Spring 5-1-2007

Characterization of Type II secretion mutants of Pseudomonas aeruginosa

Sijung Suh

Characterization of Type II secretion mutants of *Pseudomonas aeruginosa*

Sijung Suh

Candidate for B.S. Degree

in Biochemistry with Honors

May/2007

APPROVED

Thesis Project Advisor: _____

Anthony Garza

Honors Reader: _____

Scott E. Erdman

Honors Director: _____

Samuel Gorovitz

Date: _____

Abstract

Pseudomonas aeruginosa mutants PA0686, PA3102, PA3103, and PA5210 carry transposon insertions in uncharacterized genes in the PA01 background. The biofilms formed by these mutants show different phenotypes than PA01, their wild-type counterpart. BLAST searches and phylogenomic mapping tools indicate that protein products of the mutated genes are involved in *P. aeruginosa* type-II secretion, which is linked to twitching motility. In this study, 96-well microtitre plate biofilm assays, UV/visible spectrophotometer analysis, and motility assays were used to demonstrate a link between type-II secretion, twitching motility and biofilm formation. The results show that the PA5210 mutant forms a biofilm that is less compact than the wild-type, while PA0686 forms a faint biofilm. Also, the motility assays support our hypothesis that these mutants are defective in twitching motility.

Table of Contents

Introduction

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that causes persistent infection in humans by forming a highly organized microbial community called a biofilm. The biofilm is formed when planktonic (free-swimming) cells attach to biotic and abiotic surfaces under certain environmental conditions. These surface-attached cells are 10-1000 times more resistance to traditional antibiotic therapy than planktonic cells (Drenkard, 2003). *P. aeruginosa* biofilms are linked to 10% to 20% of nosocomial infections, the most common sites of biofilm formation being catheter lines, burn wounds, cystic fibrosis lung tissue, and organ transplant sites (Bodey, Bolivar, Fainstein & Jadeja, 1983; Miller & Ahearn, 1987). Also, *P. aeruginosa* biofilms are a frequent cause of otitis externa in swimmers and osteomyelitis in puncture wounds of the feet (Sarlangue, Brissaud & Labreze, 2006). The latter facts strengthen the argument that

understanding the genetics behind biofilm formation by *P. aeruginosa* is important in the medical field.

The most problematic factor in the treatment of *P. aeruginosa* infections is the resistance of its biofilm to antimicrobial agents. Thus, the treatment would be more effective if it is to include a system designed to weaken the formation, structure, or attachment of the biofilm during its development. Profound changes occur in the gene expression profile of free-living *P. aeruginosa* during biofilm formation in response to appropriate environmental signals (O'Toole & Kolter, 1998a; Sauer & Camper, 2001). Data generated using DNA microarray analysis show that about 70 *P. aeruginosa* genes (approximately 1% of genes analyzed) are differentially expressed in biofilms with respect to planktonic cells grown in chemostat cultures (Drenkard, 2003). These small numbers of important genes that are induced in biofilms include bacteriophage genes and genes that code for

proteins involved in translation, metabolism, membrane transport/secretion, and gene regulation (Drenkard, 2003).

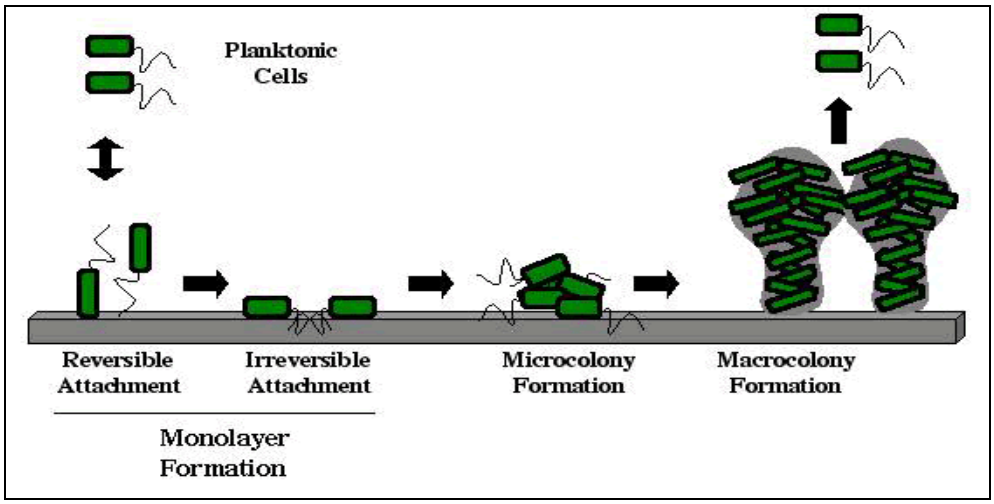


Figure 1- Steps in *P. aeruginosa* biofilm formation (O'Toole, 2007)

The development of *P. aeruginosa* biofilms under static assay conditions can be described in four processes, as shown in figure 1. At first, planktonic cells are transported and reversibly attach to a surface substrate. Then, the surface-attached cells irreversibly form a monolayer on the surface. Subsequently, the cells in the monolayer begin to migrate toward aggregation centers known as microcolonies. Lastly, these microcolonies mature into complex mushroom-shaped structures covered by

exopolysaccharide matrix and some cells break from the biofilm as planktonic cells to be dispersed (O'Toole el at., 2000; O'Toole and Kolter, 1998a).

The goal of our research is to uncover and characterize those genes required for biofilm formation in the pathogenic organism *P. aeruginosa*. Through phenotypic characterization of strains carrying disrupted genes, including an analysis of their biofilm phenotypes, a gene's role in biofilm formation can be determined. A wide range of medical applications may come from this because treatment in one organism may be effective across a broad evolutionary spectrum. There are significant morphological and genetic similarities between most biofilms, even those of very different bacterial species (Garza, 2006).

George O'Toole provided *P. aeruginosa* strain PA01 mutants that carry transposon insertions in previously uncharacterized genes. In order to

determine the function of these disrupted genes, a traditional genome annotation approach (BLAST) was used to compare each ORF within the genome to GenBank®, an official NIH genetic sequence database which contains more than 100 gigabases of an annotated collection of all publicly available DNA sequences (NCBI, 2007). The BLAST search annotated the uncharacterized clusters of genes in PA01 strain to be involved in type-II secretion. Type-II secretion is the mechanism by which Gram-negative organisms such as *P. aeruginosa* export pili components, virulence factors, toxins, and hydrolytic enzymes that may contribute to biofilm maturation (Russel, M. 1998).

Following the BLAST search, phylogenomic mapping was used to identify possible functional interactions between type-II secretion genes and other genes on the *P. aeruginosa* chromosome. The phylogenomic map groups *P. aeruginosa* genes/proteins on the basis of coinheritance, which is a

strong indicator of shared function (Srinivasan et al, 2005). According to the phylogenomic map, the genes for type-II secretion are co-inherited with genes required for twitching motility, a surface-based motility that is known to be important for biofilm formation. Thus, based on the phylogenomic map, the type-II secretion mutant strains under study are predicted to have a twitching motility and perhaps biofilm phenotypes.

Bacterial motility is required for both biofilm pathogenesis and attachment to biotic and abiotic surfaces. *Pseudomonas aeruginosa* bacteria swim in liquid by using flagella to move on surfaces by means of type IV pili, and the extension and retraction of type IV pili powers twitching motility, a surface-associated movement that propels bacteria across solid or semi-solid substrates (Semmler et al., 1999, Skerker & Berg, 2001). From the results from biofilm experiments conducted by O'Toole and Kolter (1998a) on biofilm formation by *P. aeruginosa* PA14, it is speculated that

swimming motility might enable the bacteria to overcome repulsive forces at the surface–water interface so that they reach the surface, and that the microcolonies may be formed by twitching motility-driven cell aggregation. The assessment of twitching and swarming motility of *P. aeruginosa* utilizes motility assays where the phenotype of the twitching motility defective mutants are distinguished from the wild-type on 1.5% agar media, whereas swarming motility phenotype is assessed using softer, 0.4% agar.

The 96-well microtitre plate assay is used to qualitatively characterize the progress of biofilm development. This static assay is often preferred over continuous-flow methods due to the simplicity of the protocols and its cost-effective nature (Garza, 2006). Also, the 96-well format has the capacity to screen a large number of bacterial strains at once and is a good visual indicator of adherent biomass stained in crystal violet (CV) dye (O'Toole & Kolter, 1998a and b). The results from the 96-well

microtitre plate assay indicated the influence of the type-II secretion pathway in biofilm formation.

Spectrophotometry is used for quantitative measurement of the biofilm formed in the 96-well plate assay. The spectrophotometer measures the amount of biofilm by screening the quantity of light with a specific wavelength that passes through a medium. According to Beer's law, the amount of light absorbed by a medium is proportional to the concentration of the absorbing material or solute present (Fankhauser, 2005). Thus, the concentration of a CV-stained solute in ethanol is determined by measuring the absorbency of light at wavelength of 600nm, which is the maximum absorption wavelength of CV.

The resistance of a biofilm can be determined by the antibacterial resistance assay. The quantity of the mutant *P. aeruginosa* cells after the antibacterial treatment is compared to that of the wild-type cells treated in

the same way.

Mutant strains with strongest phenotypical deviation (in both biofilm formation and antibacterial resistance) compared to the wild-type are selected for a more detailed analysis, while further characterization of the mutants with subtle biofilm defects is postponed due to time limitation.

The general impact of the research is to better understand the genetics behind pathogenic biofilm formation. The practical application of this information is the potential discovery of new targets for antimicrobial agents, and thus, new potential treatments for infections caused by *P. aeruginosa* and other biofilm-forming pathogens.

Acknowledgements

Anthony Garza, principal investigator

Kimberly Murphy, advisor

William Murtaugh, undergraduate research student

George O'Toole, collaborator

MATERIALS AND METHODS

Bacterial strains and culture conditions

The microorganism used in this study was the opportunistic pathogenic bacterium *P. aeruginosa*. The strain that was used is PA01. Four PA01 mutants were also used. *P. aeruginosa* was grown on plates containing LB and 1.5% agar. Strains were grown in a 37°C incubator overnight. Then, one colony was picked with a pipette tip and the colony was dispersed into 5-ml of liquid LB in a sterile glass tube. The liquid culture was incubated in a 37°C shaker overnight.

Microtitre plate biofilm assay

This method was designed by O'Toole and Kolter, 1998b. M63 minimal media (M63, $MgSO_4$, Glucose, CAA)) is prepared with 5 ml of 1M M63, 5 ul of 1M $MgSO_4$ (1mM dilution), 50 ul of 20% glucose (0.2% dilution), and 125 ul of Casamino acids (0.5% dilution) in a glass tube. Then, 50 ul (1:100

dilutions) of the overnight liquid cultures of the wild-type and the mutants were added to the M63 minimal media cocktail. One hundred microliterl of M63 minimal media cocktail + bacteria cells was placed into each well of 96-well microtitre plates. The microtitre plates were sealed with Parafilm® to prevent evaporation during the incubation period. The Parafilm® sealed microtitre plates were placed in the 37°C incubator for the desired amount of time (in our case, 2hr, 4hr, 6hr, 8hr, 12hr, and 24hr). By using different incubation times, we were able to determine the number of hours required for the *P. aeruginosa* strain to fully attach to the surface.

Following incubation, the planktonic cells in the wells were removed by turning over the plates and shaking the liquid into an empty plastic container. Afterwards, the 96-well plates were repeatedly submerged in a small tub of deionized water to wash out the planktonic bacteria that did not form a biofilm. The removal of unattached cells from the wells was intended to

reduce the number planktonic cells that stain with Crystal Violet (CV); planktonic cells should not be included in calculations of biofilm cell mass.

Each well was stained with 125 □l of 0.1% CV dye and the plates were incubated at room temperature for 15-30 minutes. After that, the CV in the wells was dumped into a waste plastic container and the wells were rinsed 3-4 times with deionized water. The plates were then blotted on a stack of paper towels and turned upside down to dry. A ring of CV stained biofilm cells was seen at the air-medium interface.

Quantification using UV/visible spectrophotometer

The CV in each well solubilized in a 200 ul aliquot of 95% ethanol for 15 minutes at room temperature. In order to obtain enough material for accurate quantification, 15 wells, representing 3 replicates of 5 wells, were used for each strain. A pipette tip was used to remove the CV-stained biofilm from the walls of each well. Then, the ethanol solution in 5 wells (total volume 1

ml) was transferred to a square-bottomed cuvette.

The spectrophotometer reading was taken as follows: a blank was set up with 95% ethanol for the baseline of zero. The blank and the sample cuvette containing ethanol/CV solution were read at 600 nm. For each strain and each time point, the CV in 3 cuvettes was averaged. All values obtained for the mutant strains were normalized to those of PA01 wild type. One drawback to this assay was that the CV stained not only cells, but any material that adhered to the surface of the plate, including contaminating material that was not part of the biofilm.

Twitching/swarming Motility Assay

The twitching/swarming motility assay was designed by O'Toole and Kolter (1998a). Each strain was assessed for twitching motility by stabbing microcolony cells into very thin 1.5% LB agar plates with a toothpick. Each plated included a total of 3 stabs of the same strain. These plates were

incubated for 24 hours at 37°C. Strains that were capable of twitching motility formed a haze of growth surrounding the point of inoculation.

For the swarming motility assay, 0.4% LB agar plates were used. A total of 3 inoculations of 5 ul aliquots of liquid cultures were made per plate and the plates were incubated at 37°C for 24 hours. The widest expansion of cells in diameter from the point of inoculation was measured in millimeters and the colony edges were analyzed for a haze of growth with uneven edges.

Biofilm Antibiotic Resistance Assay.

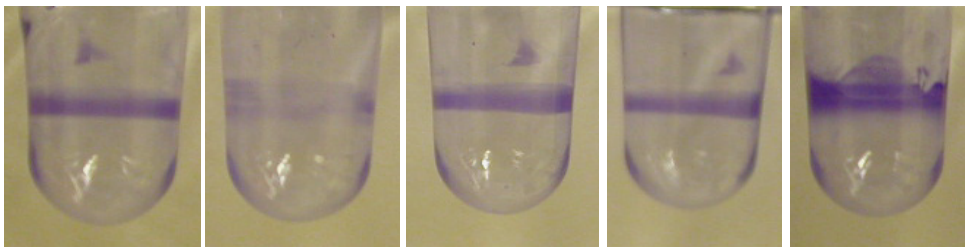
The biofilm antibiotic resistance assay followed the same protocol as the 96-well plate assay over 24-hour incubation at 37°C. The solution in the wells was removed by shaking out the liquid into an empty container and adding fresh media containing different concentrations of antibiotics into the wells (tetracyclin, ampicilin, kanamycin, and doxycycline). An additional 24 hour Incubation at 37°C was performed and the cells in the wells were

assessed for their viability by spreading the media into a clean LB plate with an L-shaped glass-rod. Antibiotic resistance was measured for the minimal anti-microbial concentration required to kill all biofilm cells.

Results and Discussion

The liquid culture for the wild-type and PA5210 often expresses a greenish-yellow shade. From the 96-well microtitre assay indicates that the PA0686 mutant does not form as strong of a biofilm as the wild-type PA01 strain and the PA5210mutant forms a biofilm that appears to be wider than the wild-type biofilm. In contrast, the biofilms formed by the PA3102 and PA3103 mutants look similar to that of the wild type.

Results of the 96-well Microtitre Assay



PA01 PA0686 PA3102 PA3103 PA5210

Figure 2- CV stain of 24-hour biofilm cells in 96-well microtitre plates (Photograph provided Dr. Kimberly Murphy, 2006).

The differences between PA5210 mutant and wild-type biofilms are more evident as the incubation time increases. At the 2-hr time point,

biofilms are barely visible to the eye and the width of biofilm ring of wild-type and PA5210 mutant biofilms look similar. As time progresses from 4-12 hours, the PA5210 mutant biofilms start to become darker and wider than wild-type biofilms. At the 24-hr time point, the biofilm ring of PA5210 mutant cells is much wider and darker than wild-type biofilm rings. At all time points after 2 hr, the PA0686 mutant biofilm are fainter in color than its wild-type counterpart.

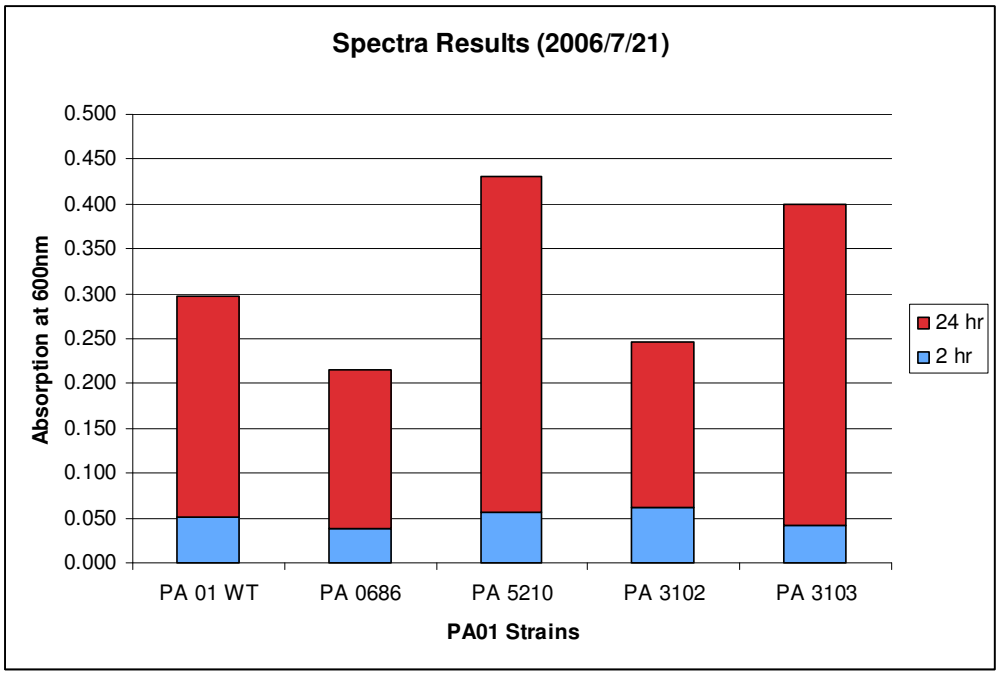


Figure 3- Spectrophotometer reading (600nm) of CV-stained biofilm mass. Wild-type and mutant biofilms were CV stained after 2 hours and 24 hours in 96-well microtitre plates.

The data shown in Figure 3 indicates that at 24 hr the PA5210 mutant and the PA3103 mutant generated more biofilm-mass than wild-type cells, while the PA686 and PA3102 mutants generated less biofilm mass than wild-type cells. For the two mutants (PA686 and PA5210) with the strongest biofilm phenotypes, this quantitative data is consistent with the qualitative data from microtitre assays. However, it should be noted a significant amount of variability is present in using these quantitative biofilm assays. For example, as shown in Figure 4, the absorbance data would sometimes show that the PA5210 mutant produced less 24-hour biofilm mass than wild-type cells. In the case of the PA0686 mutant, the absorption data consistently indicates less biofilm mass than wild-type cells.

One explanation for the variability in PA5210 mutant biofilm mass readings is that the PA5210 mutant biofilms might be attached more firmly to the plastic wells than wild-type biofilms. Therefore, it might be very

difficult to remove the PA5210 mutant biofilms and determine their mass. Also, it may be that although PA5210 forms wider biofilm than the wild-type, the biofilm is not as thick as the wild-type.

The results of the twitching and swarming motility assays are very consistent. As expected, swarming motility is not affected by disruption in the type-II secretion genes (Figure 4). In the case of twitching motility, mutations in the PA686 and PA3103 genes produced defects, whereas mutations in the PA3102 and PA5210 genes did not (Figure 5).

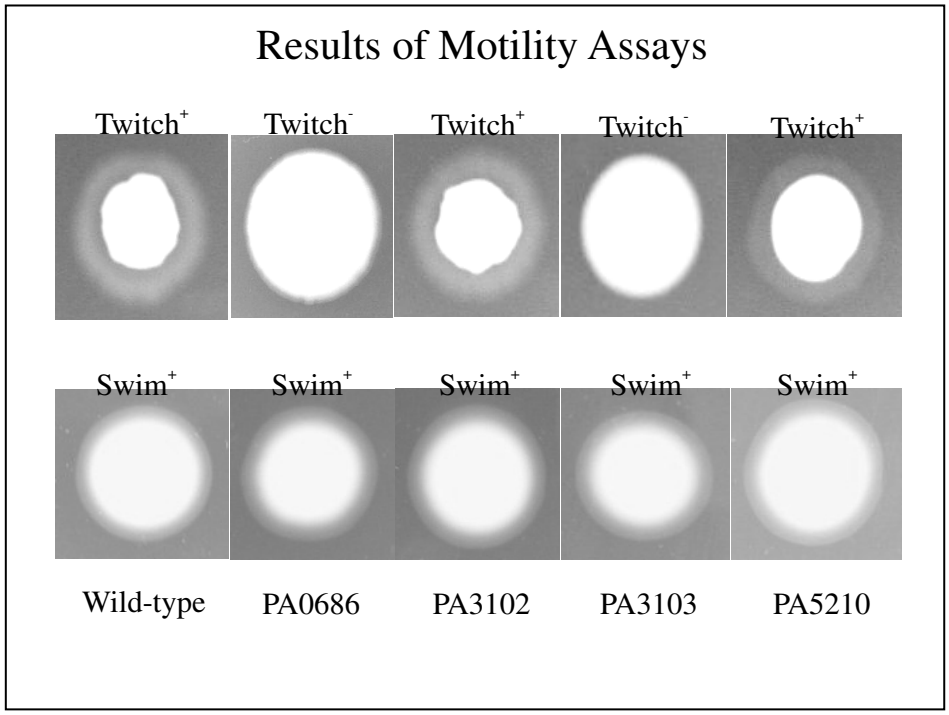


Figure 4- Twitching and swarming motility viewed under the phase-contrast

microscope (photograph provided by Dr. Kimberly Murphy, 2006). The + sign indicates that no motility defect was found, while a – sign indicates that a motility defect was found.

The motility patterns shown in figure 5 show a rounded colony without a haze of growth for mutant strains PA0686 and PA3103, which indicates that both strains are completely defective in twitching motility. On the other hand, PA5210 shows a haze of growth but the edges are smooth and not jagged as in the wild-type strain and PA3102. This result can be interpreted as that swarming motility is not affected by type-II secretion function and the type-IV pili associated movement, such as the twitching motility. However, defect in twitching motility influences the structure and development of biofilm depending on specific locus of the disrupted gene.

Conclusions

A number of proteins are differentially expressed not only between planktonic bacteria and bacteria within a biofilm, but also between bacteria within biofilms at different stages of biofilm development (Drenkard, 2003). From the microtitre plate assay, a progressive increase in biofilm mass is expected with longer incubation time. The 96-well microtitre assay results confirmed the pattern of increasing biofilm mass.

The PA5210 mutant was characterized by delayed biofilm formation since it formed a stronger biofilm than the wild-type only after 4 hours. The PA0686 mutant was characterized with weak biofilm formation from the very beginning stages. The quantification method using the spectrophotometer had conflicting data with the microtitre assay due to problems with strongly attached biofilm dispersing in ethanol.

Twitching strains of *P. aeruginosa* was found to be PA3103 which

formed a haze of growth that surrounded the point of inoculation, while PA0686 and PA3103 had none. PA5210 was special in that it formed a haze but the colony edge was rounded.

Analysis of the biofilm forming phenotype of several strains has allowed us to further understand the connection between twitching motility and biofilm formation. The types of assays designed by O'Toole and Kolter and performed in this study allow for a simple yet effective method for uncovering genes involved in biofilm formation in *P. aeruginosa* and other biofilm-forming pathogens. We found that mutant PA686 is defective for twitching motility and biofilm development using both qualitative and quantitative assays. This provides a link between type II secretion components, twitching motility and biofim development. The PA 3103 mutant also showed a twitching motility defect, but the difference in the qualitative and quantitative biofilm assay data needs to be resolved. The

PA3102 and PA5210 mutants showed no twitching motility defects, and as is the case for the PA3103 mutant, the difference in the qualitative and quantitative biofilm assay data needs to be resolved.

The next step in the study of gene expressions of *P. aeruginosa* biofilm would be to make homologous recombination mutants of the PA01 stains that correspond to the some phenotype as the transposon mutants. Homologous recombination knockout is a more direct way of gene disruption that specifies mutation in the conserved domains of the gene rather than in the unspecified regions as in transposon method. This information would allow confirmation of the role of specific genes in biofilm formation.

Due to time limitation, the antibacterial resistance assay was not done, but this assay will be a useful tool to link future experiments to *P. aeruginosa* pathogenesis. The analysis to see if the wider biofilm formed by

PA5210 is actually more resistant than the wild-type will be effected to see if biofilm structure PA5210 is as stong as the wild-type. If the PA5210 biofilm does not hold up to the same antimicrobial agent concentration as the wild-type, it will mean that the biofilm structure is actually weaker than that of wild-type due to a defect in twitching motility.

References/ Resources

1. Ball, G., Durand, E., Lazdunski, A., and Filloux, A. (2002) A novel type II secretion system in Pseudomonas aeruginosa. *Mol Microbiol* **43**: 475-485

2. Bodey, G..P., Bolivar, R., Fainstein, V., and Jadeja L. (1983) Infections caused by Pseudomonas aeruginosa. *Rev Infect Dis* **5**: 279-313.

3. Drenkard, E. (2003) Antimicrobial resistance of Pseudomonas aeruginosa biofilms. *Microbes Infect* **5**:1213-9.

4. Mikkel Klausen, Arne Heydorn, Paula Ragas, Lotte Lambertsen, Anders Aaes-Jørgensen, Søren Molin, Tim Tolker-Nielsen (2003) Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. *Molecular Microbiology* 48 (6), 1511–1524.

5. Miller, M.J., and Ahearn, D.G. (1987) Adherence of Pseudomonas aeruginosa to hydrophilic contact lenses and other substrata. *J Clin Microbiol* **25**: 1392-1397.

6. Fankhauser, D (2005) *Spectrophotometer use*. Retrieved April 15, 2005, from University of Cincinnati Clermont College, Department of Biology Web site: http://biology.clc.uc.edu/fankhauser/Labs/Microbiology/Growth_Curve/Spectrophotometer.htm

7. O'Toole, G (2007, May 8) *Pseudomonas aeruginosa*. Retrieved April 10,

2007, from Dartmouth Medical School Web site: http://www.dartmouth.edu/~gotoole/papage.html

8. O'Toole, G., Kaplan, H.B., and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**: 49-79.

9. O'Toole, G.A., and Kolter, R. (1998a) Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. *Mol Microbiol* **30**: 295-304.

10. O'Toole, G.A., and Kolter, R. (1998b) Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. *Mol Microbiol* **28**: 449-461.

11. Sarlangue J, Brissaud O, and Labreze C. (2006) Clinical features of Pseudomonas aeruginosa infections. *Arch Pediatr* **1:** S13-6.

12. Sauer, K., and A. K. Camper. 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol*. **183:**6579-6589.

13. Semmler, A.B., Whitchurch, C.B., and Mattick, J.S. (1999) A re-examination of twitching motility in Pseudomonas aeruginosa. *Microbiology* 145: 2863-2873.

14. Skerker, J.M., and Berg, H.C. (2001) Direct observation of extension and retraction of type IV pili. Proc *Natl Acad Sci USA* 98: 6901–6904.

15. Hunt, S.M., Werner, E.M., Huang, E., Hamilton, M.A., and Stewart, P.S. (2004). Hypothesis for the role of nutrient starvation in biofilm detachment. *Appl Environ Microbiol* **70**; 7418-25.

16. Russel, Marorie. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. *J. Mol. Biol* **3**; 485-499.

17. GenBank Overview (2006) Retrieved March 12, 2007, from http://www.ncbi.nlm.nih.gov/Genbank/