dissolved organic carbon, 18.4; NO_3, 0.14; PO_4, <0.001; SO_4, 13.4; Cl, 18.6; Al, 0.09; Ca, 11.6; Fe, 0.25; K, 1.0; Mg, 3.5; Na, 7.0; Cr, Cu and Ni <0.002; Cd and Zn <0.001; Co <0.01; pH 6.8. The background indigenous heterotrophic cell density for TSA is about 5 log CFU/mL.

Survival of the *P. putida* was monitored in the river water containing 0, 1500, 2600, 3000 and 4000 μM PNP. The experiment conditions were repeated as previously described in the buffer system. The most probable number (MPN) method was used to examine the *P. putida* density because selective growth medium for the *P. putida* was not available.

The MPN assay was performed in microtiter plates (96 wells Polystyrene, Costar, Fisher Scientific) filled with 180 μL (per well) of MSM supplemented with 0.02% yeast

extract, 100 μg/mL ampicillin (the *P. putida* is naturally resistant to ampicillin) and 200 μM PNP. Twenty μL of each cell sample was added to the first well of its corresponding row; 10× serial dilutions were then made up to the eleventh well. The last well was left with just MSM medium to ensure the PNP was not degraded in the absence of the *P. putida*. One row was also inoculated with non-sterile river water to ensure that PNP degradation was not due to background microflora naturally present in the river water.

Once all samples were added to the wells, the plates were covered and sealed in parafilm and then placed in sealed containers containing moist paper towel. The containers were then placed in a 30°C shaking incubator (100 rpm). After 5 days, the plates were removed and a positive result in a well was indicated by the degradation of PNP measured at an absorbance of 420 nm, using a spectrophotometer. Prior to checking absorbance, an equal volume of 0.5M NaOH was added to each well to ensure that colour change was not due to a change in pH. MPN cell counts were then determined using a MPN table (Woomer, 1994).

In order to ensure that numbers obtained using the MPN method were similar to those using the spiral plating method, a comparison between the two was performed. To compare, 20 mL of planktonic cell culture was grown using the same method as that used in the planktonic buffer experiment. Half of the washed cells were suspended in MSM and the other half were suspended in non-sterile river water. A portion of the cells in MSM was used to perform a viable cell count assay using the spiral plate method. Sixty μL of the remaining MSM solution was used to inoculate the MPN plate. Twenty μL was added to the first well of the first three rows. Ten times dilutions were then made in the same method as described above. The same was done using the cells suspended in

non-sterile river water. Data from the comparison showed that the MPN counts were not different from the spiral plate counts significantly (data not shown).

2.2.6. Survival of biofilm cells in river water

The experimental parameters in monitoring the survival of biofilm cells in river water were similar to the buffer system. The difference was that the biofilm cells were exposed to non-sterile river water containing PNP as opposed to being in MSM. The viable cell counts were determined using the MPN method described previously.

2.2.7. PNP-degradation by planktonic and biofilm cells in buffer and river water

Both planktonic and biofilm samples were prepared in the same manner as described previously. The concentrations of PNP added to each sample were 1500, 2600, 3000 and 4000 μM PNP. To monitor the dynamics of PNP-degradation, 1 mL aliquots of supernatant from both biofilm and planktonic samples were removed in triplicate at day 0, 3, 6, 9, 12, 15 and 18. The PNP mineralization of the 1 mL aliquots was determined using a spectrophotometer and reading at an absorbance of 420 nm.

2.2.8. Effect of PNP on biofilm cells

Biofilm samples were prepared as described for the survival experiments. However, a *gfp*-labeled strain of the PNP-degrading *P. putida* was used to aid the visualization of the biofilm structure. At day 0 and 3, samples treated with both 0 μM and 4000 μM PNP were washed 3x with sterile MSM. Then, the samples were exposed to 1.5 μM propidium iodide for 15 minutes in the dark, to stain. Propidium iodide was used because it stains those cells that are injured/dead or the membrane has been compromised. After staining, samples were washed twice with sterile MSM and then wet

mounted using a depression slide, to view under a confocal scanning laser microscope (CSLM, Olympus Fluoview FV300/BX51 Markham, Ontario Canada). The images were obtained with a 60x PlanApo NA1.4 oil immersion lens, using sequential scanning. An argon laser (10 mW, force air cooled), emitting blue light with at 488 nm, was used to excite the green fluorescence. While, the red fluorescence was excited using a helium-neon laser (1 mW) which emitted green light at 543 nm. Band pass emission was FVX-BA 510-530. Using CSLM, 3-D images were taken by performing z-slices (15 slices per image stack) and compiling each stack of z-slices using an ImagePro program (Media Cybernetics, Inc. Bethesda, Maryland USA). Cells that fluoresce red, represent those cells that are injured or dead, and cells that fluoresce green are the viable *gfp-P. putida* cells.

2.3. Results

2.3.1. Planktonic and biofilm survival in buffer

Survival patterns of the planktonic and biofilm *P. putida* cells were substantially different when exposed to high concentration of PNP in a sterile buffer system (Figure 1). For the 3000 or 4000 μM PNP treatment, the planktonic cell sample densities dropped from 8.25 to 5.7 and 5.0 log (CFU/mL) in 17 d, respectively. By contrast, when the biofilm samples were treated with 3000 and 4000 μM PNP for 17 days, their cell density dropped from 10.2 to 8.7 and 9 log (CFU/g glass wool), respectively (Figure 1B). Both the planktonic and biofilm *P. putida* cells survived well at 0 and 1500 μM PNP, with the planktonic cells maintaining a cell density of about 6.5 log (CFU/mL), while the biofilm cells maintained a cell density greater than 9 log (CFU/ g glass wool) at day 17.

Survival fractions were calculated by dividing biofilm and planktonic cell survival densities by their respective initial cell densities. By comparing the survival fraction of planktonic *P. putida* cells with the survival of biofilm *P. putida* cells in buffer, the survival rate of the biofilm cells was approximately 100-fold greater than that of the planktonic cells when subjected to 3000 or 4000 μM PNP for 17 days (Figure 2). In addition, when both planktonic and biofilm cells were treated with 1500 μM PNP, survival of *P. putida* biofilms was approximately 10-fold greater than that of planktonic cells after 17 days.

These results indicate that *P. putida* biofilm cells feature greater survival than their planktonic counterparts within a buffer system when treated with various concentrations of PNP.

2.3.2. Planktonic and biofilm survival in river water

The differences between the survival of *P. putida* planktonic and biofilm cells in river water samples were bigger than that of the buffer system containing the same level of PNP (Figure 3). By day 7, the planktonic *P. putida* sample densities decreased from 9.4 to 2.0 log (MPN/mL) when treated with 3000 and 4000 μM PNP. Also, when planktonic *P. putida* was treated with 0 or 1500 μM PNP for 7 days, there was a decrease in cell density from 9.4 to 5.0 and 6.4 log (MPN/mL), respectively. Additionally, after 17 days of treatment with 1500, 3000 or 4000 μM PNP, planktonic *P. putida* cell density had decreased from 9.4 log (MPN/ml) to approximately 3 log (MPN/mL).

By contrast, at day 7 the cell densities of the biofilm *P. putida* cells in the river water decreased from 10.3 to 9.4, 10.0, 9.5 and 10.0 log (MPN/ g glass wool) for 0, 1500, 3000 and 4000 μM PNP treatments, respectively (Figure 3B). After 17 days of treatment

with 1500, 3000 and 4000 μM PNP, the cell densities had dropped to 6.8, 8.0 and 8.9 log (MPN/g glass wool), respectively.

The survival fractions for *P. putida* planktonic and biofilm samples feature substantial differences in river water. At high PNP concentrations (3000 and 4000 μM), the biofilm cells exhibited approximately 350- and 450-fold greater survival than the planktonic cells at day 17, respectively. However, at lower PNP concentrations (1500 μM), biofilm cell survival was only about 100-fold greater than that of planktonic cell samples after 17 days (Figure 4).

In summary, planktonic *P. putida* cells did not survive well in river water treated with PNP, whereas biofilm *P. putida* cell survival was significantly better (based on a 95% level of confidence).

2.3.3. PNP degradation by planktonic and biofilm cells in buffer versus river water

Within the buffer system, the planktonic and biofilm cells showed similar patterns of PNP degradation (Figure 5A, B). Planktonic *P. putida* samples in the buffer system completely degraded the lowest PNP concentration of 1500 μM PNP by day 6. After 18 days, the planktonic cells degraded 2600 and 3000 μM PNP by 60% and 50%, respectively. The planktonic *P. putida* cells were did not degrade the PNP at 4000 μM (Figure 5A).

The biofilm *P. putida* samples in buffer degraded 1500 μM as well as 2600 μM PNP within 6 and 18 days, respectively. As well, biofilm cells degraded 3000 μM PNP by 55% after 18 days. However, similar to the planktonic cells, biofilm *P. putida* cell samples were unable to degrade 4000 μM PNP (Figure 5B).

Importantly, in contrast to the buffer system findings, the *P. putida* planktonic cells did not degrade PNP in the river water samples regardless of the PNP concentration used in the study (Figure 5C). The *P. putida* biofilm cells in the river water system, however, displayed the ability to completely degrade PNP at 1500 μM while not significantly degrading any of the 2600 μM, 3000 μM or 4000 μM PNP river water samples by day 18 (Figure 5D).

Overall, in both the buffer and river water systems, biofilm *P. putida* cells could degrade more PNP than planktonic *P. putida.* Nonetheless, neither planktonic or biofilm *P. putida* cells could degrade much PNP in the river water system compared to the buffer system.

2.3.4. The Effect of PNP on Biofilm Cells

Confocal laser scanning microscopy (CLSM) analysis indicates that *P. putida* biofilms in buffer at day 0, not treated with PNP had few cells stained red by the propidium iodide (PI). Since the PI enters cells through their compromised cell membranes, our results inferred that the majority of the biofilm cells were intact with little membrane damage at time 0. Similar results were observed after 3 days incubation for biofilms not treated with PNP. Again the majority of cells were intact, though a larger proportion did stain red indicating an increase in damaged cells relative to day 0 biofilms. When biofilm cells were treated with 4000 μM PNP there was a substantially greater amount of injured cells (red/stained). Those cells that were injured were mostly found on the outer layer of the biofilms. The *gfp-P. putida* cells in the interior of the biofilm samples remained green, indicating that the interior cells were not affected by the high

PNP concentration in the surrounding. This is clearly shown in the cross-section of the biofilm in Figure 6(C) (indicated by the white arrow).

In Figure 7A and B, very few injured cells were observed on the *gfp-P. putida* biofilms in river water without the PNP treatment. However, the biofilm treated with 4000 μM PNP appeared to have stained almost entirely red. A very small proportion of viable (green) *gfp-P. putida* cells remained in the interior of the biofilm. Also to be considered, injured or dead (red) indigenous microorganisms could not be distinguished from injured (red) *gfp-P. putida* cells in the river water system.

Overall, in both buffer and river water systems, PNP injured *gfp-P. putida* cells and /or indigenous river water species, primarily on the outer layer of the biofilms; while, the interior cells (green) were shown to be protected from the PNP. Also, the effect of PNP injuring cells was greater for biofilms in the river water system.

2.4. Discussion

Recently, research has shown that bacteria occurring in the environment are more frequently associated with other bacterial species in surface-associated biofilms, as opposed to living freely as planktonic cells (Costerton, 1987). This knowledge has guided the use of biofilms in bioreactors for the removal of toxic organic compounds, such as the accumulation of phenols in wastewater treatment plants (Arvin *et al.*, 1991; Israni *et al.*, 2002).

Several studies explore how biofilms in the bioreactors can be improved to increase the survival and degradation of phenols in wastewater treatment plants. One particular study, performed using a *P. putida* strain, showed that biodegradation efficiency for biofilms in a trickling bed reactor could be improved by determining

optimal temperature, monitoring biofilm thickness and biomass, and biological activity via oxygen consumption (Sá and Boaventura, 2001). However, planktonic or free-living cells are often used in the production of bioreactors to predict the degradation rate and tolerance of biofilm cells to organic compounds. The use of planktonic cells in this manner is being questioned (Mirpuri *et al.*, 1996).

In regard to these findings, biofilm *P. putida* cells in this study displayed better ability in survival and PNP degradation in high concentrations of PNP in comparison to planktonic cells in buffer; and, of utmost importance, in a natural aquatic environment, such as river water.

The results of the biofilm and planktonic *P.putida* survival in both buffer and river water suggest that the *P. putida* biofilms act to protect cells from exposure to PNP. The observations in the river water system are of particular interest because not only did the biofilm and planktonic *P. putida* cells need to survive under the stress of exposure to high levels of PNP, they also had to compete with the indigenous microbial populations in the river water samples. It is likely that the added stress of competition resulted in the poor survival of the planktonic *P. putida* in river water at all concentrations of PNP, after 17 days (Figure 3). However, the biofilm *P. putida* may be sheltering viable cells not only from the PNP but may also be from competition of other microbial species in the river water allowing biofilm samples to maintain a high cell density (Watterworth *et al.* 2006).

The *P. putida* biofilm cells had much higher survival ability than the planktonic cells for both buffer and river water systems, this in turn, allowed the biofilms to degrade substantially more PNP than planktonic cells for both experiments. However, relatively

speaking, despite biofilm *P. putida* cells maintaining a high cell density (on average 7.6 log (MPN/g of glass wool)) after 17 days in river water, they only degraded 1500 μM PNP after 17 days. Werner *et al.* (2004) showed that biofilms of *Pseudomonas aeruginosa* were primarily made up of metabolically inactive cells because oxygen only penetrated about 50 μm into the biofilm. They demonstrated that there was a small layer of cells (about 30 μm wide) at the periphery of the *P. aeruginosa* biofilms where there were actively growing cells. Therefore, if our PNP-degrading *P. putida* cells on the upper surface of the biofilm structure were not actively growing for some reason, PNP degradation would not be observed. As mentioned earlier, the biofilm *P. putida* cells in river water are stressed by both the toxicity of PNP and most likely by competition of indigenous river water microorganisms. The added stress may cause an increase of damage to the narrow layer of *P. putdia* cells that were located on the upper surface of the biofilm. This could account for the low level of PNP-degradation seen for *P. putida* biofilms in river water compared to the buffer system.

Additionally, not only is there a limitation of oxygen within biofilms but also a limitation of nutrients (Chambless *et al.*, 2005). Starvation of interior biofilm cells causes the induction of adaptive stress response genes, such as the stationary phase sigma factor gene (*rpoS*). This triggers the biofilm cells to be more resistant to toxic compounds (Walters *et al.*, 2003; Xu *et al.*, 1998). This may explain why there were a high number of viable *P. putida* biofilm cells in the river water, despite a low PNP degradation capacity.

The CLSM images of the PNP-treated 3-day old *P. putida* biofilms (Figure 6) support our hypothesis that the actively growing cells at the periphery of the *P. putida*

biofilm were injured by the stress of PNP and likely by competition of indigenous species. Both the buffer and river water systems show that cells on the outer layer of the *P. putida* biofilms were damaged by the increased stress. Some organic pollutants cause membrane damage to bacterial cells by making their membrane more fluid and permeable. It is believed that they preferentially partition in membranes (Heipieper *et al.*, 1991; Heipieper and DeBont, 1994).

Chambless *et al.* (2005), produced a 3-D computer model to show how four possible mechanisms act together to protect biofilms from antimicrobials. This computer model suggests that (1) there is slow penetration of the antimicrobials through the biofilm; (2) some biofilm cells express adaptive stress response genes; (3) an altered microenvironment is created, and (4) the persister cells exist within biofilms to allow the bacteria to survive well in response to toxic compounds. These hypothetical mechanisms may also be applied to explain the survival of the PNP-degrading *P. putida* biofilm cells when exposed to high concentrations of PNP, and competition from indigenous species.

Overall, *Pseudomonas putida* biofilms are a better choice than planktonic *P. putida* cells for the bioremediation of *p*-nitrophenols in the environment. However, even biofilm cells can only exhibit a limited ability to biodegradate organic contaminants under natural environments. Therefore, further research is required to optimize the degradation capabilities of *P. putida* biofilms in the environment.

Figure Legend

Figure 1. Survival of (A) biofilm and (B) planktonic, *P. putida* cells in MSM buffer when 0 μM ●, 1500 μM ○, 3000 μM ▼ and 4000 μM ▽ PNP is added.

Figure 2. Fraction of surviving biofilm and planktonic cells survival in MSM buffer when 0 μM, 1500 μM, 3000 μM and 4000 μM PNP is added.

Figure 3. Survival of biofilm and planktonic *P. putida* cells in river water when 0 μM ●, 1500 μM ○, 3000 μM ▼and 4000 μM ▽ PNP is added.

Figure 4. Fraction of surviving biofilm and planktonic cells survival in river water, when 0 μM, 1500 μM, 2600 μM and 3000 μM PNP is added.

Figure 5. Amount of varying concentrations of PNP degraded by planktonic and biofilm *P. putida* cells in buffer and river water, when 1500 μM ●, 2600 μM ○, 3000 μM ▼and 4000 μM, ▽PNP are added.

Figure 6. Confocal microscopy pictures of biofilms in MSM buffer for day 0 and 3 when treated with 0 μM and 4000 μM PNP, respectively. Scale = 20 microns.

Figure 7. Confocal microscopy pictures of biofilms in river water for day 0 and 3 when treated with 0 μM and 4000 μM PNP. Scale bar = 20 microns.

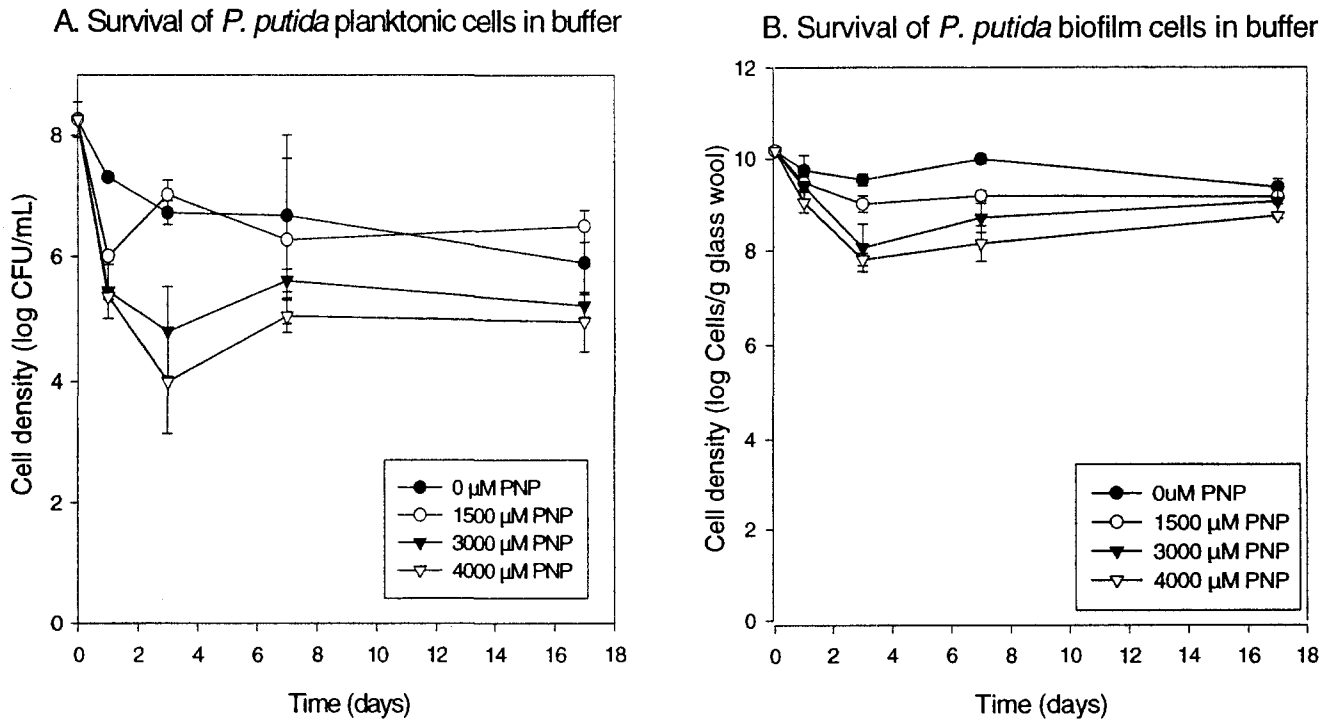

A. Survival of P. putida planktonic cells in buffer
Cell density (log CFU/mL)
Time (days)
0 µM PNP
1500 µM PNP
3000 µM PNP
4000 µM PNP
B. Survival of P. putida biofilm cells in buffer
Cell density (log Cells/g glass wool)
Time (days)
0uM PNP
1500 µM PNP
3000 µM PNP
4000 µM PNP

Figure 1

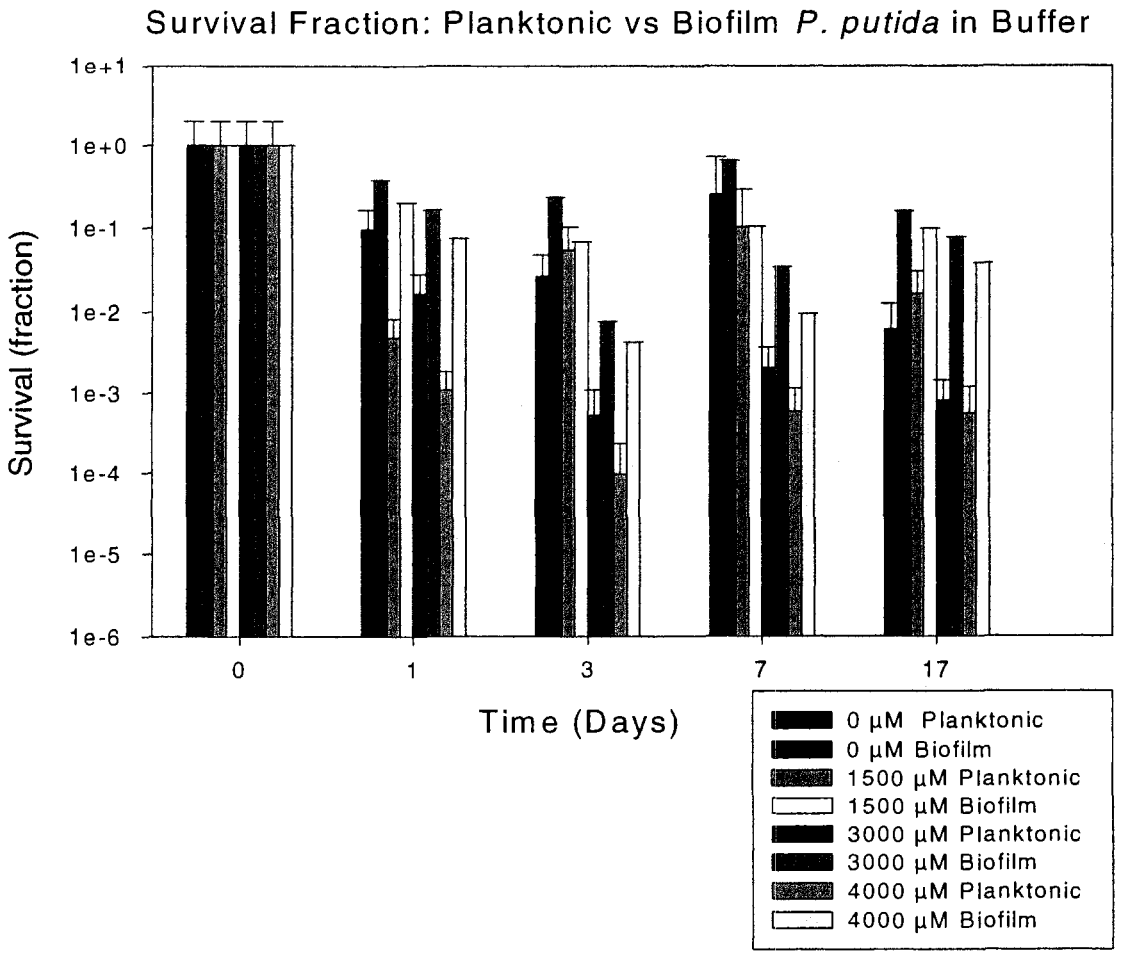

Survival Fraction: Planktonic vs Biofilm P. putida in Buffer
Survival (fraction)
1e+1
1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
1e-6
0
1
3
7
17
Time (Days)
0 µM Planktonic
0 µM Biofilm
1500 µM Planktonic
1500 µM Biofilm
3000 µM Planktonic
3000 µM Biofilm
4000 µM Planktonic
4000 µM Biofilm

Figure 2

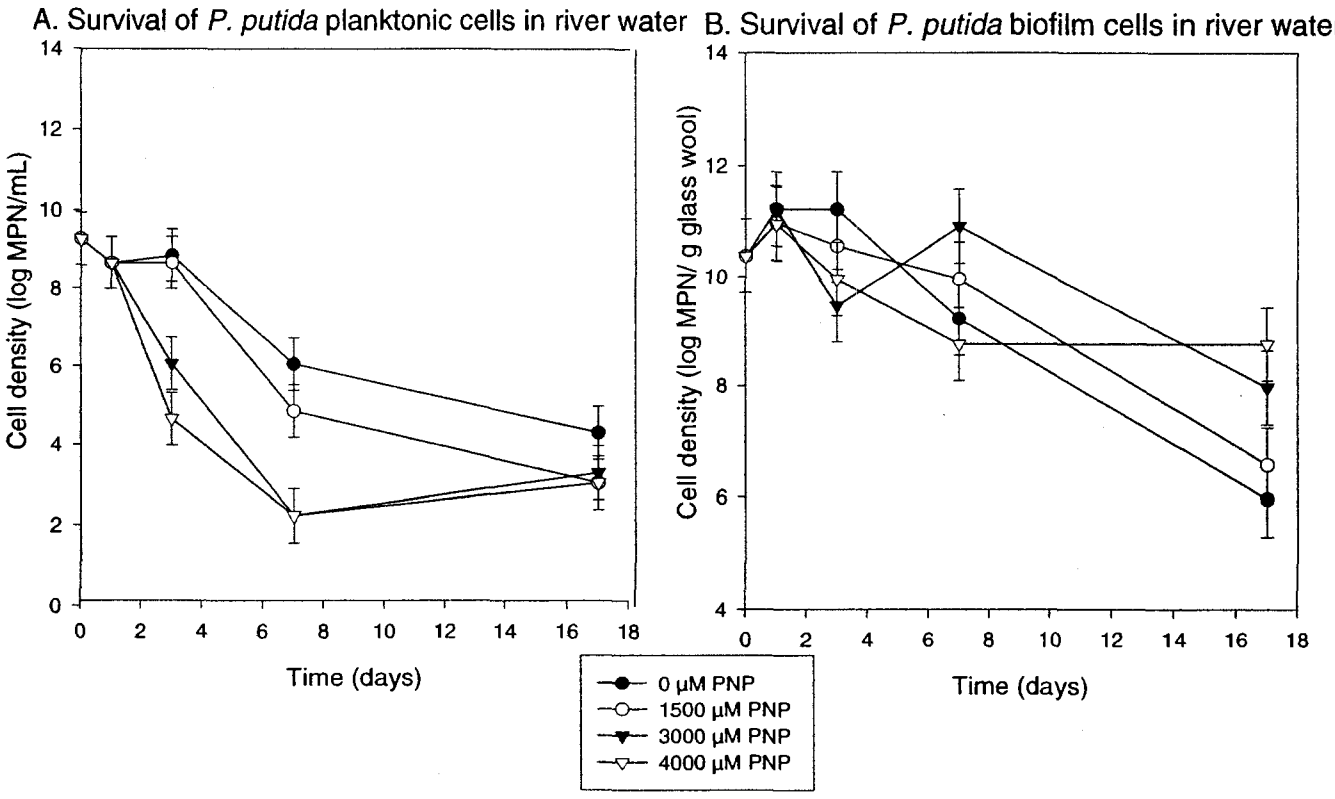

A. Survival of P. putida planktonic cells in river water
B. Survival of P. putida biofilm cells in river water
Cell density (log MPN/mL)
Cell density (log MPN/ g glass wool)
Time (days)
Time (days)
0 µM PNP
1500 µM PNP
3000 µM PNP
4000 µM PNP

Figure 3

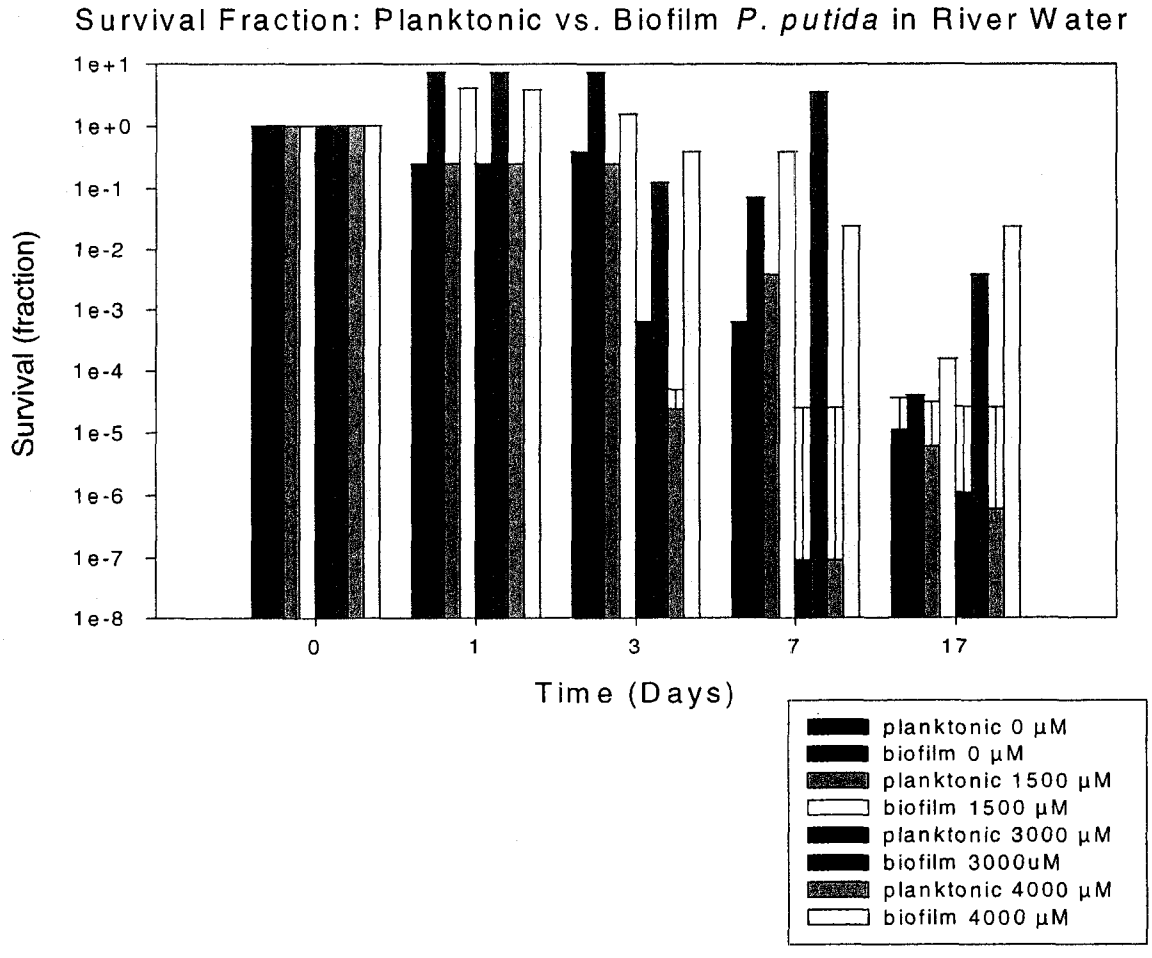

Survival Fraction: Planktonic vs. Biofilm P. putida in River Water
Survival (fraction)
1e+1
1e+0
1e-1
1e-2
1e-3
1e-4
1e-5
1e-6
1e-7
1e-8
0
1
3
7
17
Time (Days)
planktonic 0 µM
biofilm 0 µM
planktonic 1500 µM
biofilm 1500 µM
planktonic 3000 µM
biofilm 3000uM
planktonic 4000 µM
biofilm 4000 µM

Figure 4

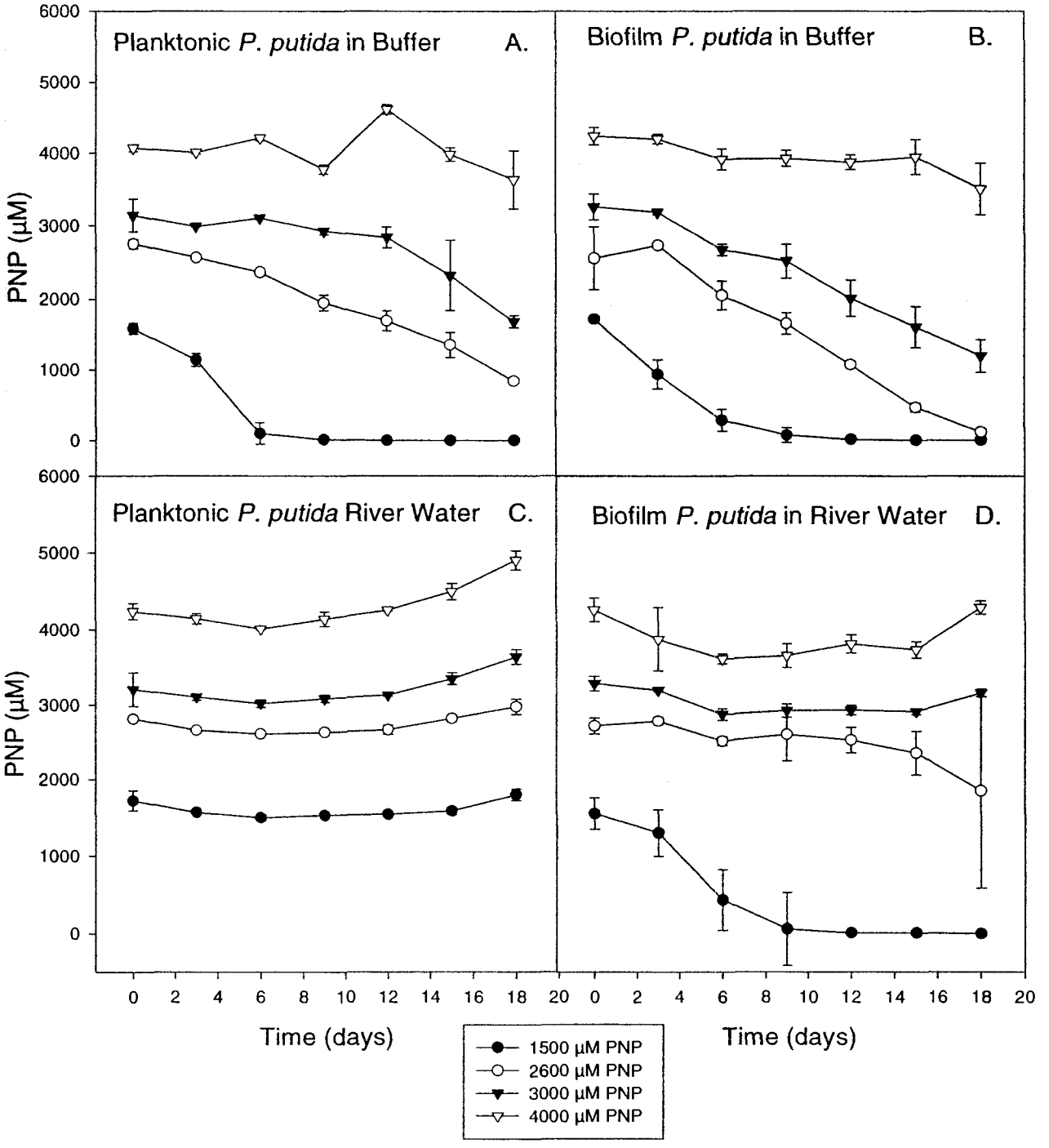

Degradation of Varying Concentrations of PNP By Planktonic and Biofilm
P. putida Cells in Buffer versus River Water
Planktonic P. putida in Buffer
A.
Biofilm P. putida in Buffer
B.
Planktonic P. putida River Water
C.
Biofilm P. putida in River Water
D.
PNP (µM)
Time (days)
1500 µM PNP
2600 µM PNP
3000 µM PNP
4000 µM PNP

Figure 5

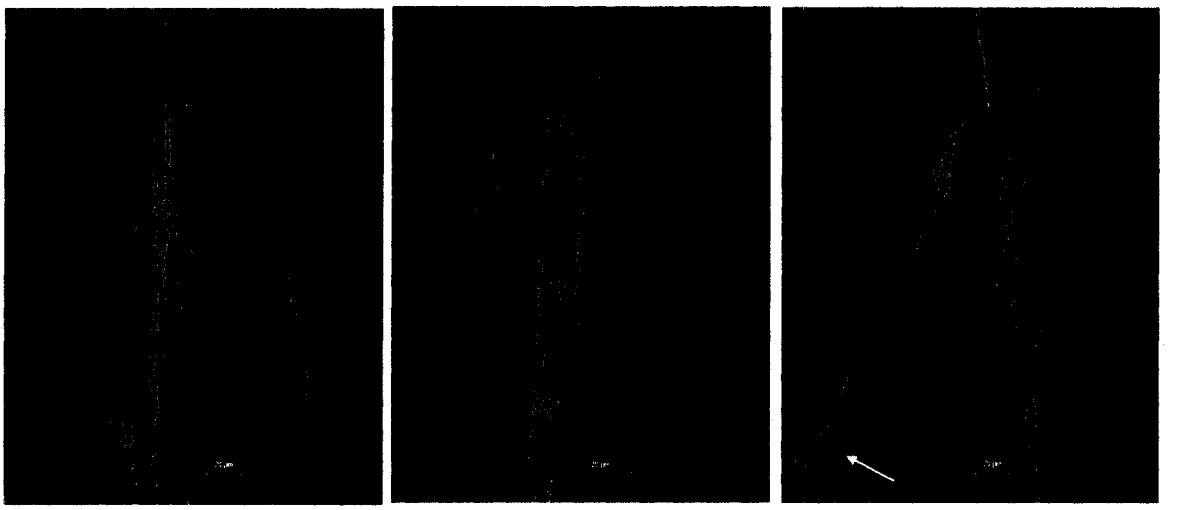


Figure 6

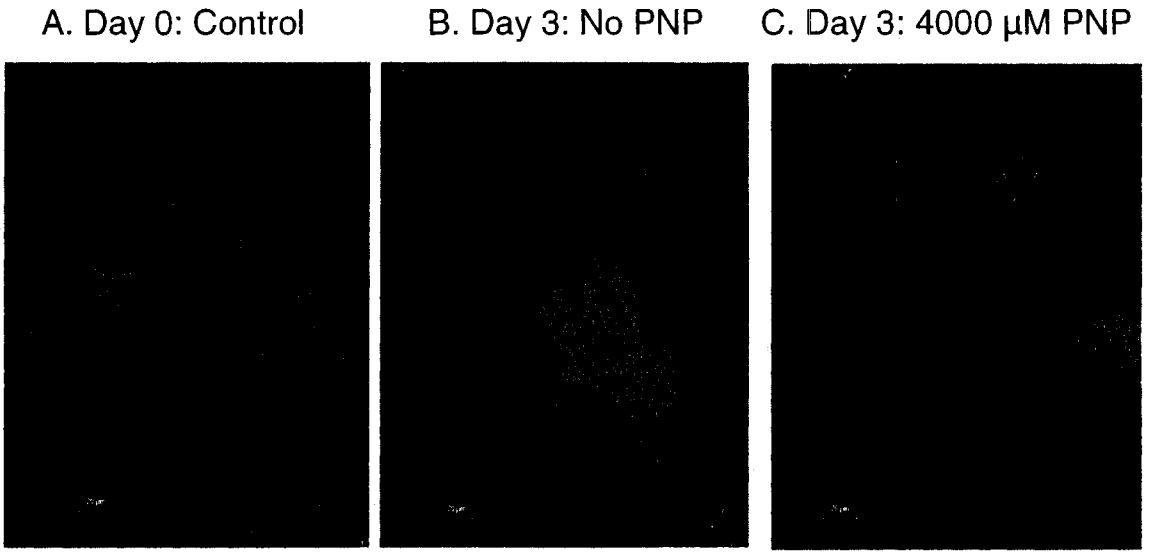


Figure 7

References

Arvin E, Jensen BK, Gundersen AT. 1991. Biodegradation Kinetics of Phenols in an Aerobic Biofilm at Low Concentrations. Water Sci Technol 23:1375-84.

Bhushan B, Chauhan A, Samanta SK, Jain RK. 2000. Kinetics of biodegradation of *p*-nitrophenol by different bacteria. Biochem Biophys Res Commun 274:626-30.

Boehncke A, Koennecker G, Mangelsdorf I, Wibbertmann A. 2000. Mononitrophenols,concise international chemical assessment document 20. [http:/www.who.int/ipcs/publications/cicad/en/cicad20.pdf.].

Brown MRW, Gilbert P. 1993. Sensitivity of biofilms to antimicrobial agents. J Appl Bacteriol 74, S87-S97.

Brown MRW, Allison DG, Gilbert P. 1988. Resistance of bacterial biofilms to antibiotics a growth-rate related effect. J Antimicrob Chemother 22:777-80.

Chambless J, Hunt SM, Stewart PS. 2005. A Three-Dimensional Computer Model of Four Hypothetical Mechanisms Protecting Biofilms from Antimicrobials. Appl. Environ Microbiol 72:2005-13.

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ.1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435-64.

Davies D. 2003. Understanding biofilm resistance to antibacterial agents. Nature Reviews Drug Discovery 2:114-22.

Gilchrist JE, Campbell JE, Donally CB, Peeler JT, Delaney JM. 1973. Spiral Plate Method for Bacterial Determination. Appl Microbiol 25:244-52.

Hanne LF, Kirk LL, Appel SM, Narayan AD, Bains KK. 1993. Degradation and induction specificity in *Actinomycetes* that degrade *p*-nitropheonl. Appl Environ Microbiol 59:3505-08.

Heipieper HJ, De Bont JAM. 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of fatty acid composition of membranes. Appl Environ Microbiol 60:440-4.

Heipieper HJ, Keweloh H, Rehm HJ. 1991. Influence of phenol on the growth and membrane permeability of free and immobilized *Escherichia coli.* Appl Environ Microbiol 57:1213-7.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 1994. Berygey's Manual of Determinative Bacteriology 9th Ed. Williams and Wilkens Publishing Ltd. Baltimore, USA. p155.

Israni SH, Koli SS, Patwardhan AW, Melo JS, D'souzaS F. 2002. Phenol degradation in rotating biological contractors. J Chem Technol Biotechnol 77:1050-7.

Jain RK, Dreisbach JH, Spain JC. 1994. Degradation of *p*-nitrophenol via 1,2,4-benzentriol by an *Arthrobacter* sp. Appl Environ Microbiol 60:3030-2.

Kadiyala V, Spain JC. 1998. A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. Appl Environ Microbiol 64:2479-84.

Kamata K, Hayashi H, Taneda S, Suzuki A, Toda N, Sakush-Ima A, Seki K, Mori Y. 2004. Isolation and identification of nitrophenols in diesel exhaust particles (DEP) that exhibit vasodilating activity using bioassay-directed fractionation. Toxicol Appl Pharmacol 197:237.

Leung KT, Tresse O, Errampali D, Lee H, Trevors JT. 1997. Mineralization of *p*-nitropheonl by pentachlorophenol-degrading *Shingomonas* spp. FEMS Microbiol Lett 155:107-14.

Lewis K. 2001. Riddle of biofilm resistance. Antimicrobial Agents Chemother 45:999-1007.

Li CM, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. 2006. Estrogenic and anti-androgenic activities of 4-nitrophenol in diesel exhaust particles. Toxicol Appl Pharmacol 217:1-6.

Madyastha KM, Shankar VN. 1994. Role of metabolites in microbial conversion of 3(beta)-acetoxy-19-hydroxycholest-5-ene into estrone. Appl Environ Microbial 60:1512-8.

Mah TFC and O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34-9.

Mirpuri R, Jones W, Bryers JD. 1996. Toluene Degradation Kinetics for Planktonic and Biofilm-Grown Cells of *Pseudomonas putida* 54G. Biotechnol Bioeng 53:535-46.

Moore M, Trevors J, Lee H, Leung KT. 2005. Stress survival responses of a carbon-starved *p*-nitrophenol-mineralizing *Moraxella* strain in river water. Can Jrnl Microbiol 51;223-9.

Munnecke DM, Hsieh DPH. 1976. Pathways of microbial metabolism of parathion. Appl Environ Microbiol 31:63-9.

Prakash D, Chauhan A, Jain RK. 1996. Plasmid-encoded degradation of *p*-nitrophenol by *Pseudomonas cepacia*. Biochem Biophys Res Commun 224:375-81.

Raymond DGM, Alexander M. 1971. Microbial metabolism and co-metabolism of nitrophenols. Pestic Biochem Physiol 1:123-30.

Sá CSA and Boaventura RAR. 2001. Biodegradation of phenol by *Pseudomonas putida* DSM 548 in a trickling bed reactor. Biochem Eng Jrnl 9:211-9.

Simpson JR and Evans WC. 1953. The metabolism of nitrophenols by certain bacteria. Biochem J 55(320th Meeting):xxiv.

Spain JC, Gibson DT. 1991. Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl Environ Microbiol 57:812-9.

Sterjiades R, Pelmont J. 1989. Occurences of two different forms of protocatechuate 3,4-digoxygenase in a *Moraxella* sp. Appl Environ Microbial 55:340-7.

Stewart PS. 2002. Mechanisms of antibiotic resistance in bacterial biofilms. Int Jrnl Med Microbiol 292:107-13.

Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358:135-8.

Tallon P, Leung KT. 2005. The impact of *rpoS* deletion on biofilm formation of a *p*-nitrophenol degrading *Moraxella* strain. Proc Can Soc Microbiol. 55th conference, Halifax, Nova Scotia, Canada. p63.

Tremp J, Mattrel P, Fingler S, Giger W. 1993. Phenols and nitrophenols as tropospheric pollutants: emissions from automobile exhaust and phase transfer in the atmosphere. Water Air Soil Poll 68:113-23.

US Environmental Protection Agency (1976) Water quality criteria. USEPA, Washington, DC.

Van Veen JA., van Overbeek LS., van Elsas JD. 1997. Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61:121-35.

Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrobial Agents Chemother 47:317-23.

Watterworth L, Rosa B, Schraft H, Topp E, Leung KT. Survival of various ERIC-genotypes of Shiga toxin-producing *Escherichia coli* in well water. Water Air Soil Pollut 177: 367-82.

Werner E, *et al.*. 2004. Stratified Growth In *Pseudomonas aeruginosa* Biofilms. Appl Environ Micriobiol 70:6188-96.

Wittich RM, Rast HG, Knackmuss, H.J. 1988. Degradation of naphthalene-2,6- and naphthalene-1,6-disulfonic acid by a *Moraxella* sp. Appl Environ Microbiol 54:1842-7.

Woomer PL. 1994. Most Probable Number Counts. Weaver *et al.*. Methods of Soil Analysis: Part 2 Microbiological and Biochemical Properties. Soil Science Society of America. Michigan, USA. p71-75.

Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl Environ Microbiol 64:4035-9.

Chapter 3: The role of *rpoS* on the survival of a *p*-nitropheol degrading *Pseudomonas putida* strain in planktonic and biofilm phases

Abstract

The expression of the stationary phase sigma factor gene (*rpoS*) and biofilm formation are important bacterial stress-survival strategies. To further explore the relationship between these two factors, the survival and competitiveness of the PNP-degrading *P. putida* strain as well as its *rpoS*$^-$ mutant were examined in both planktonic and biofilm phases. To distinguish wild-type (WT) and knock-out (KO) strains in mixed samples, they were labeled with a red fluorescent protein-gentamycin resistant and a green fluorescent protein-gentamycin resistant gene cassette via a Tn7 transposon system, respectively. In single cultures, the KO-*gfp* biofilm featured a greater than 4-fold increase in biovolume than the WT-*rfp*. However, mixed biofilm biovolume revealed that KO-*gfp* was reduced by 75.6% compared to the single culture KO-*gfp* biofilm biovolume, suggesting that the WT-*rfp* suppressed KO-*gfp* biofilm formation. Competitiveness studies showed that carbon-starved planktonic WT-*rfp* single cultures achieved a 3.5-fold greater survival rate in 0.85% saline than their KO-*gfp* counterparts, and out-competed them by more than 13-fold in mixed culture samples. Conversely, there were no significant survival differences between the KO-*gfp* and WT-*rfp* strains in biofilm samples. Finally, differences in cellular cohesiveness were evident, after 60 min washing with 0.2% SDS resulted in 32 and 89% cells detached from the KO and WT samples, respectively. These results indicate that although the KO produced more biofilm than the WT, it may in fact not confer an advantage in survival because the KO

biofilm has a lower percentage of surviving cells and cannot readily detach to colonize new habitats. Overall, these findings provide new perspectives towards a more complete understanding of the role of *rpoS* in biofilm formation.

3.1. Introduction

In many natural environments, microorganisms are often found living in the form of complex multicellular biofilm structures adhering to surfaces. The advantage of biofilm formation is that it provides a protective extent of homeostasis and stability in continuously changing environments (Hall-Stoodley and Stoodley, 2005). Biofilms have significant relevance to medical, industrial and environmental settings because of their increased survival or tolerance to compounds such as antimicrobials and antibiotics (Brown and Gilbert, 1993). Biofilms have also been shown to have an increased access to nutrients adhered to surfaces (Flemming 1993; Geesey *et al.*, 1992).

It has been shown that formation of a bacterial biofilm is through a series of developmental stages, including attachment, formation of microcolonies, proliferation and production of exopolymeric substances (EPS), maturation of the biofilm and finally detachment or dispersal of bacterial cells from the biofilm structure. Detachment is considered as one of the crucial steps of biofilm development because it is important for bacteria to colonize new surfaces as nutrients are exhausted by a mature biofilm (Allison *et al.*, 1990; Bester *et al.*, 2005).

The stationary phase sigma factor (RpoS) in *Escherichia coli*, coded for by the *rpoS* gene, is known to be the central regulator for the expression of more than 50 genes for cells in stationary phase (Hengge-Aronis, 2002a, b; Kolter *et al.*, 1993). In *Pseudomonas aeruginosa,* RpoS level is known to increase upon entering stationary

phase and is similar in many other gram negative bacteria (Fujita *et al.*, 1994). For planktonic cells, such as the *p*-nitrophenol (PNP)-degrading *Moraxella* strain (re-classified as *Pseudomonas putida* in this study) and the *Pseudomonas fluorescens* Pf-5, it has been shown that carbon starvation triggers an increase in cellular resistance to oxidation, high temperature shock, osmotic stresses and many other environmental stresses (Moore *et al.*, 2005; Stockwell and Loper, 2005). During glucose starvation there will be an increase in RpoS levels, and the RpoS-RNA polymerase complex will be able to transcribe greater than 50 genes related to stress response and protection (Hengge-Aronis, 2002a,b). Knocking out *rpoS* has been shown to affect the stress responses as well as the expression of virulence factors in *Pseudomonas aeruginosa* (Suh *et al.*, 1999). However, there are contradicting observations in the role of RpoS with respect to biofilm development.

Observations in a study of *Escherichia coli* have shown that the *rpoS* knockout mutant produces a considerably smaller biofilm compared to the wild-type (Adams and McLean, 1999). Similarily, it has also been shown that *rpoS*$^-$ strains are incapable of attachment to surfaces (Prigent-Combaret *et al.*, 2001; Schembri *et al.*, 2003). In contrast to these observations, recent studies on an *rpoS*$^-$ of *E. coli* and *Pseudomonas aeruginosa* have shown better biofilm formation for the *rpoS*$^-$ mutants than the their wildtype counterparts (Corona-Izquierdo and Membrillo-Hernandez, 2002; Whiteley *et al.* 2001). As well, a study by Corona-Izquierdo and Membrillo-Hernandez (2002) suggested that *rpoS*$^-$ *E.coli* secretes a chemical signal that causes an increase in biofilm production of the wildtype bacteria (Corona-Izquierdo and Membrillo-Hernandez, 2002).

The formation of biofilms and the expression of *rpoS* have been shown to increase survival of bacteria in the environment (Brown *et al.*, 1988; Lange and Henggearonis, 1991; Mah and O'Toole, 2001; Stewart and Costerton, 2001). These factors are important for survival of microorganisms including those related to remediation of organic pollutants in the environment. *p*-Nitrophenol is found extensively in the environment as it is a precursor of pharmaceuticals, a breakdown product of insecticides and a waste product in diesel fuel exhaust (Spain, 1995). Because of the widespread nature of *p*-nitrophenol and other nitrophenolic compounds, they are considered a major group of pollutants by the USEPA (1976). In our previous studies, we have shown that a *Pseudomonas putida* strain (formerly classified as a *Moraxella* strain) is able to remove PNP from environmental samples (Moore *et al.*, 2005) and survives significantly better in biofilm phase (Maki *et al.*, 2005). However, little is known about the role of *rpoS* on the survival of pollutant-degrading bacteria in biofilm settings.

In this study, the *p*-nitrophenol-degrading *Pseudomonas putida* strain and its *rpoS*$^-$ mutant are used to examine the role of *rpoS* in biofilm development, specifically their competitiveness in both the planktonic and biofilm phases. In addition, the biofilm cohesiveness of both the *P. putida* wild-type and the *rpoS*$^-$ mutant is also compared.

3.2. Materials and Methods

3.2.1 Bacterial strains

Bacterial strains and plasmids used in this study are listed in Table 1. The *P. putida* strain (WT) used in this study (formerly identified as a *Moraxella* species) was isolated from an activated sludge sample and has the ability to degrade *p*-nitrophenol

(PNP) (Spain and Gibson, 1991). The 16S rDNA sequence of this bacterium shows more than 99% homology to known *Pseudomonas putida* 16S rDNA sequences in the GenBank (Tallon and Leung, 2005). API 20E test was used (Biomerieux, St. Laurent, Quebec) which resulted in a good-likelihood selection for *Pseudomonas putida*. Further confirmative biochemical tests verified that the strain was in fact a *Pseudomonas putida*, included positive test results for catalase, oxidase, fermentation and motility (Holt *et al.*, 1994). The *P. putida* strain is grown optimally in Trypticase Soy Broth (TSB; Becton Dickinson and Co., Sparks, MD) medium at 30°C and was stored in sterile 25% glycerol at -80°C before use.

An *rpoS*⁻ mutant strain (KO) was also used. The *rpoS* gene of the KO mutant was disrupted by a kanamycin resistant gene inserted to a *Sal*I site located near the middle of the *rpoS* gene (Tallon and Leung, 2005). This strain is also grown optimally at 30°C in TSB medium with the addition of kanamycin (Km 50 $\mu g\ mL^{-1}$).

Escherichia coli S17-1λ containing the transposon plasmid pMiniTn7(Gm)$P_{PA1/04/03}$*gfp-a* or the pMiniTn7(Gm)PPA1/04/03*DsRedExpress-a* was grown in Luria-Bertani medium (LB, Fischer Scientific, Fair Lawn, New Jersey) with 10 $\mu g\ mL^{-1}$ gentamycin at 37°C. Also, another *E. coli* S17-1λ containing the pUX-BF13 helper plasmid was grown in LB with 100 $\mu g\ mL^{-1}$ ampicillin (Sigma-Aldrich, address) (Lambertsen *et al.*, 2004). An *E. coli* MM294 containing the delivery/conjugation plasmid pRK2013 was grown in LB medium with 50 $\mu g\ mL^{-1}$ kanamycin (Moore *et al*, 2005).

The two genotypes of the PNP-degrading *P. putida* strain used in this study are the wild-type strain labeled chromosomally with the *rfp* (WT-*rfp*) and the *rpoS*⁻ knockout

mutant strain labeled chromosomally with the *gfp* (KO-*gfp*). These strains were grown at 30°C in Trypticase Soy Broth (TSB; Becton Dickinson and Co., Sparks, MD) medium. The WT-*rfp* was grown in TSB with gentamycin (Gm 10 μg mL^{-1}) and the KO-*gfp* with gentamycin (Gm 10 μg mL^{-1}) and kanamycin (Km 50 μg mL^{-1}).

3.2.2. gfp and rfp labeling

The WT and the KO strains were chromosomally labeled with the *rfp* and *gfp*, respectively, using a mini-Tn7 insertion system (Lambertson *et al.*, 2004). To perform the labeling protocol, tetraparental conjugation was used with a protocol adopted from De Bruijn and Rossbach (1994). Before conjugation, spontaneous rifampicin resistant (Rif^r) WT and KO strains were produced from the WT and KO parent *P. putida* strains, respectively (Ramirez *et al.*, 1998). This allowed for selection of the *P. putida* transconjugants against the *E. coli* strains. Both the WT-Rif^r and the KO-Rif^r *P. putida* strains tested positive for PNP-degradation. The biodegradation was determined by measuring the disappearance of PNP using spectrophotometry at OD_{420nm} (Moore *et al.*, 2005). The WT-Rif^r and the KO-Rif^r were recipients of the MiniTn7 (Gm)PPA1/04/03*DsRedExpress-a,* and pMiniTn7(Gm)$P_{PA1/04/03}$*gfp-a,* respectively. The helper plasmid (pUX-BF13) consists of the transposase genes required for transposition of *gfp* and *rfp* in the WT-Rif^r and the KO-Rif^r recipients, respectively. A delivery plasmid (pRK2013) which induced conjugation between the *P. putida* strains and the *E. coli* strains containing the appropriate MiniTn7 and helper plasmids. *E. coli* cell cultures containing the plasmids: pMiniTn7(Gm)$P_{PA1/04/03}$*gfp-a,* pMiniTn7(Gm)PPA1/04/03*DsRedExpress-a,* pUX-BF13, and pRK2013 were grown in 30 mL of LB containing appropriate antibiotics (refer to table 1) to an OD_{600nm} 1. Cells

were then harvested using 10 mM sterile $MgSO_4$ solution and resuspended to an OD_{600nm} of 1 in 10 mM $MgSO_4$. The donors and recipients were mixed in a 1:1:1:1 ratio. Proceeding, 50 μL of cell mixture was added to a sterile polycarbonate membrane filter (2 μm pore size) placed on a TSA plate. The samples were incubated at 30°C overnight. The filters were then removed and vortexed in 5 mL sterile 10 mM $MgSO_4$. After resuspension, the cell suspension was serially diluted and plated on Tryptic Soy Agar (TSA; Difco Detroit, Michigan) with appropriate antibiotics (refer to Table 1), to select for WT-*rfp* and KO-*gfp P. putida* transconjugants. Colonies grown were viewed using a 4x objective lens with an Olympus Fluoview FV300/BX51 confocal scanning laser microscope (CSLM). Colonies that exhibited the brightest red or green fluorescence for the WT-*rfp* or the KO-*gfp* were selected, respectively, for further study.

The WT-*rfp* and the KO-*gfp P. putida* strains were also tested for PNP-degradation using spectrophotometry described previously. As well, both the WT-*rfp* and the KO-*gfp* were tested for biochemical similarity to their parent strains (WT and KO) using API 20E test strips (Biomerieux, St. Lawrent, Quebec, Canada). Furthermore, growth curves for the WT-*rfp* and KO-*gfp* and, mixed WT-*rfp* and KO-*gfp*, were determined and were used to compare to the growth rate of the WT and KO *P. putida* strains, respectively. The WT-*rfp*, KO-*gfp*, WT and KO parent strains were inoculated into sterile TSB to an initial cell density of OD_{600nm} 0.5, incubated at 30°C and shaken at 150 rpm. To examine whether the WT-*rfp* and KO-*gfp* would compete with each other in a mixed culture condition, mixed WT-*rfp*:KO-*gfp* samples were created by inoculating sterile TSB with a 1:1 ratio of WT-*rfp* and KO-*gfp* until an initial cell density of OD_{600nm} 0.5 was reached. Every two hours, 1 mL from each of the 4 samples was removed,

serially diluted, and plated on TSA containing appropriate antibiotics (refer to Table 1). Cell densities (CFU/mL) were determined from colony counts.

Finally, to ensure the correct insertion of both the *rfp* and *gfp* labels into the *att*Tn7 intergenic region, which is about 25 bp downstream of the *glmS* gene in *P. putida*, a PCR assay was adapted to validate correct insertion of the marker genes (Lambertsen *et al.*, 2004). The *glmS* primer (5'-ATTCTGGCCAAGTCGGTGAC-3') anneals to a target sequence that is about 30 bases from the 3' end of the *glmS* gene, while the Tn7R109 primer (5'-CAGCATACCTGGACTGATTT-3') binds to the 109th nucleotide of the mini-Tn7. Correct amplification will give a DNA fragment of about 160-200 bp. For PCR parameters, the initial denaturing temperature was held for 2 min at 94°C followed by 30 cycles of 1 min denaturing at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C. Completion of these cycles was followed by a final extension at 72°C for 5 min. Following electrophoresis, the PCR products were purified and sequenced (MOBIX, Hamilton, Ontario, Canada). The sequencing results were evaluated using the GenBank database to verify the incorporation of the fluorescent genes at the intergenic region of the *Pseudomonas* strains.

3.2.3. Survival of planktonic WT-rfp and KO-gfp

Overnight cultures of each WT-*rfp* and KO-*gfp* *Pseudomonas putida* strains was used to inoculate three flasks containing 150 mL of sterile TSB to an OD_{600nm} 0.2. The cells were grown for approximately 2.5 hours to OD_{600nm} 0.5, harvested by centrifugation (3000 x g for 10 min), and re-suspended in same volume of sterile distilled water. The cells were washed three times and re-suspended in 0.85% sterile saline to an optical density of 0.5 at 600 nm. WT-*rfp* and KO-*gfp* planktonic samples were prepared by

aliquoting 30 mL of the cell suspension in sterile 250 mL Nalgene centrifuge bottles. Mixed culture (WT-*rfp* and KO-*gfp*) samples were prepared by placing 15 mL of each cell suspension into the same bottles. The samples were then placed in a 22°C shaking incubator at 150 rpm. The WT-*rfp*, KO-*gfp* and mixed samples were sampled on days 0, 3, 6 and 13. The experiment was done in triplicate.

To prepare the carbon-starved WT-*rfp* and KO-*gfp* samples for the survival study, WT-*rfp* and KO-*gfp* cell suspensions were prepared as described earlier. However, the cell cultures were harvested at mid-log phase (OD_{600nm} of 0.5-0.7) and resuspended to an OD_{600nm} 0.5 in minimal salt medium (MSM; 1.294 mmol/L KH_2PO_4, 3.73 mmol/L K_2PO_4, 0.4 mmol/L $MgSO_4$. $7H_2O$, 0.02 mmol/L $FeSO_4$, 1.4 mmol/L NH_4Cl, pH7). The resuspended samples were then placed in a 30°C shaking incubator (150 rpm) and harvested after 2-day carbon starvation (Moore *et al.*, 2005).

Carbon-starved and non-starved planktonic cell survival was determined based on a culturing assay. This was accomplished by sampling 1 mL from each sample, serially diluting in sterile phosphate buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 and pH 7.4 in 1 L of double distilled H_2O) and drop plating on TSA plates containing appropriate antibiotics (refer to Table 1). The plates were incubated for 24 h at 30°C and cell survival was calculated from the CFU counts.

3.2.4. Biofilm growth, characterization and analysis

Separate WT-*rfp* and a KO-*gfp* biofilms were prepared by diluting appropriate overnight cultures, to an OD_{600nm} of 0.5 in sterile deionized water. 200 µL of the diluted cultures were used to inoculate 25 mL of sterile TSB in sterile disposable, polystyrene Petri dish (100 x 15 mm, Fisher Scientific) containing a sterile 22 x 22 mm (Fisher

Scientific) glass coverslip. The mixed culture (WT-*rfp* and KO-*gfp*) biofilms were prepared by inoculation 100 μL of each of the diluted WT-*rfp* and KO-*gfp* cultures to the same coverslip-Petri dish system. Prior to use, the coverslips were acid-washed in 70% nitric acid for 15 min, then aseptically washed 3 times in sterile double deionized water and finally rinsed with 100% methanol. The coverslips were then autoclaved before used as a substratum for the WT-*rfp*, KO-*gfp* or mixed biofilm samples.

Biofilms were grown at 30°C, shaking at 25 rpm and the growth medium was changed at 24 h intervals to ensure a continuous supply of nutrients. The biofilm samples were analyzed at 3, 9, 24, 48 and 72 h. Triplicate biofilm samples with a total of 10 images were analyzed for each treatment at each time point and the experiment was repeated in three separate trials. The WT-*rfp*, KO-*gfp* and mixed biofilm coverslip samples were removed using sterile forceps and washed once (by slowly dipping in a container of sterile double de-ionized water). Each washed biofilm coverslip sample was placed on a concave microscope slide with the biofilm facing downward in the well of the microscope slide. The biofilm samples were kept hydrated in sterile water held in the concave well of the slide and the coverslip samples on the slides were sealed with nail polish.

Biofilms were immediately viewed using an Olympus FV300 Confocal Scanning Laser Microscope (CSLM). The images were obtained with a 60x PlanApo NA1.4 oil immersion lens, using sequential scanning. An argon laser (10 mW, force air cooled), emitting blue light with at 488nm, was used to excite the green fluorescence of the KO-*gfp*. While, the red fluorescent protein of the WT-*rfp* was excited using a helium-neon laser (1 mW) which emitted green light at 543nm. Images were then analyzed for total

biovolume using a biofilm image analysis program called PHLIP (Phobia Laser Imaging Processor; Xavier *et al.*, 2003). Biovolume data points for each strain were obtained from 7 images per 3 repetitions.

3.2.5. Survival of biofilm cells

WT-*rfp*, KO-*gfp* and mixed (WT-*rfp* and KO-*gfp*) biofilms were grown on glass coverslips for 48h at 30°C as described previously. The glass coverslips used were 24 x 60 mm, (Fisher Scientific) and a black line was drawn (with a wash and heat resistant marker, Fisher Scientific) to section-off a 24 x 24 mm area where biofilm cells would be removed for enumeration by drop plating. After 48 h of growth, the biofilms were washed once by dipping in sterile distilled water and submerged in 25 mL of 0.85% sterile saline. The biofilm samples were then placed in a 22°C shaking incubator at 25 rpm. The coverslips were sampled at days 0, 3, 6, and 13 for cell survival study.

Biofilm cell survival was determined based on cultivation method. This was accomplished by washing the biofilm samples once, by gently dipping the coverslip in 200 mL of sterile double distilled water. Then, the biofilm cells were removed from the 24 x 24 mm sectioned-off area by scrubbing with a sterile toothbrush (Nandakumar *et al.*, 2004). To do so, the coverslip was held on an angle in a sterile plastic Petri dish and 1 mL aliquot of sterile distilled water was placed at the base of the coverslip. Using a sterile toothbrush, the water was scrubbed over the 24 x 24 mm area to remove biofilm cells from the surface of the coverslip. The cells would then be collected at the base of the coverslip in a sterile plastic Petri dish. After discarding the used coverslip, the toothbrush was then rinsed with 1 mL of sterile distilled water into the Petri dish, to remove any cells adhering to the bristles. The two mL of distilled water were collected

and serially diluted with a sterile PBS. Drop plating was performed on TSA containing 10 μg/mL of gentamycin for the WT-*rfp* and 10 μg/mL gentamycin and 50 μg/mL kanamycin for the KO-*gfp*. The plates incubated for 24 h at 30°C before counting colonies to calculate biofilm cell survival.

3.2.6. Cohesiveness of WT-rfp and KO-gfp biofilms

The cohesiveness of the WT-*rfp* and the KO-*gfp P. putida* biofilms were examined under flow cell conditions. A flat plate flow cell consisted of a polycarbonate base with an acid-washed rectangular glass coverslip top (24 x 60 mm) was constructed. The size of the flow cell chamber was 40.0 x 10.0 x 3.2 mm. A continuous flow system was set up using a Watson Marlow 205S peristaltic pump (Watson Marlow, Cornwall, England). Medium samples were directed into and out of the flow cell through silicon tubing (ID 1.6 mm and OD 3.2 mm, Cole-Parmer, Anjou, Quebec, Canada). The flow system was sealed with a non-corrosive silicone sealant (Dow Corning 3140 RTV Coating, World Precision Instruments Inc., Sarasota, Florida, U.S.A.) to prevent any leakage. The flow cells were sterilized by submerging in a 10% Clorox bleach for one hour and rinsed thoroughly with sterile deionized water for three times. The silicone tubing and polypropylene fittings were autoclaved at 121°C and 15 psi for 15 min.

To establish a bacterial biofilm (either the WT-*rfp* or KO-*gfp* strain) on the coverslip of the flow cell, two mL of an overnight WT-*rfp* or KO-*gfp* culture were transferred to 200 mL of sterile TSB medium with an appropriate antibiotic (Table 1) and the diluted cell suspension was pumped through the sterile flow cell system at 0.25 mL/min for two hours. After the cell attachment process, the cell suspension was

replaced by a sterile TSB medium and the TSB was continuously pumped through the flow cell at 0.25 mL/min for 24 h. The flow cell system was operated at 22°C.

To determined the cohesiveness of the 24-h WT-*rfp* and KO-*gfp* biofilms, a sterile 0.2 % sodium dodecyl sulphate (SDS) solution was pumped through either the WT-*rfp* or KO-*gfp* flow cell at 9.5 mL/min and detachment of biofilm cells was examined by a Confocal Scanning Laser Microscope that was set with a 60X objective and interfaced with a CCD imaging system (Davies *et al.*, 1998). Because the SDS washing solution reduced the fluorescent intensity of the bacteria, a DNA fluorescent dye (Syto 9 for the KO-*gfp* and Syto 85 for the WT-*rfp*) was injected into the flow cell system prior to the CSLM analysis to enhance the fluorescent intensity of the biofilm samples. The areas covered by the WT-*rfp* and KO-*gfp* biofilms were analyzed by an image analysis program (Scion Image 1.62, Scion Corporation, Frederick, Maryland, USA) before exposure to the SDS solution, and at 10 and 60 min washing by the detergent. Ten biofilm images around the middle of the flow cell were randomly chosen and recorded at each time point. The average area covered by the biofilm samples at each time point was calculated. The cohesiveness experiment was repeated in three separate trials. An ANOVA was used to compare the cohesiveness of the WT-*rfp* and KO-*gfp* biofilms.

3.3. Results

3.3.1. Labeling of the WT and KO parent strains

The WT-Rif^r and KO-Rif^r were successfully labeled with the *rfp* and *gfp* by the Tn7 transposon system, respectively. Using confocal scanning laser microscopy, the WT-*rfp* fluoresced red when excited with the HeNe Green (1mW, 543nm) laser and the KO-*gfp* fluoresced green when excited by the Argon (10mW, force air cooled, blue

488nm) laser with FVX-BA 510-530 band pass emission (Figure 1). A PCR assay using the *glmS* and Tn7R109 primers showed that the WT-*rfp* and KO-*gfp* produced an amplicon between 160-200 bp (Figure 2), indicating that the fluorescent genes were inserted downstream of the *glmS* gene of the bacteria. Sequence analysis of both the WT-*rfp* and KO-*gfp* amplicons using the Tn7R109 primer further confirmed that the Tn7-gfp insertion was indeed at the *att*Tn7 intergenic region downstream of the *glmS* gene.

3.3.2. Characterization of rpoS mutant in planktonic and biofilm phases

The growth patterns of WT, KO, WT-*rfp* and KO-*gfp* were similar to each other in TSB (Figure 3). When the WT, KO, WT-*rfp* and KO-*gfp* were cultured individually, their generation times were, 2.5 h, 2.0 h, 2.38 h and 2.25 h, respectively, and all four strains reached an early stationary phase at approximately 2 x 10^9 CFU/mL. Even in the mixed WT-*rfp* and KO-*gfp* culture, the growth rate of the two genotypes remained similar (2.56 h/generation and 2.61 h/generation, respectively) and neither of the genotypes could out-compete the other. However, a completely different growth pattern was observed in the biofilm phase of the WT-*rfp* and KO-*gfp P. putida* (Figure 1).

CSLM images in Figure 1 show that the WT-*rfp* formed considerably less biofilm than its KO-*gfp* counterpart at 9, 24, 48 and 72 h of growth. Upon mixing the WT-*rfp* and KO-*gfp* in the growth medium, the resulting mixed culture biofilm produced was much smaller than that of the biofilm produced by the KO-*gfp* single culture. Using the PHLIP image analysis program, the biofilm biovolumes for each of the WT-*rfp*, KO-*gfp* and mixed biofilms were analyzed (Figure 4). The KO-*gfp* biofilm grew continuously for 48 h before reaching a plateau at 12900 $\mu m^3/mm^2$. On the other hand, the growth of the WT-*rfp* biofilm was substantially slower than that of the KO-*gfp* and did not reach its

plateau at in 72 h. After 2 days of incubation, the biovolume of the KO-*gfp* biofilm was 6.5 times greater than that of the WT-*rfp*. The total biovolume of the mixed culture biofilm (WT-*rfp* + KO-*gfp*) was similar to that of the WT-*rfp* pure culture biofilm throughout the experiment and the final biovolume of the mixed culture biofilm was substantially smaller (about 6 times smaller) than the KO-*gfp* pure culture biofilm at 72 h. In the mixed culture biofilm, the presence of the WT-*rfp* decreased the growth of the KO-*gfp* biofilm substantially. However, the KO-*gfp* strain still out-competed the WT-*rfp* in a ratio of 3:1 ratio at the end of 72 h, with the biovolumes of the WT-*rfp* and the KO-*gfp* at 93.0 and 314 $\mu m^3/mm^2$ in the mixed biofilm, respectively.

3.3.3. Planktonic and biofilm survival

The survivability of the non-starved (i.e. bacterial cells were harvested at log phase) WT-*rfp* and KO-*gfp* were compared in a nutrient depleted 0.85% saline. Regardless of the experimental treatment, single WT-*rfp* or KO-*gfp* culture or mixed WT-*rfp* and KO-*gfp* culture, the declines of the two non-carbon starved *P. putida* genotypes were not substantially different from each other (Figure 5A). This is reasonable that under exponential growth (i.e. log phase) conditions, the amount RpoS would be low in either the WT-*rfp* or KO-*gfp* genotypes. However, when the carbon-starved WT-*rfp* and KO-*gfp* cells were incubated separately in sterile 0.85% saline, there was a higher decline in cell viability on the C-starved KO-*gfp* than the C-starved WT-rfp. After 13-d incubation, the WT-*rfp* survived about 4.8 times better than the KO-*gfp*. In the 1:1 mixed culture of the WT-*rfp* and KO-*gfp*, the WT-*rfp* survived substantially better than the KO-*gfp* in the 0.85% sterile saline and out-competed the KO-*gfp* mutant by 13.2 times in 13 d (Figure 5B). In the biofilm samples, the survival of both WT-*rfp* and KO-

gfp, either in single or mixed culture, were not substantially different from each other (Figure 6). The samples had an approximately 2 log reduction in cell density in the first 6 days' of incubation. After the initial decrease, the biofilm cell densities of the samples remained steady between 3 x 10^5 and 1 x 10^6 cfu/cm^2.

3.3.4. Biofilm cohesiveness

The percentage area coverages by the WT-*rfp* and KO-*gfp* on the glass coverslips of the flow cells were 12.1 and 16.1 %, respectively, after 24 hours of growth. Cohesiveness of the 24-h biofilms of two genotypes was determined by monitoring the percentage attachment of the bacteria relative to the initial area covered by the 24-h biofilms. The area covered by the WT-*rfp* biofilms decreased significantly to 44.8 and 11.4 % of the initial biofilm after washed by a 0.2% SDS solution for 10 and 60 min, respectively (Figure 7). For the KO-*gfp* biofilm, detachment of the bacteria was not significantly different from that of the WT-*rfp* in the first 10 min, but a significant decrease in attachment (61.2%) was observed after 60 min of washing. An ANOVA test shows that the detachment of the WT-*rfp* was significantly higher than that of the KO-*gfp* at $p<0.05$.

3.4. Discussion

Despite the fact that the *rpoS* did not have any impact on the growth of the PNP-degrading *Pseudomonas* strain in liquid nutrient medium, it had a significant impact on the biofilm development of the bacteria. The volume of the KO-*gfp* biofilm was about 7 times larger than its wild-type (WT-*rfp*) counterpart. In a mixed biofilm condition, the growth of the WT-*rfp* was not enhanced by the KO-*gfp*. Instead, the WT-*rfp* suppressed

the development of KO-*gfp* biofilm substantially. This contradicts to the findings of Corona-Izquierdo and Membrillo-Hernandez (2002) that the spent medium of an *E. coli rpoS* knockout mutant increased biofilm formation of its wild-type parent. Our observations suggest that the *rpoS* of the PNP-degrading *P. putida* strain is related to the down regulation of biofilm production by the bacteria.

Based on studies of the *Pseudomonas aeruginosa* PAO1 strain, there is a complex relationship between the *rpoS* and the quorum sensing (QS) regulons (Schuster *et al.*, 2004). This is partly because of the inconsistent observations on the effect of *rpoS* on the two QS regulons (i.e. *lasR-lasI* and *rhlR-rhlI* systems) (Schuster *et al.*, 2004; Schuster *et al.*, 2003; Whiteley *et al.*, 2000) and due to the lack of data on the direct effect of *rpoS* on biofilm synthesis gene(s) (Sakuragi and Kolter, 2007). As a result, the genetic mechanism that links *rpoS* to biofilm formation is not clear. Nevertheless, the *P. aeruginosa* PAO1 *rpoS* knockout mutant develops a significantly larger biofilm matrix than its wild-type strain and it is consistent to our observations on the PNP-degrading *P. putida*. Another study by Whiteley *et al.* (2000) showed that the PAO1 *rpoS* mutant had a significant increase in *rhlI* expression in comparison to its PAO1 wildtype strain. Since *rhlI* encodes for N-butyryl-homoserine lactone (C4-HSL), an increase in *rhlI* expression may explain the increase in biofilm formation by the PAO1 *rpoS*$^-$ mutant. Furthermore, the study also showed that neither the *rhlR* nor the *lasR-lasI* system was affected by the mutation of the *rpoS*. In this study, the fact that the WT-*rfp* suppressed biofilm development of the KO-*gfp* in the mixed culture biofilm suggests that the *rpoS* may induce the production of a diffusible molecular signal molecule that suppresses the expression of the *rhlI* of the bacteria and this in turn limits the size of the WT-*rfp* single

biofilm and WT-*rfp*/KO-*gfp* mixed biofilm structures. It may be a survival strategy of the bacteria to optimize nutrient uptake of the biofilm matrix by limiting the size of the biofilm structure. Plate count assay on the 2-d old biofilm samples showed that the CFU/cm^2 of the KO-*gfp* and WT-*rfp* were similar while the biovolume of the KO-*gfp* was about 7 times higher than that of the WT-*rfp* strain. This infers that a high percentage of the larger KO-*gfp* biofilm population is unculturable in standard growth medium as compared to the smaller WT-*rfp* biofilm samples. This further supports our theory that the larger biofilm structure of *rpoS* knockout mutants may not be optimal for the survival of the bacteria.

Our previous study has shown that pre-exposing the PNP-degrading *P. putida* to carbon starvation increased the viability and PNP-degrading ability of the bacteria in artificial and environmental water samples (Moore *et al.*, 2005). This study shows that the *rpoS* of the PNP-degrading *Pseudomonas* is important for the survival of the C-starved *Pseudomonas* (in planktonic phase) in nutrient limiting conditions. Although *rpoS*-dependent stress survival responses are seen in other Gram-negative bacteria such as *E. coli*, *P. fluorescens*, and *P. aeruginosa* (Jorgensen *et al.*, 1999; Lange and Hengge-aronis, 1991; Murakami *et al.*, 2005; Stockwell and Loper, 2005), we show that the difference between the survivability of a *rpoS*$^-$ mutant and its wild-type counterpart will widen in a mixed culture sample compared to a single culture *rpoS*$^-$ mutant or wild-type (Figure 5A). This indicates that not only does the *rpoS* protect the bacteria from physicochemical stresses, it is also important for the competitiveness of the bacteria under planktonic conditions.

Both in this and other studies, it is shown that knockout *rpoS* bacterial mutants survive poorly in nutrient limited or stressful planktonic environments (Jorgensen *et al.*, 1999; Murakami *et al.*, 2005; Stockwell and Loper, 2005). However, little is known about the survival of the *rpoS* mutants in biofilm conditions. When it was monitored by plate counting, survival (or rate of decline) of the sessile WT-*rfp* and KO-*gfp*, either in single or mixed biofilm cultures, were not substantially different from each other. This suggests that survival behaviors of *rpoS*⁻ mutant are different in biofilm and planktonic conditions.

Also important to survival of microorganisms in the environment, is the role of detachment. The cohesion of biofilms can be directly correlated to how well the biofilm cells detach. This study shows that cohesiveness of the *P. putida* biofilm increased when the *rpoS* gene was mutated. This indicates that RpoS up-regulates detachment of the bacteria. Through the use of computer modeling, Hunt *et al.* (2004) showed that nutrient starvation in biofilms of *P. aeruginosa* triggers detachment of biofilm cells. Considering the fact that nutrient starvation will up-regulate *rpoS* expression, these findings suggest that *rpoS* plays a role in detachment. Studies perfomed on *Vibrio cholerae* also show that *rpoS* plays a key role in the detachment of biofilm cells from biotic and abiotic surfaces, allowing the dispersal and colonization of new hosts or habitats (Müller *et al.*, 2007; Nielson *et al.*, 2006). These findings further support our observations that *rpoS* has a key role in the dispersal of *P. putida* biofilm cells.

In conclusion, our findings suggest that not only is the RpoS important to the stress survival responses of planktonic bacterial cells, it also has an important role in

optimizing the fitness of biofilm bacteria by regulating the size and the cohesiveness of biofilm structure.

Table 1. Bacteria strains or plasmids used in this study with relevant characteristics for growth.

Strain or plasmid	*Relevant characteristics*	**Reference**
Pseudomonas putida strains		
WT	Indigenous	Spain and Gibson (1991)
KO	*rpoS*::Tn5Km^r 50 μg mL^{-1}	Tallon and Leung (2005)
WT- Rif^r	Spontaneous Rif^r 50 μg mL^{-1}	This study
KO- Rif^r	Spontaneous Rif^r 50 μg mL^{-1}, Km^r,50 μg mL^{-1}	This study
WT-*rfp*	*rfp*, Gm^r 10 μg mL^{-1}	This study
KO-*gfp*	*gfp*, Gm^r 10 μg mL^{-1}, Km^r 50 μg mL^{-1}	This study
Escherichia coli plasmids		
pMiniTn7(Gm)P$_{PA1/04/03}$*gfp-a*	Gm^r, *gfp*	Lambertson *et al.* (2004)
pMiniTn7(Gm)P$_{PA1/04/03}$*DsRedExpress-a*	Gm^r, *DsRedExpress*	Lambertson *et al.* (2004)
pUX-BF13	Helper plasmid carrying transposase genes, Ap^r 100 μg mL^{-1}	Lambertson *et al.* (2004)
pRK2013	Helper plasmid for mobilization, Km^r 50 μg mL^{-1}	Moore *et al.* (2005)

Figure Legend

Figure 1. Confocal images of hydrated, WT-*rfp*, KO-*gfp*, and mixed WT-*rfp* and KO-*gfp* biofilms at 3, 9, 24, 48 and 72 hour incubations. Scale bar = 20 microns.

Figure 2. (A) The structure of the pMiniTn7(Gm)$P_{PA1/04/03}$*gfp-a* transposon plasmids, similar to the pMiniTn7(Gm)$_{PPA1/04/03}$*DsRedExpress-a* (not shown here) (Dr. Søren Molin, Technical University of Denmark, Lyngby) that was introduced into *P. putida*. The transposon contains a gentamycin resistance gene, Gm R; the *gfp-a*, is a green fluorescent protein which is constitutively expressed by $P_{PA1/04/03}$ promoter; termination sequence, T_oCmRT_1; on the outside two transposon flanking sequences, Tn7R and Tn7L. (B) 25 bp downstream from the *glmS*. The PCR primers used to verify correct insertion are shown as Tn7R109 and Tn7GlmS. (C) A 1% agarose gel shows the presence of a 164 bp PCR product for the WT-*rfp* and the KO-*gfp*, and the absence of the product in the parent WT and KO strains. Marker = 100 kb ladder.

Figure 3. Growth curves for the transformed *Pseudomonas putida* strains of uniculture WT-*rfp* ● and KO-*gfp* ○, mixed WT-*rfp* ▼ and KO-*gfp* ▽ and, WT ■ and KO □.

Figure 4. PHLIP analysis of the biovolume ($\mu m^3/\mu m^2$) of a WT-*rfp* ●, KO-*gfp* ○, mixed▼, mixed WT-*rfp* ▽and mixed KO-*gfp* ■, *Pseudomonas putida* biofilms. Values given represent the mean of 25 z-slice confocal images from 3 separate trials.

Figure 5a. Survival of non-starved, WT-*rfp* ●, KO-*gfp* ○, and mixed WT-*rfp*▼ and KO-*gfp*▽, planktonic *P. putida* cells in 0.85% saline at 22°C.

Figure 5b. Survival of carbon-starved, WT-*rfp* ●, KO-*gfp* ○, and mixed WT-*rfp* ▼ and KO-*gfp* ▽planktonic *P. putida* cells, in 0.85% saline at 22°C.

Figure 6. Survival of WT-*rfp* ●, KO-*gfp* ○, and mixed WT-*rfp* ▼ and KO-*gfp* ▽ biofilm *P. putida* cells, in 0.85% saline at 22°C.

Figure 7. Confocal Scanning Laser Microscope images of (A) KO-*gfp* and (B) WT-*rfp P. putida* cells attached to glass coverslip after 0, 10 and 60mins washing in 0.2% SDS. (C) Percentage of KO-*gfp* ● and WT-*rfp* ○, *P. putida* cells attached to glass coverslip after 0, 10 and 60mins washing in 0.2% SDS.

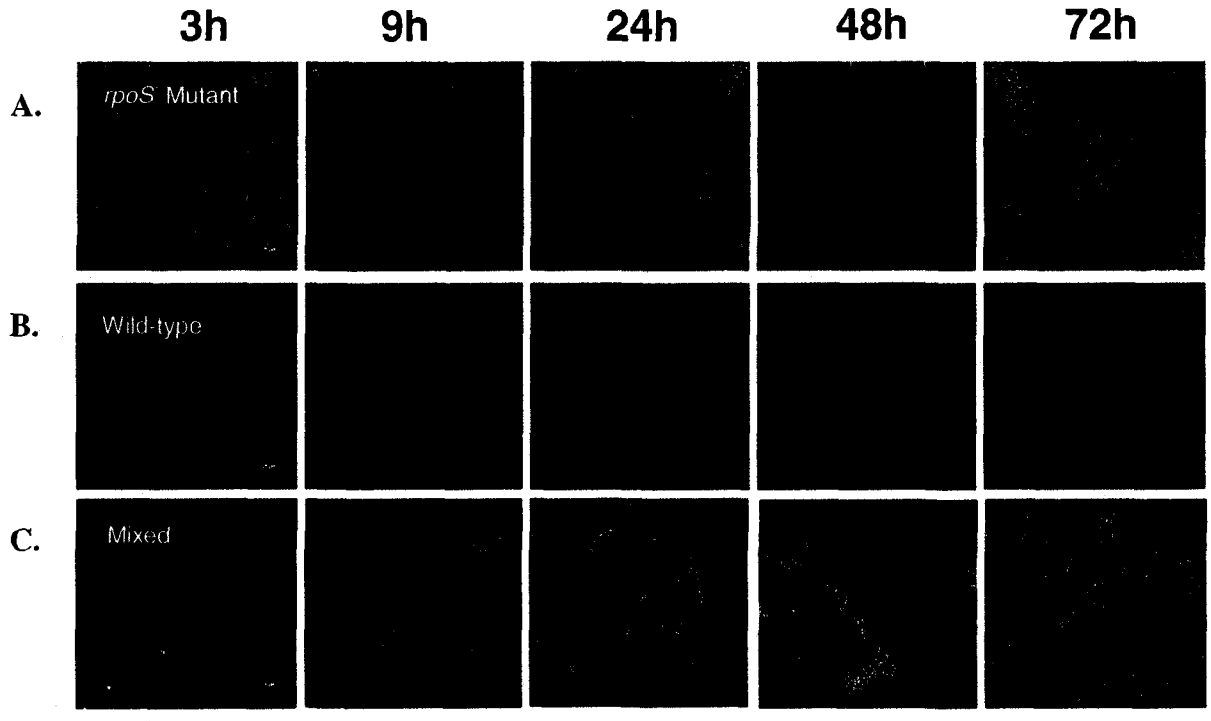


Figure 1

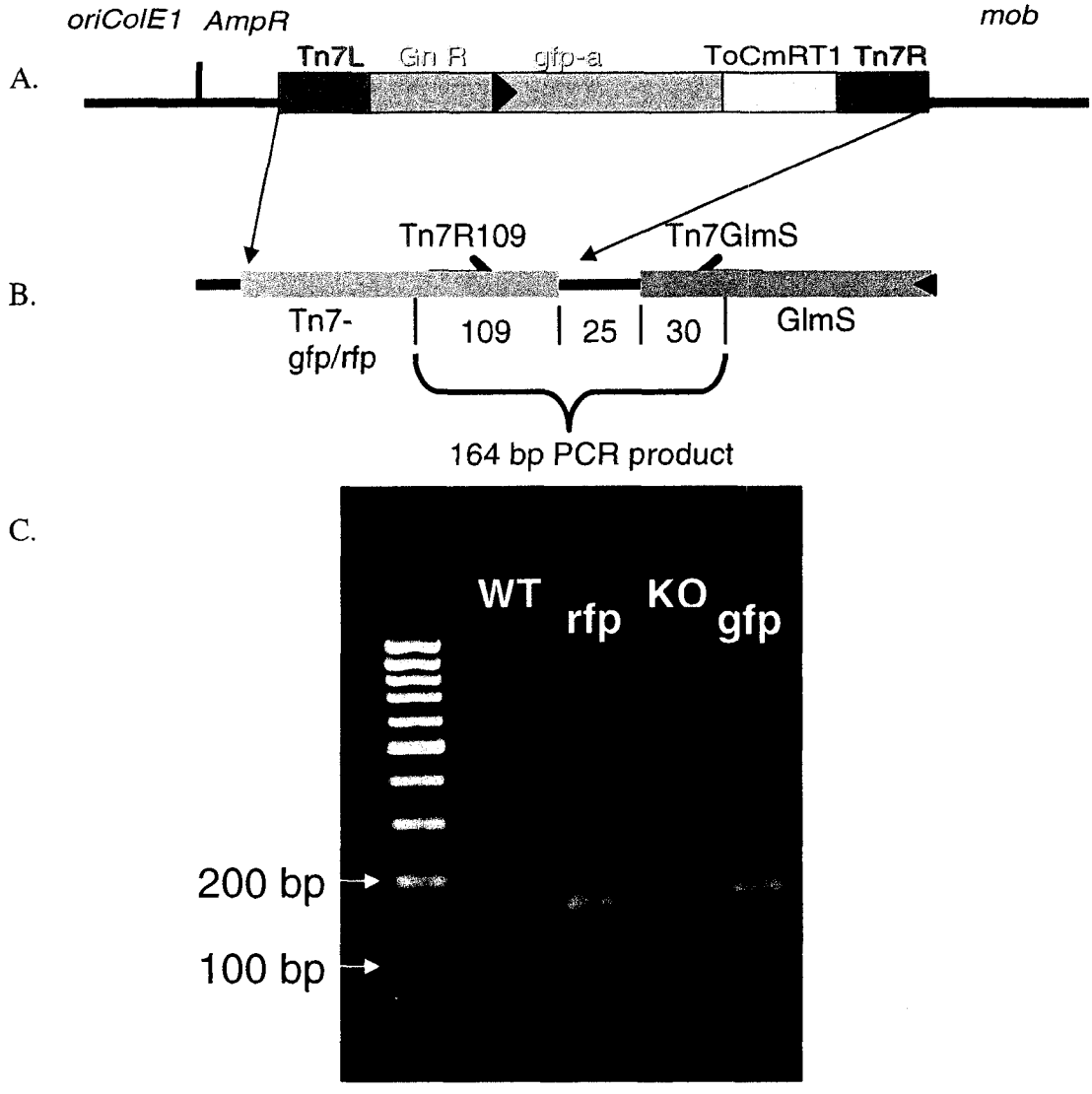

A.
oriColE1
AmpR
mob
Tn7L
Gm R
gfp-a
ToCmRT1
Tn7R
B.
Tn7R109
Tn7GlmS
Tn7-
gfp/rfp
109
25
30
GlmS
164 bp PCR product
C.
WT
rfp
KO
gfp
200 bp
100 bp

Figure 2

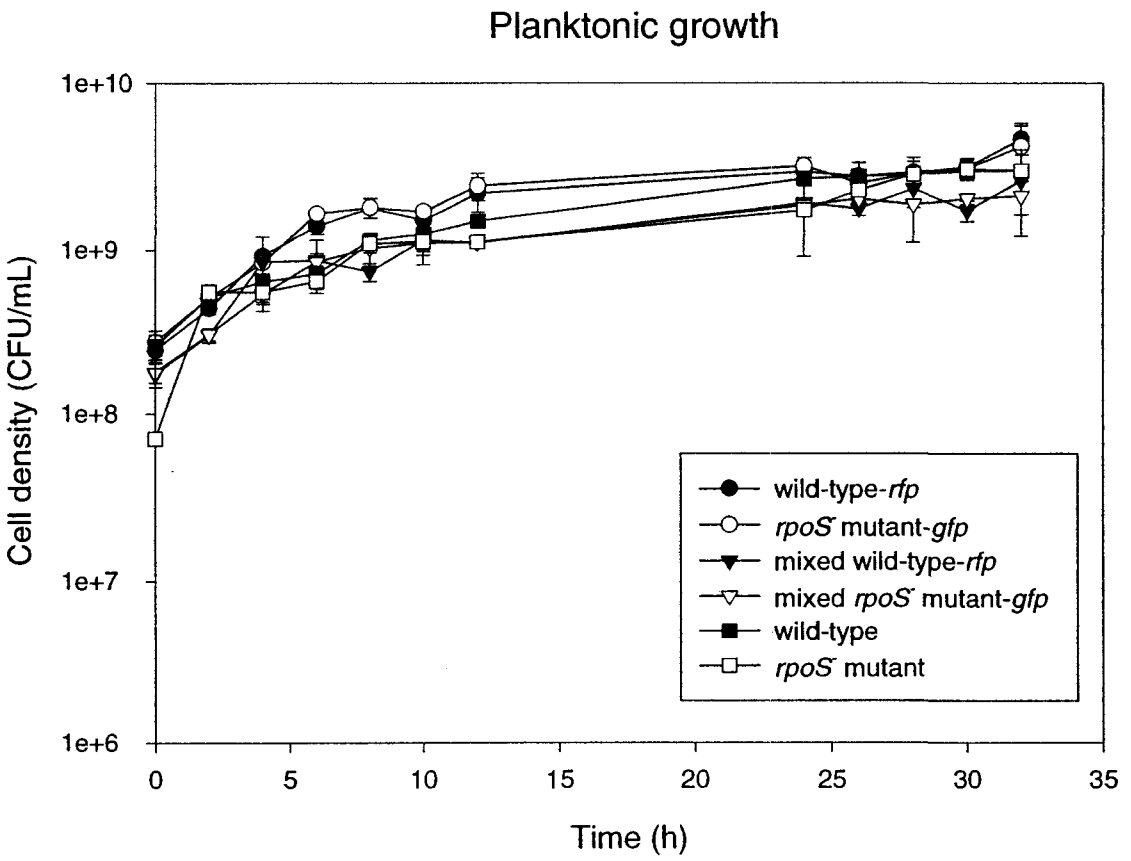

Planktonic growth
Cell density (CFU/mL)
1e+10
1e+9
1e+8
1e+7
1e+6
0
5
10
15
20
25
30
35
Time (h)
wild-type-rfp
rpoS mutant-gfp
mixed wild-type-rfp
mixed rpoS mutant-gfp
wild-type
rpoS mutant

Figure 3

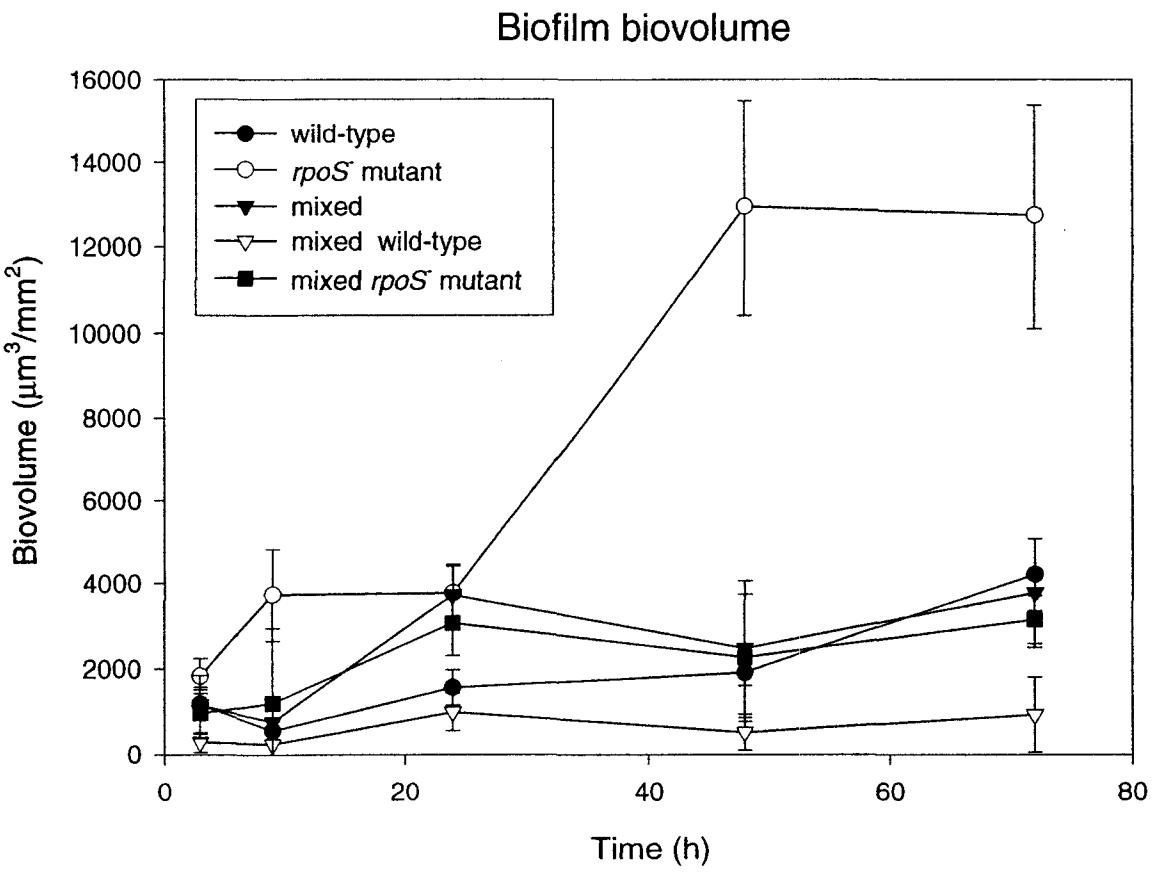
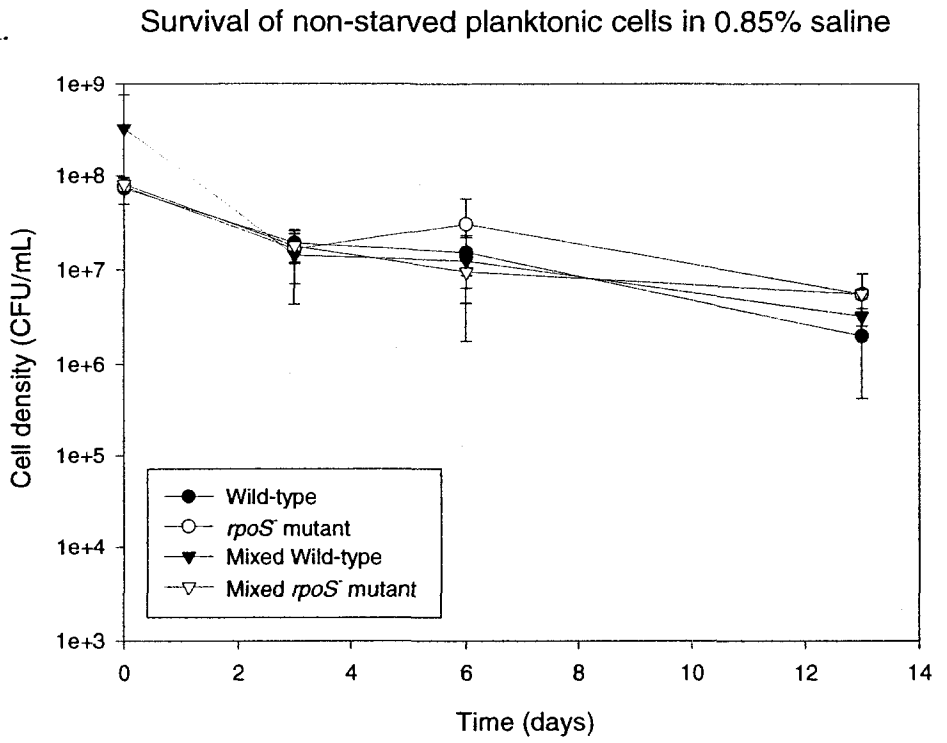

Biofilm biovolume
wild-type
rpoS⁻ mutant
mixed
mixed wild-type
mixed rpoS⁻ mutant
Biovolume (µm³/mm²)
Time (h)
16000
14000
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80

Figure 4

A.


Survival of non-starved planktonic cells in 0.85% saline
Cell density (CFU/mL)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
2
4
6
8
10
12
14
Time (days)
Wild-type
rpoS⁻ mutant
Mixed Wild-type
Mixed rpoS⁻ mutant

B.

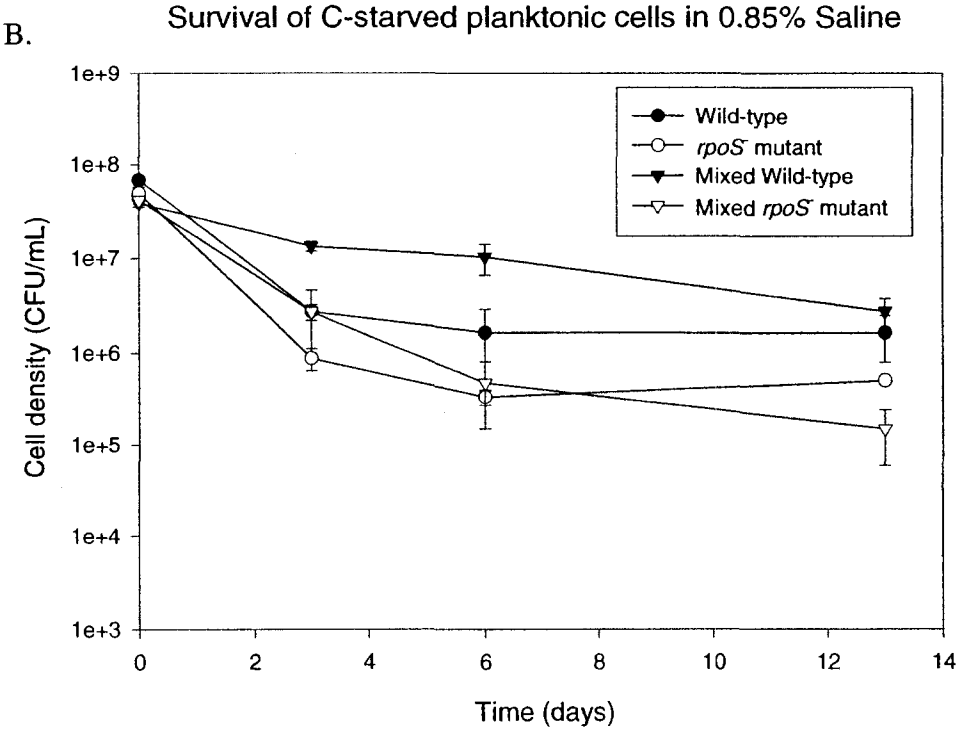

Survival of C-starved planktonic cells in 0.85% Saline
Cell density (CFU/mL)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
2
4
6
8
10
12
14
Time (days)
Wild-type
rpoS⁻ mutant
Mixed Wild-type
Mixed rpoS⁻ mutant

Figure 5

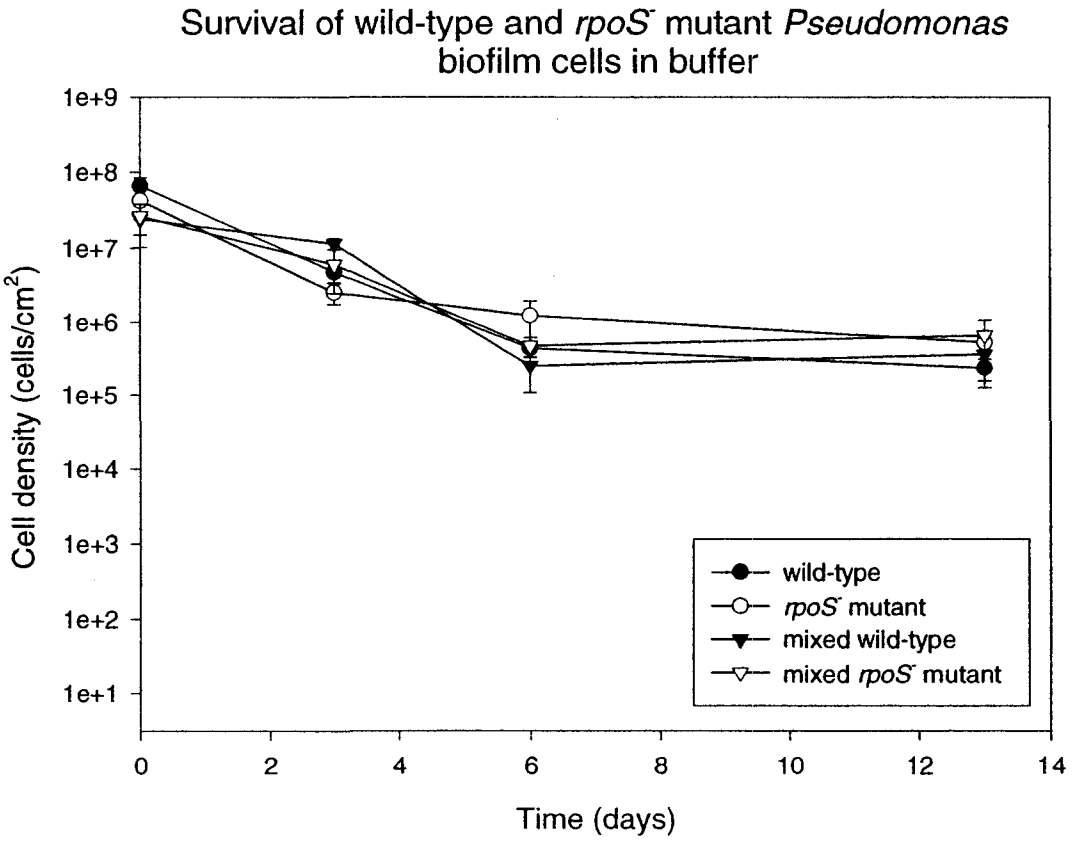

Survival of wild-type and rpoS⁻ mutant Pseudomonas biofilm cells in buffer
Cell density (cells/cm²)
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
0
2
4
6
8
10
12
14
Time (days)
wild-type
rpoS⁻ mutant
mixed wild-type
mixed rpoS⁻ mutant

Figure 6

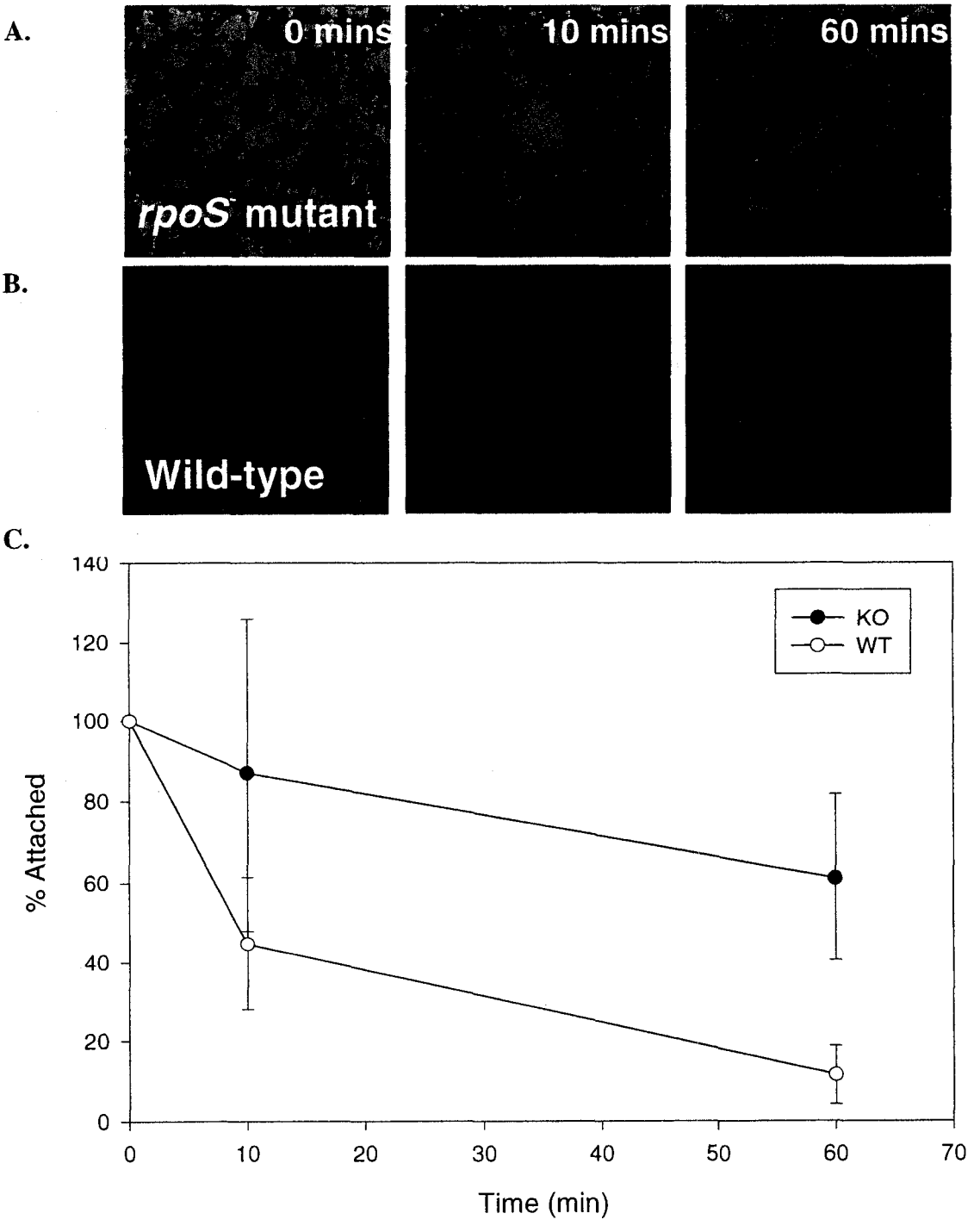

A.
0 mins
10 mins
60 mins
rpoS mutant
B.
Wild-type
C.
140
120
100
80
60
40
20
0
% Attached
KO
WT
0
10
20
30
40
50
60
70
Time (min)

Figure 7

References

Adams JL, McLean RJC. 1999. Impact of *rpoS* deletion on *Escherichia coli* biofilms. Appl Environ Microbiol 65:4285-7.

Allison D.G., Evans D.J., Brown M. R. W., Gilbert P. 1990. Possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. J Bacteriol 172:1667-9.

Bester E, Woolfaardt G, Joubert L, Garny K, Saftic S. 2005. Planktonic-cell yield of a Pseudomonad biofilm. Appl Environ Microbiol 71:7792-8.

Brown, M.R.W. and Gilbert, P. 1993. Sensitivity of biofilms to antimicrobial agents. J Appl Bacteriol 74:S87-S97.

Brown MRW, Allison DG, Gilbert P. 1988. Resistance of bacterial biofilms to antibiotics - a growth-rate related effect. J Antimicrob Chemother 22:777-80.

Corona-Izquierdo FP and Membrillo-Hernandez J. 2002. A mutation in *rpoS* enhances biofilm formation in *Escherichia coli* during exponential phase of growth. FEMS Microbiol Lett 211:105-10.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-8.

De Bruijn, F.J., and Rossbach, S. 1994. Transposon mutagenesis. *In* Methods for general and molecular bacteriology. *Edited by* P, Gerhardt, R.G.E. Murray, W.A. Wood, and N.R. Kreig. American Society of Microbiology, Washington, D.C. pp.387-405.

Flemming HC. 1993. Biofilms and environmental-protection. Water Sci Technol 27:1-10.

Fujita M, Tanaka K, Takahashi H, Anemura A.1994. Transcription of the principal sigma factor genes, *rpoD* and *rpoS*, in *Pseudomonas aeruginosa* is controlled according to the growth phase. Mol Microbiol 13:1071-7.

Geesey GG, Stupy MW, Bremer PJ. 1992. The dynamics of biofilms. Int Biodeterior Biodegrad 30:135-54.

Hall-Stoodley L and Stoodley P. 2005. Biofilm formation and dispersal and the transmission of human pathogens. Trends Microbiol 13:7-10.

Hengge-Aronis R. 1993. Survival of hunger and stress: The role of *rpoS* in stationary phase gene regulation in *Escherichia coli*. Cell 72:165-8.

Hengge-Aronis R. 2002b. Signal transduction and regulatory mechanisms involved in control of the δ^S (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev 66:373-95.

Hengge-Aronis R. 2002a. Recent insights into the general stress response regulatory network in *Escherichia coli.* J Mol Microbiol Biotechnol 4:341-436.

Holt JG, Krieg NR, Sneath PHA., Staley JT, Williams ST. 1994. Berygey's Manual of Determinative Bacteriology 9th Ed. Williams and Wilkens Publishing Ltd. Baltimore, USA. p.155.

Hunt SM, Werner EM, Huang BC, Hamilton MA, Stewart PS. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol 70:7418-25.

Jorgensen F., Bally M., Chapon-Herve V., Michel G., Lazdunski A., Williams P., Stewart G.S.A.B..1999. RpoS-dependent stress tolerancein *Pseudomonas aeruginosa.* Microbiol 145:835-44.

Kolter R, Siegele DA, Tormo A. 1993. The stationary phase of the bacterial life cycle. Annu Rev Microbiol 47:855-74.

Lambertsen L, Sternberg C, Molin S. 2004. Mini-Tn7 transposons for site-specific tagging of bacteria with fluorescent proteins. Environ Microbiol 6:726-32.

Lange R and Henggearonis R. 1991. Identification of a central regulator of stationary-phase gene-expression in *Escherichia-coli.* Mol Microbiol 5:49-59.

Mah TFC and O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9:34-9.

Maki M, Schmerk C, Nix E, Leung KT. 2005. Comparing survival and *p*-nitrophenol-degradation of planktonic and biofilm *Moraxella* cells. Proc Can Soc Microbiol. 55th conference, Halifax, Nova Scotia, Canada. [http://www.csm-scm.org/english/abstracts/public/view_abs.asp?id=1397]

Moore M, Trevors J, Lee H, Leung KT. 2005. Stress-survival responses of a carbon-starved *p*-nitrophenol-mineralizing *Moraxella* strain in river water. Can J Microbiol 51:223-9.

Müller J, Miller M, Nielsen A, Schoolnik G, Spormann A. 2007. *vspA*- and *luxO*-dependent biofilms of *Vibrio cholerae.* FEMS Microbiol Lett 275:199-206.

Murakami K, *et al.*. 2005. Role for RpoS gene of *Pseudomonas aeruginosa* in antibiotic tolerance. FEMS Microbiol Lett 242:161-7.

Nandakumar K, Obika H, Utsumi A, Ooie T, Yano T. 2004. In-vitro laser ablation of laboratory developed marine biofilm. Biotechnol Bioengineer 86:729-36.

Nielsen AT, Dolganov NA, Otto G, Miller MC, Wu CY, Schoolnik GK. 2006. RpoS Controls the *Vibrio cholerae* Mucosal Escape Response. PloS Pathog 2:934-48.

Prigent-Combaret C, *et al.*. 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. J Bacteriol 183:7213-23.

Ramirez ME, Israel DW, Wollum AG. 1998. Using spontaneous antibiotic-resistant mutants to assess competitiveness of bradyrhizobial inoculants for nodulation of soybean. Can J Microbiol 44:753-8.

Sakuragi Y, Kolter R. 2007. Quorum-Sensing Regulation of the Biofilm Matrix Genes (*pel*) of *Pseudomonas aeruginosa*. J Bacteriol 189, 5383-6.

Schembri MA, Kjaergaard K, Klemm P. 2003. Global gene expression in *Escherichia coli* biofilms. Mol Microbiol 48:253-67.

Schuster M, Hawkins A. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. Mol Microbiol 51(4): 973-85.

Schuster M, Lostroh P, Ogi T, Greenburg EP. 2003. Identification, Timing, and Signal Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome Analysis. J Bacteriol 185, 2066-79.

Spain JC. 1995. Biodegradation of Nitroaromatic Compounds. Annu Rev Microbiol. 49:523-55.

Spain JC and Gibson DT. 1991. Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl Environ Microbiol 57:812-9.

Stewart PS and Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358:135-8.

Stockwell VO and Loper JE. 2005. The sigma factor RpoS is required for stress tolerance and environmental fitness of *Pseudomonas fluorescens* Pf-5. Microbiol 151:3001-9.

Suh S, *et al.*. 1999. Effect of *rpoS* Mutation on the Stress Response and Expression of Virulence Factors in *Pseudomonas aeruginosa*. J Bacteriol 181:3890-7.

Tallon P, Leung KT. 2005. The impact of *rpoS* deletion on biofilm formation of a *p*-nitrophenol degrading *Moraxella* strain. Proc Can Soc Microbiol. 55th conference, Halifax, Nova Scotia, Canada p.63.

US Environmental Protection Agency (1976) Water quality criteria. USEPA, Washington, DC.

Whiteley M *et al.* 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413:860-4.

Whiteley M, Parsek MR, Greenberg EP. 2000. Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa*. J Bacteriol 182:4356-60.

Xavier JB, White DC, Almeida JS. 2003. Automated biofilm morphology quantification from confocal laser scanning microscopy imaging. Water Sci Tecnol 47:31-7.