Biofilm Production in Response to DNA Methylation in *Pseudomonas Aeruginosa*

Avanish Madhavaram

Abstract— *Pseudomonas aeruginosa* bacterium play a comprehensive role in the development and persistence of harmful biofilms in many cystic fibrosis patients. This study is an attempt to analyze a possible genetic source for that biofilm production. With a rise in the popularity of epigenetics in bacteria, we proposed a regulation in the role of DNA methylation in *P. aeruginosa* through the extraction of the enzyme, DNA Methyltransferase, by its gene- *WspF*. Through doing so, we hoped to analyze the varying degrees of biofilm production in the mutant and control bacterial strain. Through our tests, however, the primer region indentified for extraction proved very difficult to identify and excise. This prevented the ability of testing the bacterial mutant initially. The remainder of this study operated in a much less conservative manner. Through ligation and electroporation techniques, we began preparation of this model. By staining the DNA and measuring its absorbance, the discovery of a clear difference in biofilm levels in this mutant strain became eminent. This led to the belief that DNA methylation has a direct impact in the biofilm production of *P. aeruginosa* and could directly correlate to the harmful effects of the cystic fibrosis condition. However, this belief cannot be substantiated thoroughly without the implementation of future research and analysis. Several measures and opportunities for error in the study must be noted in order to note the validity this bacterial relationship. In order to record the variance of the *wsp* operon on gene expression in a more thorough manner, further study must be implemented.

Index Terms— Biofilm, Cystic Fibrosis, DNA Methylation, DNA Methyltransferase, epigenetics, *Pseudomonas Aeruginosa*, *wsp* operon

1 Introduction

Pseudomonas aeruginosa is a proteobacteria known for causing disease in animals. It thrives in moist environments, while also having a high versatility of infecting host cells. These colonies persist in areas with low immunity and infected tissue. They are particularly dangerous and prevalent in clinics and hospitals because of the danger associated with cross-infection through medical equipment [1].

Even with these dangers, *P. aeruginosa* becomes most notorious for its bacterial participation in the condition of cystic fibrosis in the lungs. Cystic fibrosis patients suffer from chronic lung infections through the continued persistence of specific bacterial strains. These strains are able to persist even through heightened antibiotic responses and phagocytosis. This resistance in turn leads to inflammation and is the primary cause for direct lung tissue damage [2].

Cystic Fibrosis is a hereditary recessive disorder that excessively targets the exocrine glands; it then causes a mutation in the gene- Cystic Fibrosis Transmembrane Conductance regulator (CFTR). This gene is necessary for proper mucus accumulation, sweat, and digestion. Therefore, CF develops into a dangerous condition when heavy mucus begins to build in the respiratory tract and lungs of patients. This mucus is of an abnormally sticky and thick consistency. This condition is also characteristic of infection and permanent respiratory damage, with the development of scar tissue and cysts of the lungs. Cystic fibrosis is still considered a fatal disease; however, improved treatment techniques have allowed patients to live well into adulthood [3].

P. aeruginosa becomes extremely prevalent and involved in cystic fibrosis patients because the bacterium essentially accelerates an aggressive decline of the pulmonary function of the CF lung. Studies also demonstrate that there is a significant increase in lung disease of cystic fibrosis patients associated with *P. aeruginosa* infections. *P. aeruginosa* operates this way because of the complex structure of the matrix-enclosed communities that these bacteria implement [4]. As this matrix grows, it becomes known as a biofilm- the primary damaging tool in the CF condition. Biofilms develop in CF patients through a polysaccharide alginate agent that becomes embedded in the bacterial consortium. Polysaccharides, proteins, and DNA concentrations remain in this matrix as they develop into a tough mucoid substance [2].

Biofilms are harmful because they have slower growth. This in turn leads to an adaption of bacteria to the host lung and a higher mutation count. Through these properties, a biofilm assumes a high level of antibiotic resistance- becoming a unique threat to the body's innate immune system [2]. If biofilms fail to be properly regulated by the body, their threat multiplies. However, the disruption and direct attacking of biofilm levels could potentially allow for the condition and destructive effects of cystic fibrosis to be minimized.

In order to analyze the nature of the expression of the biofilm matrix in *P. aeruginosa*, a complex understanding of the bacterial epigenetics must be initially documented. Epigenetics mechanisms operate by regulating expression of the genome to different cell types without an alteration to the DNA sequence [6]. These mechanisms orchestrate cellular responses to external stimuli by selecting for various genes [7]. Epigenetic control is the mechanism that allows for the DNA sequence to be unfolded and regulated for development and interpretation [5]. The primary and most notable form of bacterial epigenetics in known as DNA Methylation. Methylation occurs after DNA synthesis and acts by attaching a specific methyl group to adenine and cytosine residues from the genome [8].

This control is dictated by the enzyme DNA Methyl-

• *Avanish Madhavaram is a class of 2016 graduate from Marvin Ridge High School in Waxhaw, North Carolina. He will attend the University of North Carolina at Chapel Hill in the fall of 2016. This research project was conducted with the North Carolina Summer Ventures in Science and Mathematics program at East Carolina University.*

transferase, which creates specific inhibition patterns by maintaining active and silent chromosomal states. The action of DNA Methyltransferase is highly contributory to the overall epigenetic response of bacterial cultures. It enables unicellular organisms to respond efficiently to environmental factors and in turn adjusts replication and cell cycle progression according to various extracellular stresses [8].

DNA methylation has already been studied based on its microbial properties, where the correlation between methylation patterns and virulence is thought to exist. One such test was presented with Salmonella typhimurium, in which methylation pathways were mutated and defected. This in turn caused the pathogen to be rendered entirely avirulent, and presented a genomic state that could be used as a vaccine 9. If the same such notion could be applied to the CF condition, in which the harmful effect of the *P. aeruginosa* infection could be subdued through the deletion of Methyltransferase- advancement in cystic fibrosis and biofilm development research could be a reality.

This study intends to illustrate how the removal of the protein coding DNA Methyltransferase gene from *P. aeruginosa* directly affects the biofilm production levels of the bacteria. We propose that if DNA Methylation is hindered in a mutated *P. aeruginosa* cell, there will be a visible difference in the biofilm production of the bacteria. If a correlation is found to exist, we realize that methylation in *P. aeruginosa* has a direct effect on the organization and communal properties of the bacterial culture, which is notable in biofilm development. This study is essential because it provides a unique perspective on a medically relevant subject in a conservative and economical manner. The modern day applicability of epigenetics is also crucial to the evaluation of this study and its potential for success, as intensive, acclaimed research continues in this dynamic field

2 PROCEDURE

2.1 Pseudomonas Aeruginosa Gel Extraction

Materials

PA01 DNA Sample
WspF Forward and Reverse Primers
Taq DNA Polymerase Supermix
PCR Tubes
Distilled Water
Thermal Cycler
Gradient Cycler
Agarose
TAE Buffer
Gel Tray (Electrophoresis Unit)
UV Light Source (Gel Imaging Unit)
1 KB DNA Ladder
Wash/Binding/Elution Buffer
Scalpel
GeneJET Purification Column
Microcentrifuge

Fig 1*- Eight PCR Tubes prepared for gradient thermal cycling- solution previously prepared*

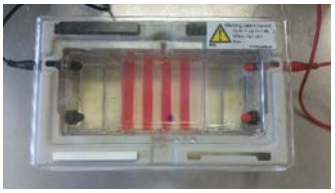

Fig 2- *Gel box with agarose solution thoroughly ran*

Fig 3- *Gel Imaging Unit used to emit UV radiation and observe the various band structure after electrophoresis*

Methods

In order to accurately extract the proper segment of Pseudomonas aeruginosa DNA, we made use of the chromosomal sample- PA01. PA01 is a comprehensive gene collection for the organism *P. aeruginosa*, which allows researchers to analyze the specific annotated genome of the species. This DNA sample has grown incredibly pertinent, and the bacteria's complex genome has been noted to compare relatively to simple eukaryotes [10]. 1 µl of the PA01 solution was imputed into 2 separate PCR tubes. These tubes allowed for 2 distinct PCR models to take place in order to ensure accuracy. Then, the forward and reverse primers for the regulatory protein, *WspF*, were added. The *wsp* operon found in *P. aeruginosa* is responsible for the receptor-signaling response associated with growth. It is linked to adaptive mutations and activates a response regulator [13]. The *WspF* primer acts as a global inhibitor. It is part of the feedback look that is present in controlling the activity of methyltransferase. It is also able to exercise control of the rest of the operon. When these primers are present, *WspF* begins to be limited in its ribosomal properties, and the protein is unable to be created [11]. *WspF* has been promisingly studied to increase cell aggregation when mutated, providing added optimism to the success of this study [12]. 1 µl of the forward and reverse reactants were added to both tubes at this stage. Then, 12.5 µl of Taq Polymerase Supermix were added to both solutions. This solution inhibits polymerase activity, as well as permitting varied temperature set-ups necessary for amplification. After this addition, 9.5 µl of distilled water were included in order to achieve a total concentration of 25 µl.

These two tubes were then placed in a thermal cycler machine where they underwent the polymerase chain reaction. This machine is able to simulate extracellular conditions at various temperatures in order to amplify the DNA sequence in an intermittent fashion. This initial PCR was completed at the following temperature gradient:

95^{o} for 1 minute
95^{o} for 15 seconds
58^{o} for 15 seconds

72° for 10 seconds
72° for 2 minutes

This cycle was then repeated 40 times in order to maximize the amount of time the primers had to efficiently bind and operate on the genetic sequence. After this PCR was ran to its duration, the solutions were placed onto an agarose gel and were ran through electrophoresis. Upon analysis of this gel, there was not enough band presence available for active extraction of the DNA segment- forcing experimentation through a new, gradient PCR technique.

Gradient PCR is a process where researchers can test the same solution of chromosomal DNA and primer sets, but at a varied temperature range for each tube. In this test, each chromosomal DNA set was initially diluted 1 to 10 with distilled water. This ensured that the DNA wasn't as highly concentrated as before. Then, the 1:10 chromosomal DNA, primers, supermix, and distilled water were all imputed into 9 separate PCR tubes. The input levels of all of these factors remain the same as in the initial PCR steps above, with a total concentration of 25 µl in every tube.

When these samples were reacted in the cycler machine from a range of 53° to 68° and struck onto a gel, conclusive and promising DNA streaking began to appear. This gel plate, when ran under a UV light source, revealed considerable chromosomal development and bar presence in 3 different sample wells. These streaks all occurred in the 5 µl chromosomal concentration sample and developed at the temperatures- 53°, 59.4°, and 63.7°. These values were a uniquely wide range of temperatures that all happened to show similar promise. The 59.4° sample, however, showed the most reactive and changing state on the gel because it was able to manipulate a portion of its primer region in the visual. Due to the promise of that sample, a future PCR test was again narrowed to fit that temperature scale.

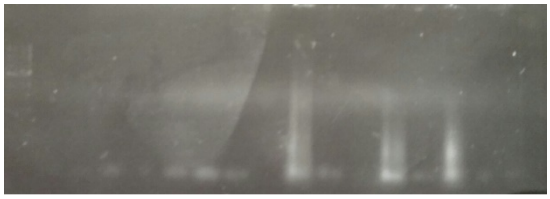

Fig 4- *This image documents the initial gradient PCR test, showing promising chromosomal fragment visualization. It clearly demonstrates a relationship between the chromosomal dilution factor and DNA migration.*

Another gradient cycler reaction was then prepared. This test used only three PCR tubes, with the 5 µl chromosomal DNA dilution again. This form of PCR operated directly at 59°, while also commencing a highly variable PCR sequence. This sequence began with a very high temperature gradient in order to allow for the primers to cross and come into place. Then, the cycler was programmed to sharply drop the machine temperature in order to allow for the primers to create a strong hold on the chromosomal fragment. This would allow for a more accurate and strong primer presence, increasing the opportunity for a clearer plasmid visual. A final gel electrophoresis test was then run with these three sample concentrations. The results are shown in ***Fig 6***, where the forward and reverse primer regions shows a great amount of illumination. This visual promotes the idea that there may be a presence of plasmid formation at that base range. The primers begin right at the 2,000 base pair range, where the gene is expected to be targeted at. This targeted gel sample provided encouraging enough results to allow for procession into gel extraction.

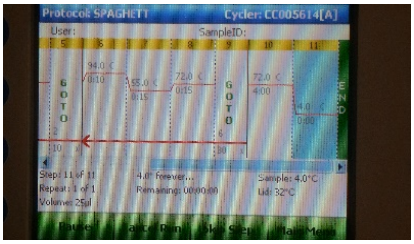

Fig 5- *Conditions for the highly variable, gradient PCR test. Note the differences between the peak and bottom temperature in the beginning of every cycle*

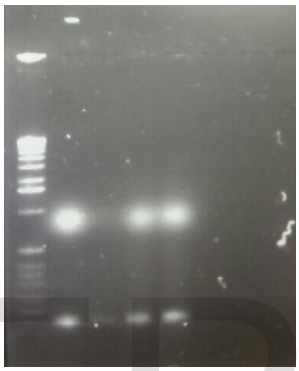

Fig 6- *This figure represents the visual of the final gel ran prior to extraction. The three brightest regions illustrate where the primer had the strongest hold and is the site of plasmid formation. Chromosonal DNA presence is strong enough to warrant extraction*

Gel extraction began by excising the primer region of the DNA at the 2,000 base pair location. The three bands were physically removed through a scalpel and placed into two 1.5 ml tubes. They were then washed with binding buffer and incubated between 50 and 60°. After the gel was entirely dissolved, it was entered into two columns that were then washed and centrifuged multiple times. After a final 1-minute spin with elution buffer, the purified DNA sample was extracted into a fresh collection tube and stored at -20° C.

2.2 Ligation and Transfection of *P. Aeruginosa* Bacteria with pUC19 DNA

Materials
pUC19 DNA sample
10x Digestion Buffer
Distilled Water
Sma1 Enzyme
Ligation Buffer
Incubator

Methods
pUC19 DNA was implemented to be used as a suicide plasmid. This plasmid was intended to replace the locus where the gel was initially extracted. In order to do so, a pUC19 digest was necessary to be set up. This digest required the addition of 10 µl of substrate DNA, 2 µl of 10x stock digestion buffer, 1 µl of the restriction enzyme- Sma1, and 7 µl of distilled water. After this solution was made, it was incubated for close to 10 minutes at 37°.

This solution was then heat inactivated at 65 degrees for 10 minutes. Afterwards, a ligation DNA procedure was enacted. This procedure required a 2x ligation buffer, the Sma1 enzyme, as well as the vector from the digest previously mentioned and the original purified DNA insert. The solution consisted of 10 µl of buffer, 4 µl of digest vector, 5 µl of *WspC* insert, and 1 µl of the enzyme. Two tubes were used for collection of the two differing inserts from the three original gel bands.

Then, a plated bacterial specimen from *P. aeruginosa* was presented. In order to get all of the DNA precipitate, the bacteria were suspended in distilled water. This solution was vortexed and then centrifuged for 1 minute. The supernatant was then pipetted out, and the solution was resuspended in 300 µl of distilled water again. Both tubes were then gently mixed.

Finally, this sample was run through electroporation, a conductive process that transfers an electrical impulse across the cell suspension. The electrical potential across the cell membrane causes charged molecules like DNA to be driven through the membrane pores- initiating transfection of the pUC19 DNA and the *P. aeruginosa WspC* plasmid. These resultant bacterial samples were then plated and incubated overnight.

2.3 Testing for Biofilm Development

Materials

Luria Broth
Glass Tubes
Mutant *P. aeruginosa* sample
Wild *P. aeruginosa* sample
Phosphate Buffered Saline
Shaking Incubator
Spectrophotometer with cuvettes

Methods

In this test, we began by observing the mutant bacterial sample from the previous day. There was a numbered few alien colonies on those plates. These colonies were then extracted and placed in 1 ml of PBS (phosphate buffered saline) solution. Wild *P. aeruginosa* colonies were also isolated from a separate plate and placed in another 1 ml PBS tube. These solutions were vortexed completely.

Then, six glass tubes were set up with 1 ml of Luria broth each. In three of the tubes, 100 µl of the mutant DNA solution were added. In the other three, 100 µl of the wild type DNA were integrated. These solutions were again vortexed and then placed in a shaking incubator at 37°. Using glass tubes allows for a greater visualization of biofilm levels because we can evaluate accumulation at the sides of the tube. The shaking incubator allows for this staining to take effect, thoroughly integrating the bacterial sample with the solution in order to observe results completely.

This solution was placed in the incubator for several hours. When the solution was removed, the entire culture was poured off. This was because the biofilm levels accumulate at the cusp of the glass tube in the sample. Afterwards, the dye solution, Crystal Violet, was inserted into the tube just over the fluid line from before. This solution was incubated at 37° for 10 minutes. The dye was then washed off with water gently. The sample was tapped in order to remove the excess liquid, and filled with 30% acetic acid. This sample was then tested in a spectrophotometer for absorbance rates. 1 ml of solution was filled into cuvettes for testing.

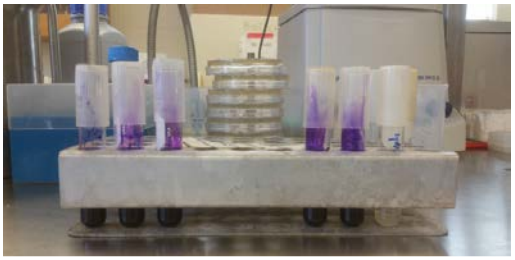

Fig 7- *Process of adding dye to the cusp of tube*

3 Results

When analyzing both samples in this test, with the mutant DNA and the wild type bacteria- several observations can be made. The first is the visual film at the cusp of the glass tube in the mutant and wild samples. There was a definite difference in the amount of staining at the base of the tubing, calling attention to the effect of *WspF* gene on the product of biofilm development.

Fig 8- *Staining differences in mutant and wild type bacteria*

Fig 8, to the left, shows a clear difference in the level of biofilm development between both samples. These samples show the greatest visual of biofilm difference, and the quantitative spectrophotometer data below further substantiates this. The mutant DNA does seem to undergo severe change that occurs when the gene encoding for methylation is removed. This entirely disrupts the ability of the sample to generate harmful biofilms, which could render the condition less effective and reduce irritation.

Table 1- The spectrophotometer data is documented below.

	M1	M2	M3	W1	W2	W3
Absorbance	.840	.980	1.606	2.233	1.055	1.438

**M1, M2, M3- Mutant 1, Mutant 2, Mutant 3*
**W1, W2, W3- Wild 1, Wild 2, Wild 3*

These levels of absorbance are highly indicative of major biofilm production differences. Although Mutant 3 did prove to have a much higher absorbance rate than the other mutant samples, it should be observed as an outlier- with the greater trend of the data showing a much smaller value among the test group. Again, these results ensure that biofilm levels trend differently with this one plasmid mutation- calling for a deeper discussion of methylation and epigenetic patterns.

4 DISCUSSION

4.1 Analysis

The *WspF* gene, when excised, provided a strong phenotypic difference in this study. This gene encodes for the entire *wsp* operon in *Pseudomonas aeruginosa* bacteria. Therefore, it acts in an all-inclusive fashion over DNA Methylation and the enzyme, DNA Methyltransferase. It can then be predicted that there is a direct correlation in the engineering of epigenetics with the virulence factors in the respiratory pathogen *P. aeruginosa*. Biofilm development is a major harm associated with the cystic fibrosis condition. This study proves that the methylation factors of the *P. aeruginosa* bacteria has a direct involvement in the harmful and deadly nature of the disease. The *wsp* operon still can be studied greatly within the lab in order to recognize more methylation factors associated with its function. Due to the harsh nature of *P. aeruginosa* as a pathogen, these factors can continue to be tested in order to understand more about the nature of this operon on harmful bacterial functions. *P. aeruginosa*, more than most bacterial specimen, seems to rely on the overall function of various genes controlled by a similar source. This observation can be directly related to methylation and epigenetic mechanisms.

This study was a simple observation about epigenetic control of virulence factors, as well as an in depth and highly applicable study of the development of biofilms- highly destructive in a medical, respiratory condition. We hope to use these observations to further the understanding of *P. aeruginosa* in the CF case, as well as to provide the opportunity for other virulence studies.

4.2 Error Examination

This study portrayed several considerable trends that could be attributed to experimental error. The primary cause of these were the time restraints implemented. The smaller timeframe present did not promote a complete, double cross examination with the pUC19 plasmid and the *WspF* primers, therefore a much less revealing single cross approach was used. This format could have caused the phenotypic expression of the biofilm levels to be only partially altered. Also, because of the time commitment to a lengthy PCR test in this study, the remainder of the test became much more variable and flux. This led to the lack of multiple controls into the stages of experimentation following gel extraction. Therefore, the digest and ligation mixtures were run without a comparative sample. By running the study in this manner, there becomes the possibility that these results lack substantiation because error could have been introduced in some stages of experimentation. Because of this, any acceptance or negation of the hypothesis must be made hesitantly due to the chance of preparation mistakes.

4.3 Further Study

The sample tested in this study was mutated to be represented without a methylation pathway. While there were great decreases in the level of biofilm development in the test tube, there was still banding with the dye at the bottom of the sample. This is definitely not expected to occur because the presence of oxygen causes these biofilms to normally persist at a higher level, just above the fluid line. This occurrence in the mutant strain is particularly interesting and can be studied further in order to prove the effect of methylation and the tested gene on the altered location of biofilms. Also, by using a double cross technique, we could observe how much more developmental differences persist in both samples. Finally, because of the varied effect of the *wsp* operon on multiple gene expressions- there could be many, more specified tests ran to detail its widespread impact on virulence through different forms of primer control.

ACKNOWLEDGMENT

Avanish Madhavaram would like to sincerely thank North Carolina Summer Ventures in Science and Mathematics for providing an opportunity to attend a cost free, immersive, four-week program at a state university in order to strive towards a career in the scientific field. Madhavaram would also like to thank his lead professor, Dr. Eric Anderson, for his devotion to this project and his continued strategical and resourceful help while in the laboratory. The help of Mr. Ian Davison and Ms. Rebecca Nickle must also be noted for their continued assistance and added knowledge. Finally, Madhavaram would like to greatly thank East Carolina University and the Biology department for their devotion to the Summer Ventures program and their generous donation of resources and the laboratory space necessary in making this study presentable.

REFERENCES

[1] Zhang, Z., Louboutin, J. P., Weiner, D. J., Goldberg, J. B., & Wilson, J. M. (2005). Human airway epithelial cells sense Pseudomonas aeruginosa infection via recognition of flagellin by Toll-like receptor 5. *Infection and immunity*, 73(11), 7151-7160.

[2] Høiby, N., Ciofu, O., & Bjarnsholt, T. (2010). Pseudomonas aeruginosa biofilms in cystic fibrosis. *Future microbiology*, 5(11), 1663-1674.

[3] NEW, W. S. Encyclopedia> Cystic fibrosis.

[4] Costerton, J. W. (2001). Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends in microbiology*, 9(2), 50-52.

[5] Bierne, H., Hamon, M., & Cossart, P. (2012). Epigenetics and bacterial infections. *Cold Spring Harbor perspectives in medicine*, 2(12), a010272.

[6] Holliday, R. (2006). Epigenetics: a historical overview. *Epigenetics*, 1(2), 76-80.

[7] Razin, A., & Riggs, A. D. (1980). DNA methylation and gene function. *Science*, 210(4470), 604-610.

[8] Casadesús, J., & Low, D. (2006). Epigenetic gene regulation in the bacterial world. *Microbiology and molecular biology reviews*, 70(3), 830-856.

[9] Heithoff, D. M., Sinsheimer, R. L., Low, D. A., & Mahan, M. J. (1999). An essential role for DNA adenine methylation in bacterial virulence. *Science*, 284(5416), 967-970.

[10] LaBaer, J., Qiu, Q., Anumanthan, A., Mar, W., Zuo, D., Murthy, T. V., & Brizuela, L. (2004). The Pseudomonas aeruginosa PA01 gene collection. *Genome research*, 14(10b), 2190-2200.

[11] Bantinaki, E., Kassen, R., Knight, C. G., Robinson, Z., Spiers, A. J., & Rainey, P. B. (2007). Adaptive divergence in experimental populations of Pseudomonas fluorescence. III. Mutational origins of wrinkly spreader diversity. *Genetics*, 176(1), 441-453.

[12] Hickman, J. W., Tifrea, D. F., & Harwood, C. S. (2005). A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proceedings of the National Academy of Sciences of the United States of America*, 102(40), 14422-14427.

[13] Spiers, A. J. (2014). A mechanistic explanation linking adaptive mutation, niche change, and fitness advantage for the Wrinkly Spreader. *International journal of evolutionary biology*, 2014.