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Identification of Transcription Regulators of the AlgZ/R Two-Components Regulatory System in Pseudomonas aeruginosa

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Identification of Transcription Regulators of The AlgZ/R Two-Components Regulatory System in *Pseudomonas aeruginosa*

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by

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ABSTRACT

Identification of Transcription Regulators of the AlgZ/R Two-Components Regulatory System in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that express a plethora of virulence components controlled through two-component regulatory systems that allow for sensing and responding to environmental stimuli. This study was aimed at identifying transcription regulators of *algZ* that encodes the histidine sensor kinase (AlgZ) of the AlgZR two-component regulatory system. To understand how the *algZ* gene is transcriptionally controlled, transposon mutagenesis was used to create a mutant library with varying *algZ* expression based on their β-Galactosidase activity. The gene *PA3327* was identified as a potential regulator of *algZ* expression using arbitrary PCR. This gene encodes a probable non-ribosomal peptide synthetase responsible for the biosynthesis of secondary metabolites such as antibiotics. Further experiments are required to understand how *PA3327* transcriptionally regulates *algZ* expression and its physiological role in the organism. Because the AlgZ/R system regulates virulence, it is possible to attenuate virulence by targeting the expression of *algZ* gene.

DEDICATION

This work is dedicated to my two beautiful nieces Yaa Asantewaa Donkor and Akua Boamah Donkor. These two have been the main reason for my academic journey. This work is also dedicated to my mum who has been a pillar in my life.

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CHAPTER 1: INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative opportunistic bacteria and a common nosocomial pathogen known to cause life threatening infections in immunocompromised people (Gellatly & Hancock, 2013). Patients with cystic fibrosis, HIV and people recovering from burn wounds are usually at risk of *P. aeruginosa* infection (Gonzalez et al., 2016; Meynard et al., 1999; Yetkin et al., 2006). *P. aeruginosa* is metabolically versatile and ubiquitous in the environment, surviving even in the harshest of environments. This characteristic makes it possible for *P. aeruginosa* to inhabit any tissue in the human body. *P. aeruginosa* possesses a wide array of virulence factors that enables it to colonize, survive and damage host tissue while evading detection and elimination by host immune system and antibiotic treatment. It is capable of establishing an acute infection (Pankhaniya et al., 2004) characterized by the presence of pili which mediates adherence, and twitching motility (Bradley, 1980). *Pseudomonas aeruginosa* exhibit two types of virulence factors. Factors involved in acute (early stage) infection are either on the surface or secreted. The type 4 pili responsible for adherence to epithelium, the exoenzyme S and other adhesins reinforce the adherence to the epithelium. Factors involved in in chronic infection includes siderophores (pyoverdine and pyochelin), which allows bacteria growth in the absence of ferrous ions (Konings et al., 2013; Takase et al., 2000). Strains isolated from patients with cystic fibrosis produce alginate that protects the bacterium from host immune system, dehydration and antibiotics. Alginate production improves adherence to solid surface forming biofilm. Development of alternative therapies for treatment of *P. aeruginosa* infections is important because of its intrinsic and growing resistance to current antibiotics. One important such alternative therapy is to target regulators of virulence factors using anti-virulence drugs

(Heras et al., 2015). This option can be used to inhibit the expression and activity of virulence factors, facilitating eradication of infections by the host immune response (Leid et al., 2005) and antibiotics (Alkawash et al., 2006). Understanding virulence factor regulation in *P. aeruginosa* is essential to enable us to target regulatory signal responsible for modulating the numerous virulence factors associated with acute and chronic infecting strains.

Virulence Factors

Bacterial virulence factors are cell surface and secreted proteins or biological molecules such as exotoxins, flagella, type IV pili (T4P) and alginate produced by pathogenic bacteria that enable them to colonize a niche in the host, evade and suppress host immune response, damage host tissue, and also obtain nutrients from host (Ben Haj Khalifa et al., 2011; Fleitas Martínez et al., 2019). *P. aeruginosa* virulence factors enables it to initiate and sustain infections or diseases in different hosts and host tissues. Acute infecting strains are motile and are characterized by the possession of early state virulence factors for tissue invasion, tissue necrosis, and attachment to epithelium (Moradali et al., 2017). Chronic infecting strains are sessile, form biofilm and produce alginate (Laventie et al., 2019).

In chronic infecting strains isolated from the lungs of patients with cystic fibrosis, the biosynthesis and secretion of alginate is upregulated (May et al., 1991). This is due to the increase in transcription of enzymes involved in alginate biosynthesis, maturation and secretion. Alginate production and biofilm formation in chronic infecting strains makes them resistant to host immune response and antibiotic treatments (Franklin et al., 2011; May et al., 1991).

Acute infecting strains cause rapid cell death or tissue damage. This is due to the presence of the T3SS used to inject effector proteins directly into eukaryotic cells and exotoxin

A responsible for tissue necrosis and four protease responsible for bleeding and tissue necrosis (Ben Haj Khalifa et al., 2011).

P. aeruginosa is capable of swimming, swarming and twitching motility. Virulence factors mediating these forms of translocation are the T4P and flagella (O'Toole & Kolter, 1998; Persat et al., 2015). These factors enable the bacteria to colonize new niches, as in the case of acute pneumonia when the bacteria moves deeper into lungs from the endotracheal tubes (Hauser, 2009; Sadikot et al., 2005). It has been demonstrated that when this bacterium encounters a solid surface, c-di-GMP levels increases and stimulates pili assembly (Laventie et al., 2019).

Regulation of virulence is important in pathogenic bacteria for survival and continuous presence of a disease condition in a host (Wout et al., 2015) . Depending on the environmental cues, the expression of certain virulence factors are upregulated and others repressed. When *P. aeruginosa* is grown on a solid surface, planktonic cells transition into sessile cells upregulating the expression of genes involved in biofilm formation and alginate biosynthesis (Petrova & Sauer, 2011). *P. aeruginosa* virulence factors are tightly regulated using two-component systems (TCS). This allows the bacteria to sense and respond to environmental cues through signaling pathways which terminates in either activation or repression of gene transcription (Stephenson & Hoch, 2002). The quorum sensing system is another means by which *P. aeruginosa* regulate the expression of virulence genes necessary to the survival and proliferation in host (Whiteley et al., 1999).

Two-Component Regulatory Systems (TCS)

Bacterial survival depends on the ability to detect changes in the environmental and mount the appropriate response by upregulating the expression of genes necessary for survival and proliferation in the host (Tiwari et al., 2017). This survival instinct is mediated by two-component systems consisting of a membrane bound sensor (histidine) kinase and a cytoplasmic response regulator. PilS-PilR system is an example of a quorum sensing system that regulates type IV pilus expression, biofilm formation, twitching and swarming motility (Ishimoto & Lory, 1992; Kilmury & Burrows, 2016). The sensor kinase picks up environmental cues, autophosphorylates and then relays this information into the cell via phosphorylation of the response regulator (Francis et al., 2018). The phosphorylated response regulator then mediates gene expression through DNA binding. Response regulators have been shown to have transcription regulatory effect in both phosphorylated and unphosphorylated states (Deretic et al., 1992; Ma et al., 1998). This is evident in *P. aeruginosa* AlgZ/R TCS where phosphorylated AlgR drives the expression of the *fimU* operon leading to type IV pili assembly and unphosphorylated AlgR drives the biosynthesis of alginate through activation of *algC* and *algD* expression (Mohr et al., 1992; Zielinski et al., 1992). An example of response regulator phosphorylation in *P. aeruginosa* is when AlgR phosphorylation by AlgZ results in the expression *fimU* operon encoding minor pilins and the PilY1 protein necessary for twitching motility and biofilm formation (Whitchurch et al., 2002) (Luo et al., 2015). In summary, pathogenic bacteria rely on the use of the two-component transcriptional regulatory system for survival and proliferation in the host (Ben Haj Khalifa et al., 2011). The genome of *P. aeruginosa* encodes one of the largest complements of TCS regulatory proteins found in bacteria and are responsible for the different phenotypes in *P. aeruginosa (Rodrigue et al., 2000)*.

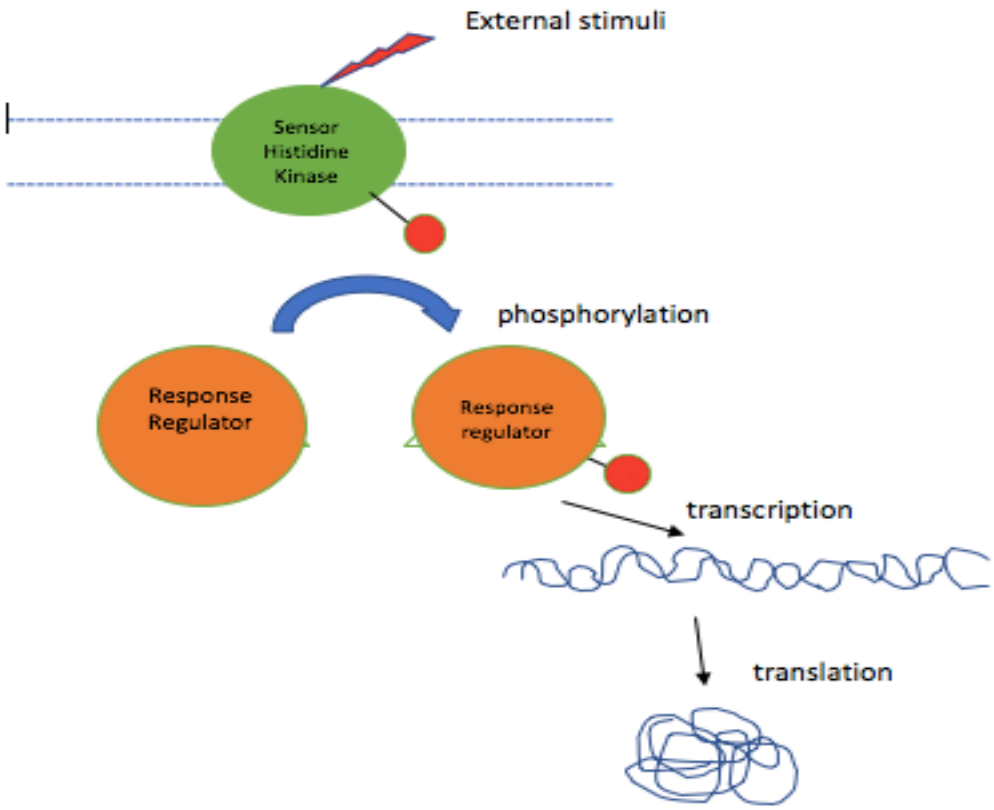


Figure 1. Schematic representation of a generic bacteria two-component system. Phosphorylation of response regulator results in downstream expression of desired genes

AlgZ/R Two-Component System

In *P. aeruginosa*, the AlgZ/R two-component system is of great importance because it coordinates multiple phenotypes (Okkotsu et al., 2014). In this system, AlgZ is the membrane bound sensor kinase, and AlgR is the cytoplasmic response regulator that acts as a transcriptional regulator via DNA binding(Stephenson & Hoch, 2002). During signal transduction, phosphorylation activate transcriptional regulators (Lukat et al., 1992). This is not the case with AlgR since both phosphorylation states are capable of activating gene transcription (Okkotsu et al., 2014). In the phosphorylated state, AlgR regulates twitching motility by activating the

transcription and expression of the *fimU* operon encoding minor pilins important in pilus biogenesis (Whitchurch et al., 1996).

In summary, the AlgZ/R TCS is essential in regulating important virulence factors in *P. aeruginosa* and elucidating the regulation of the different components of the system (AlgZ and AlgR) could uncover potential anti-virulence therapies for effectively combating *P. aeruginosa* infections.

Regulation of algZ/R Operon

The genes encoded in the *algZ/R* operon are co-transcribed occasionally. However , the presence of different and independent promoters for *algZ* and *algR* indicates the presence of independent transcription regulation mechanisms for these genes (Pritchett et al., 2015). Two independent promoters for *algR* are found within the *algZ* coding region. Recent studies have identified two transcription initiation start sites for *algZ* upstream of the *algZ* coding region(Pritchett et al., 2015) .

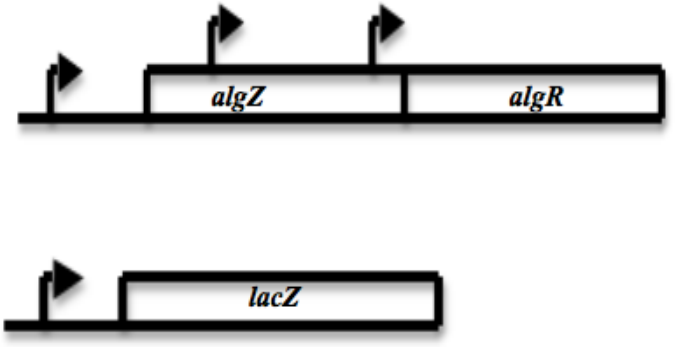


Figure 2. Schematic representation of AlgZ/R operon and the algZ transcriptional fusion. Arrows denote promoters for *algZ*, algR, and *algZ-lacZ* transcriptional fusion. The transcriptional fusion encodes β-Galactosidase.

Although *algZ* and *algR* are sometimes co-transcribed, the promoters presented in Figure 2 indicate that regulation of *algR* expression can occur independently of *algZ* expression.

Transcriptional regulation of *algR* has been well studied in previously and is known to be modulated by sigma factor AlgU and RpoS (Pritchett et al., 2015). Transcription of *algZ* on the other hand is known to be regulated by virulence factor regulator (Vfr), which binds upstream of *algZ* to intiate transcription. Previous studies have shown that the type IV minor pilins and PilY1 regulate virulence by modulating the activity of AlgZ/R two-component system in *P. aeruginosa* (Marko et al., 2018). However, the exact mechanism of this regulation is still being investigated. Our study aims to determine the regulators of *algZ* expression and their physiological roles in the pathogenesis of *P.aeruginosa*. Since the PilY1 protein encoded in the *fimU* operon negatively regulate *algZ* expression and this regulatory effect is not due to activity through cAMP-Vfr signaling pathway, this unlocked the possibility for the potential discovery of novel regulators of *algZ* expression. Promoter region upstream of *algZ* has an increased activity in a *pilY1* mutant but has no know regulators (Pritchett et al., 2015). This mutant background serve as reference strain for identifying positive regulators of the promoter upstream of *algZ* since these positive regulators repress or inhibit the promoter activity

CHAPTER 2: MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa PAO1 wild-type strain was used as the reference strain in all experimental procedures. All strains of *P. aeruginosa* (wild-type and mutants) used in all experiments were grown at 37ºC on Pseudomonas Isolation Agar (PIA) and PIA supplemented with the appropriate antibiotics when necessary. For conjugation, *P. aeruginosa* was incubated at 42°C for 2 hours to inactivate the restriction modification system to allow for successful integration of foreign DNA into its genome. The *E. coli sm10 λpir* strain containing the pBT20 plasmid was used for conjugation with Δ*pilY1algZ*TF-*lacZ* to generate transposon mutants. *E. coli* strain containing the pBT20 plasmid were grown at 37˚C on LB plate supplemented with gentamicin when appropriate. Conjugation reactions were performed on LB plate and incubated at 30˚C. For certain experimental procedures, *P. aeruginosa* strains were grown in LB broth supplemented with antibiotics at 37˚C with shaking between 8 to 16 hours. Freezer stocks of all bacteria strains were made by mixing 360uL of 50% glycerol, 240uL of LB broth and 400uL of bacterial culture in a freezer tube for storage at -80ºC. Plasmids used in this study are listed in Appendix C. Growth media and antibiotic concentrations are listed in Appendix A.

Transposon Mutagenesis

The *pilY1 P. aeruginosa* mutant containing the *algZ* transcriptional fusion (Δ*pilY1algZ*TF-*lacZ*) was grown on a PIA plate at 42ºC overnight. The sm10 λpir *E. coli* strain containing the mariner transposon on pBT20 plasmid was grown on LB agar supplemented with 15ug/uL gentamicin and incubated at 37ºC overnight. Both strains of bacteria were scraped from their plates, and resuspended in 500uL of sterile 0.85% saline in a 1.5mL microfuge tube and plated on LB agar for 2hrs at 30ºC to allow for conjugation. After 2hrs conjugants were scraped,

resuspended in 500uL of sterile 0.85% saline and plated PIA plate supplemented with 150ug/uL gentamicin. Irgasan in PIA kills any remaining *E. coli* whiles gentamicin kills any *P. aeruginosa* strain that does not take up the pBT20 plasmid during the conjugation. These plates were incubated at 37˚C for 16 – 24 hours. Isolated colonies representing mutants were then used for High Throughput β-Galactosidase assay to identify mutants with significantly different reporter activity.

High Throughput β-Galactosidase Assay

Isolated mutant colonies on PIA gentamicin plates were inoculated into 200uL LB broth in a 96-well plate with the reference strain Δ*pilY1algZ*TF-*lacZ* inoculated in well A1. The 96-well plates were incubated for 8hrs at 37^{o}C with shaking. Cells incubated more than 8hrs showed clumping and biofilm formation leading to inaccurate results from the β-Galactosidase assay. After the 8hrs incubation, optical density (OD) of each well was measured at 600nm using a microtiter plate reader. To rule out the effect of a growth defect on our assay, mutant cells with OD_{600} readings significantly reduced from neighboring cells were excluded from the screening. Using a new 96-well, 100uL of permeabilization buffer was added to each well. From the initial 96-well plate, 10-30uL of 8hrs cultures was transferred into the 100uL permeabilization buffer and mixed thoroughly by pipetting up and down several times. The plates were left for 10 minutes to settle. To each well 30uL of ONPG solution was added and reaction stopped with 70uL of 1M Na_2CO_3 solution when a yellow color developed. The reaction time from addition of ONPG to cessation of reaction with Na_2CO_3 was noted and recorded for the first horizontal line of wells (A1 to A12). The process was repeated for the remaining wells using the same reaction time. The plates were centrifuged at 8000G and optical density of each well measured at 420nm using the microtiter plate. Miller units were calculated using the formula below:

$$1000* (OD_{420})/(OD_{600}*\text{volume of lysate}*\text{time})$$

Note, time is in minutes, and lysate volume is milliliters.

Confirmation of β-Galactosidase Assay

Using 1:100 dilution factor, 50uL of significant mutants from the High Throughput β-Galactosidase assay were inoculated into 5mL LB broth supplemented with 150ug/ul gentamicin and grown at 37^{o}C with shaking for 8hrs. For confirmation assay, 750ul of cultures were spun at 8000G for 5 minutes. Supernatant discarded and pellet resuspended in 1mL chilled Z-buffer plus 2.7% β-mercaptoethanol (BME). Optical density was measured at 600nm and recorded. Then 50ul chloroform and 50ul 10% SDS were added to each reaction tube and vortexed to permeabilize the cells. Mixture was allowed to sit for 10 minutes to settle and allow for maximum permeabilization. Then 10-200uL of cell lysate was added to Z-buffer plus BME solution and 200uL of ONPG added to make a volume of 1mL. Each reaction was stopped with 500uL of 1M Na_2CO_3 when a yellow color developed. Optical density of each reaction tube was measured and recorded at 420nm and 550nm. Miller units were calculated using the following equation:

$$1000* (OD_{420}-(OD_{550}*1.75)/(OD_{600}*\text{volume of lysate}*\text{time})$$

Note, time is in minutes, and lysate volume is milliliters.

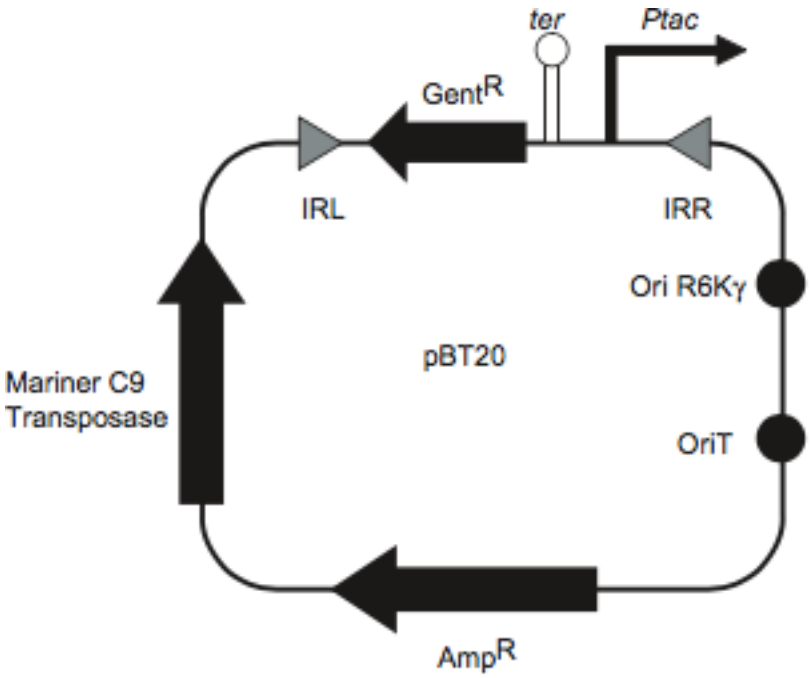
Colony PCR

To screen for the presence of gentamicin gene in the transposon, colony PCR was performed to confirm insertion of the gent gene into the genome of *P. aeruginosa*. Colony PCR master mix was assembled on ice and 24uL aliquoted into PCR tubes. A single colony of mutant of interest was transferred from a plate using a sterile tooth pick into the PCR tubes.

Table 1. Colony PCR for Gentamicin Gene

Master Mix	PCR Program
Per sample: 19uL ddH2O 2.5uL 10X PCR Buffer 1uL DMSO 0.5uL 10mM dNTPs 0.5uL 10uL GentF primer 0.5uL 10uL GentR primer 0.1uL Taq DNA polymerase	95°C – 3 minutes 95°C – 30 seconds 60°C – 30 seconds 72°C – 2 minutes Repeat steps 2-4 30 times 72°C – 5 minutes 4°C – Hold

Arbitrary PCR

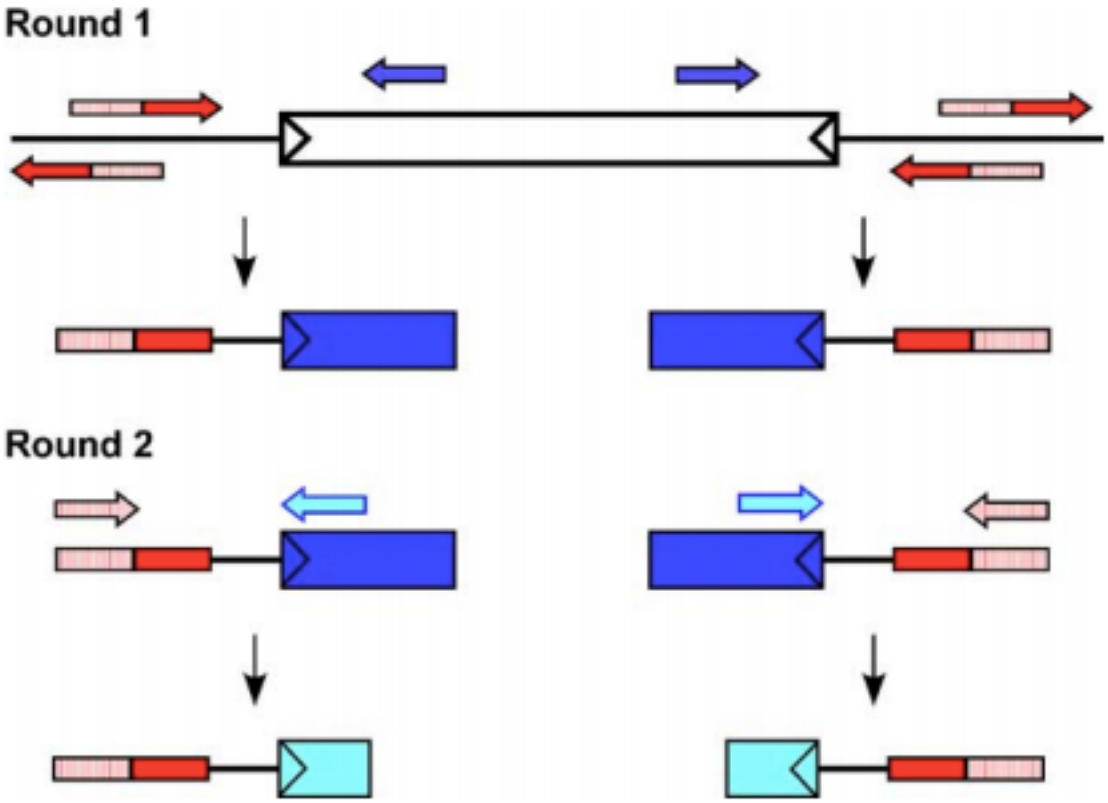


(Kulasekara et al., 2005)

Figure 3. Shows the schematic representation of pBT20, the mariner transposon mutagenesis vector. IRL and IRR: inverted repeats for trans position; Gent^R: *aaC1* gene encoding gentamicin resistance for positive selection of transposon insertions; Amp^R: bla gene encoding ampicillin resistance for plasmid conjugation; *Ptac*: the outward directed tac promoter; Mariner C9 Transposases: encodes transposases for recombination; ter: transcription termination; Ori R6Kγ origin of replication; *OriT*: origin of transfer from broad host range plasmid.

Genes interrupted by transposon were identified using arbitrary PCR. Arbitrary PCR is a two-step PCR process which involves the arbitrary primers, two different PCR processes. A single colony from a plate of a mutant of interest was inoculated into 3mL LB broth supplemented with 150ug/ul gentamicin and incubated at 37^{o}C for 16hrs with shaking. For DNA template, 750ul of 16hrs culture was spun at 8000G for 5 minutes. Supernatant was discarded and pellet resuspended in 1mL of arbitrary PCR lysis buffer. This solution was then heated at

95ºC for 10 minutes and centrifuged at 8000G for 10 minutes. The resulting supernatant was then used as template DNA in the arbitrary PCR reaction. While the cell lysing process was occurring, the master mix for arbitrary PCR was assembled on ice. All primers used in the arbitrary PCR are shown in Appendix C



(Das et al., 2005)

Figure 4. Principle of Arbitrary PCR. Arbitrary primers depicted as solid and striped red bind randomly in the genome. The transposon specific primers depicted blue bind to the transposon and create amplicons in conjunction with the arbitrary primers. Products from round one PCR are used as templates for round two PCR. In the round two PCR, sequence specific primer (turquoise) paired with the Arb2 primer identical to the 5' sequence of the round one arbitrary primers. Round two amplicons are subjected to gel electrophoresis and DNA sequencing.

Table 2. Arbitrary PCR #1

Master Mix (ARB1)	ARB1 PCR Program
Per reaction: 36.25uL ddH_2O 1uL DMSO 1uL 10nMdNTPs 5uL Thermopol buffer 2uL 10uM ARB1 primer (RdPa1, RdPa2, RdPa3, or combination of these primers) 2uL 10uM internal specific primer (R1TnM) 0.25uL Taq DNA polymerase	1. 94°C – 3 minutes 2. 94°C – 3 minutes 3. 49°C – 30 seconds (reduce temp by 1°C for each subsequent round) 4. 72°C – 3 minutes 5. Repeat steps 2-4 15 times 6. 94°C – 30 seconds 7. 60°C – 30 seconds 8. 72°C – 3minutes 9. Repeat steps 6-8 20 times 10. 12°C – Hold

To each PCR reaction tube, 46uL of the master mix was added along with 1.5uL of supernatant from lysed cultures. Samples were transferred to a thermal cycler and run using the ARB1 PCR program. At the end of the ARB1 program, master mix for ARB2 PCR was assembled on ice.

Table 3. Arbitrary PCR #2

Master mix (ARB2)	ARB2 PCR Program
Per reaction: 28.25 ddH_2O 1uL DMSO 5uL Thermopol buffer 5uL 10uM ARB2 primer (Rd2Pa) 5uL 10uM external specific primer (R2TnM) 0.25uL Taq DNA polymerase	1. 94°C – 3 minutes 2. 94°C – 30 seconds 3. 60°C – 30 seconds 4. 72°C – 2 minutes 5. Repeat steps 2-4 30 times 6. 72°C – 5 minutes 7. 12°C – Hold

For ARB2 PCR reactions, 43uL of master mix was aliquoted into PCR reaction tubes and 1.5uL of ARB1 PCR product added as template. The tubes were transferred to the thermal cycler and ARB2 PCR program run.

At the end of the ARB2 PCR program, products were analyzed using agarose gel electrophoresis to determine the band sizes of amplicons (appendix C). PCR products fromARB2 were cleaned up using the Monarch DNA Gel Extraction Kit (New England BioLabs®) according to manufacturer's instructions and sent to the ETSU Molecular Biology Core Facility for sequencing.

Growth Curves

To rule out the effect of a growth defect contributing to differences in *algZ* gene expression, growth curve experiments were performed. Mutants and wild-type PAO1 *P. aeruginosa* cells were inoculated into tubes containing 5mL LB broth. Tubes were incubated overnight at 37^{O}C with shaking. Cells were sub-cultured by taking 1% volume of parent culture and diluting it in a flask containing 250mL LB broth and incubated at 37^{O}C with shaking. At 4, 6, 8, 10, 12, and 24 hours, 1mL aliquots of cultures were removed and used for OD_{600} measurements.

Density at 600nm were measured and recorded at 6 time points. Results were graphed and analyzed analyzed to determine growth defects between mutants and wild-type *Pseudomonas aeruginosa*.

CHAPTER 3: RESULTS

Beta-Galactosidase Assay

PilY1 Represses *algZ* Expression in *Pseudomonas aeruginosa*

Aside from playing an important role in mechanosensing, minor pilins and PilY1 protein are involve in the regulation of AlgZ (Marko et al., 2018). For this reason, a *pilY1* mutant was constructed and used as a reference strain in a beta-galactosidase screening. Expression of *algZ* was measured in the reference strain and transposon mutant strains using the *algZ-lacZ* transcriptional fusion in a beta-galactosidase assay.

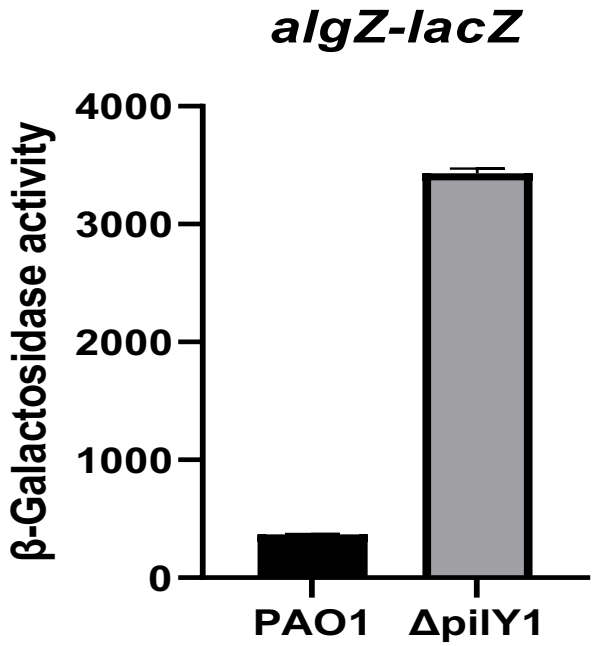


Figure 5. *algZ* expression increases in *ΔpilY1* relative to PAO1. β-Galactosidase activity in these strains were measured in triplicates after growth of strains in LB broth for 8 hours. Statistical analysis (ttest) of expression in PAO1 and *ΔpilY1* has a $p<0.001$

In the *pilY1* mutant background, a significant increase in *algZ* expression was observed, indicating that PilY1 represses *algZ* expression. Previous studies have shown that other minor

pilins such as *pilW*, *pilV* represses *algZ* expression (Marko et al., 2018). Effect of *pilY1* on *algR* expression was not assessed in this experiment.

Transposon Mutants Screen

To determine genes responsible for the increase in *algZ* expression in the *pilY1* mutant strain, a transposon mutagenesis screening was performed. *E. coli* strain *sm10 λpir* containing pBT20 plasmid (Kulasekara et al., 2005) was conjugated with the *Pseudomonas aeruginosa ΔpilY1algZTF-lacZ* strain. During the conjugation, the mariner transposon contained in the pBT20 plasmid (Appendix C) inserts randomly in the chromosome of the *P. aeruginosa*, inactivating genes resulting in changes in *algZ* expression in the transcriptional fusion. *Pseudomonas* cells not transformed during conjugation die from the bactericidal effect of gentamicin in the growth plate.

Approximately 5000 transposon mutants were screened during this experiment, out of this number, 5 mutants were found to have significant decrease in *algZ* expression relative to the *ΔpilY1algZTF-lacZ* strain.

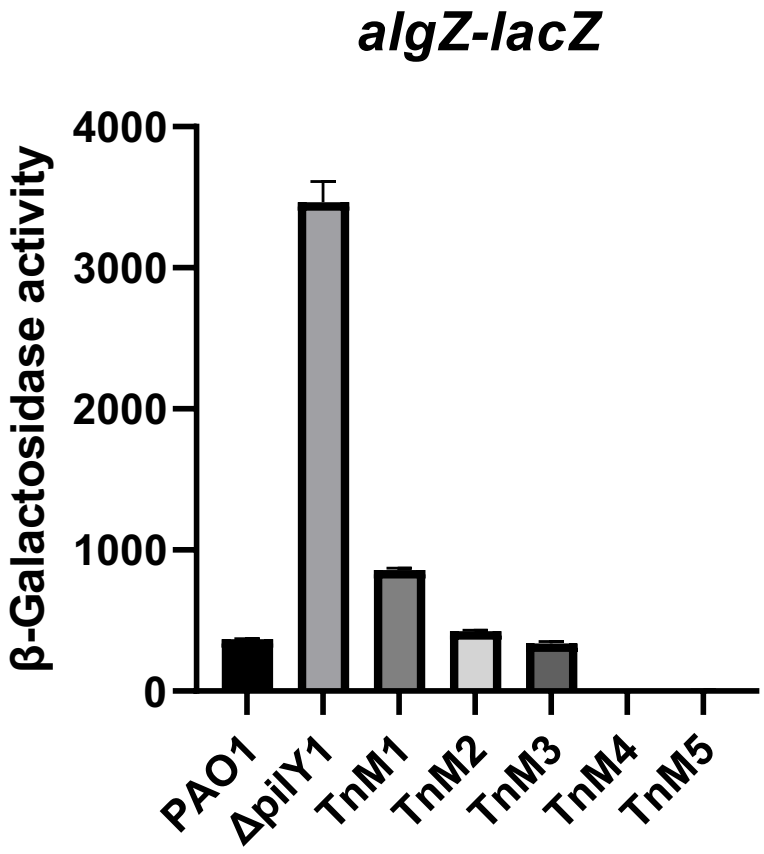


Figure 6. *algZ* expression in transposon mutants. Mutants generated had decreased *algZ* expression relative to *pilY1* mutant strain. TnM4 and TnM4 are hits corresponding to the *lacZ* gene. TnM2 correspond to *algR* and TnM1 corresponds to PA3327 in *P. aeruginosa* genome.

Comparing the β-Galactosidase activity of TnM1 and TnM2 (*PA3327* & *algR*) to the wild type PAO1 strain and the *ΔpilY1* strain, TnM1 and TnM2 have significant reduced *algZ* expression relative to the *ΔpilY1* and similar *algZ* expression relative to the wild type PAO1 strain. Under the *pilY1* mutant background, *PA3327* acts as a potential positive regulator of *algZ* expression.

The *lacZ* gene encoding the beta-galactosidase enzyme was interrupted twice, these mutants (TnM4 & TnM5) had no *algZ* expression because the insertion of the mariner transposon into the *lacZ* gene inhibited the expression of the enzyme resulting in no measurement in the beta-gal assay.

Gentamicin Resistance Screen

To select for transformed *P. aeruginosa* strains after conjugation with pBT20, the cells were cultured on PIA gent plates (Appendix A). In some instances, *Pseudomonas* strains were able to grow on PIA gent plate due to random insertions of gentamicin gene into their genome. For this reason, colony PCR was used to screen for the presence of gentamicin gene in the *Pseudomonas* mutants capable of growing on the PIA gent plates. The primers used for the colony PCR were gentF and gentR (Appendix C). The *E. coli sm10λ*-pBT20 was used as a positive control for the colony PCR. Mutants with PCR products equal to the positive control were used for downstream applications. All transposon mutants were screened for the insertion of the mariner transposon in their chromosome prior to β-Galactosidase assay.

Arbitrary PCR

Genes interrupted by the transposon were identified using arbitrary PCR. For round one arbitrary PCR in this experiment, RdPa1, RdPa2, and R1TnM (Appendix C) were used. Products from the round one reaction were used for the round two reaction. In the round two reaction, the primers Rd2Pa and the transposon specific primer R2TnM (Appendix C) were used. PCR products were cleaned up using the Monarch DNA Gel Extraction Kit (New England BioLabs®) as per instructions provided with the kit. Concentration and purity of round two PCR products were determined using nanodrop.

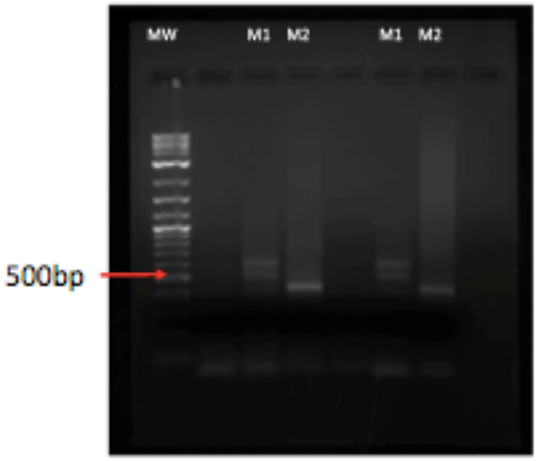


Figure 8. Gel electrophoresis of successful arbitrary PCR products. Round two PCR results on an agarose gel. TnM1 shows two bands with sizes of approximately 500bp and 700bp. TnM2 shows a single band with a size of approximately 500bp. Samples were screened and sequenced to identify the genes involved.

Growth Curves

Mutants were subjected to growth curve assay to determine if the genes interrupted encoded proteins necessary for growth or biochemical processes involved in growth and development.

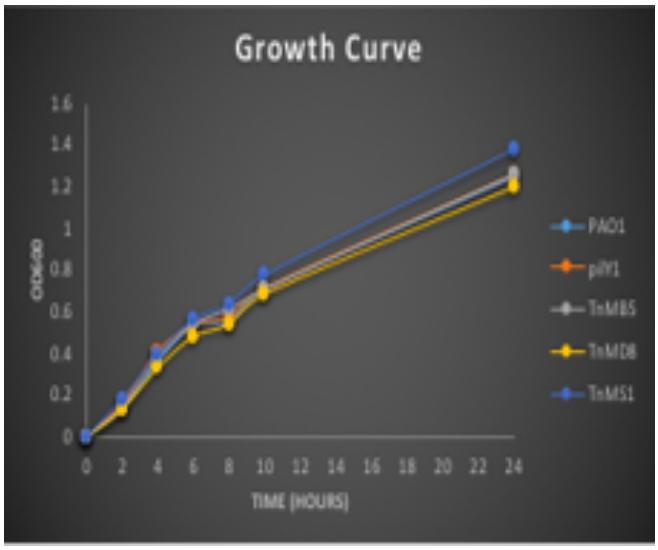


Figure 11. Growth curve assay. Transposon mutant had a similar growth rate compared to the wild type PAO1 and *ΔpilY1* strains after 24 hours of growth at 37°C.

The mutants with *lacZ* interrupted had no *algZ* expression when they were subjected to beta-Galactosidase assay. All other mutants had relative reduced *algZ* expression compared with the screening reference strain. A mucD mutant was identified in the screens. This mutant was mucoid and protease deficient but had a relatively increased algZ expression compared with the reference so was not added to our significant mutants.

CHAPTER 4: DISCUSSION

The opportunist pathogen *Pseudomonas aeruginosa* is highly adaptable to different environmental conditions due to the presence of a plethora of virulence factors at its disposal. These virulence factors make *Pseudomonas aeruginosa* resistant to immune clearance and antibiotic treatment, particularly in immunocompromised patients such as those undergoing cancer treatment or those with cystic fibrosis or severe burn wounds. These virulence factors are subject to complex regulation through two-component regulatory systems allowing sensing and responding to environmental cues. Therefore understanding regulation of virulence factors in *Pseudomonas aeruginosa* has become imperative for determining potential targets and development of new anti-virulence therapies. *Pseudomonas aeruginosa* uses the AlgZ/R TCS to regulate virulence gene expression in response to external stimuli. AlgR, the response regulator encoded by the *algR* gene has been studied extensively with known regulators such as the Vfr, Rpos, or AlgU but the signals sensed by AlgZ, the sensor kinase, remain unknown and regulation of *algZ* expression has not been extensively studied though Vfr is known to regulate *algZ* expression (Pritchett et al., 2015). The aim of this study was to identify novel regulators of *algZ* expression and how they contribute to virulence. The potential for elucidating the regulation of *algZ* expression includes attenuation of virulence through inhibition of *algZ* expression and also contribute to the understanding of AlgZ signaling.

Regulators of *algZ* Identified via Transposon Mutagenesis

The use of transposon mutagenesis screens remain a powerful tool for the identification of novel genes involved in controlling essential biological processes in bacteria. In *Pseudomonas aeruginosa*, transposon mutagenesis has been used in the identification of virulence genes encoding proteins necessary for disease causation and survival. The significance of transposon

mutagenesis technique was shown in our screen for regulators of *algZ* expression (Kulasekara et al., 2005). In the work, approximately 5000 mutants were screened and ten genes were identified to as potential regulators of *algZ* expression. The genes identified include *fimS*, *algR*, *lacZ* and *PA3327*. Of all the genes identified through transposon mutagenesis, the most interesting ones were *algR* and *PA3327*.

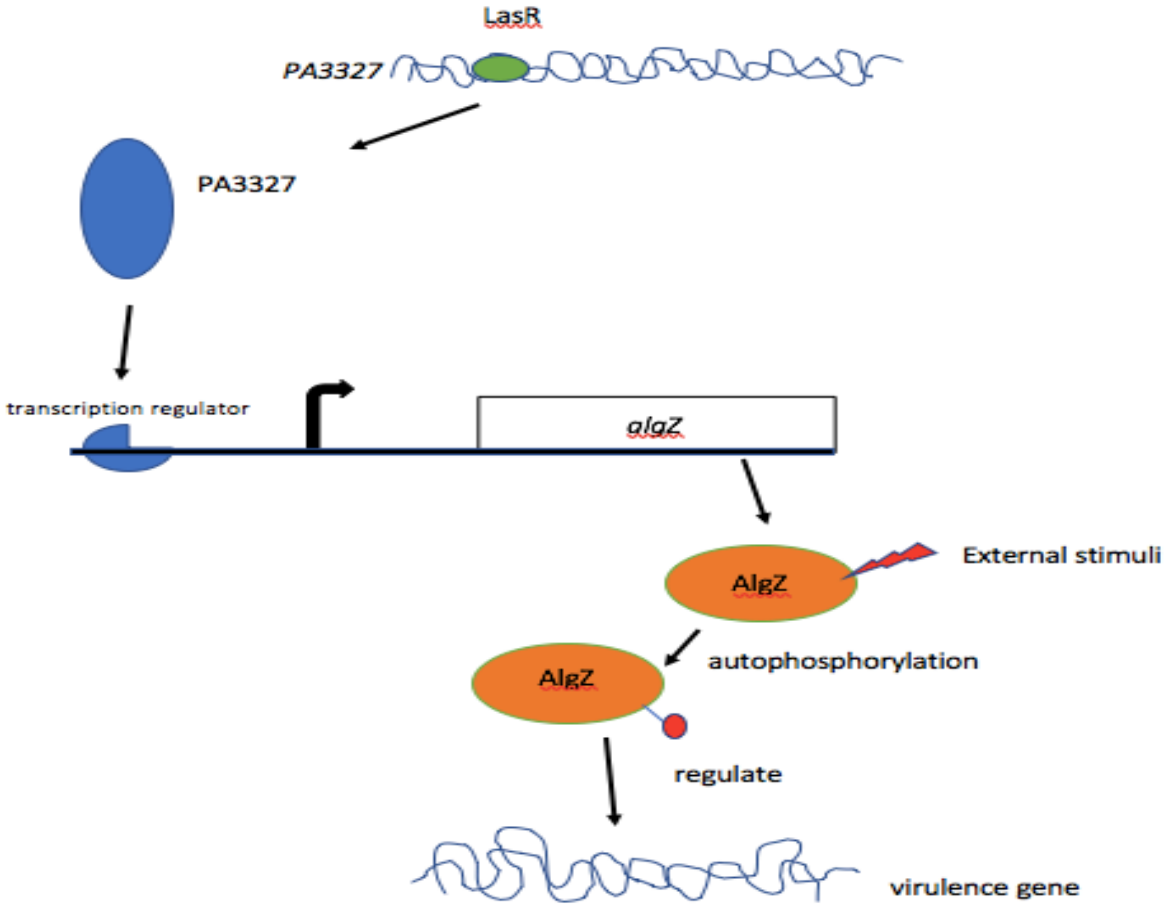
Non-Ribosomal Peptide Synthetase (PA3327)

Pseudomonas aeruginosa genome encodes four uncharacterized Non-ribosomal Peptide Synthetases (NRPSs) which play significant roles including the biosynthesis of secondary metabolites (non-ribosomal peptides) such as siderophores, antibiotics, pigments and harmful toxins (Martínez-Núñez & López, 2016). These non-ribosomal peptides play important roles in cellular signaling, intra- and interspecies communication, and virulence. In the transposon mutagenesis screening, *PA3327* was identified and encodes a putative non-ribosomal peptide synthetase and is part of an eight member gene cluster that includes a gene encoding an FAD-dependent monooxygenase. In a *pilY1* mutant background and using beta-galactosidase assay, inactivation of the NRPS encoded by *PA3327* repressed the expression of *algZ*. To rule out polar effects of *PA3327* inactivation of downstream genes in the cluster, deletion mutants of all the genes in the cluster should be made and their corresponding effects of *algZ* expression determined using β-galactosidase activity. Although the *PA3327* mutant did not exhibit any phenotypic characteristic, it is expected to regulate the expression of some virulence factors due to its regulatory effects on *algZ* expression leading to AlgR phosphorylation and subsequent expression and repression of known virulence factors in *P. aeruginosa*.

Previous studies have shown that *algZ* expression is activated by the virulence factor regulator (Vfr) which binds to sequences upstream of the transcriptional start site (Kanack et al.,

2006). In another study it was shown that Vfr activates *lasR* (transcription regulator) expression and a blast analysis revealed a LasR binding site upstream *PA3327* transcriptional start site. LasR acts as a transcriptional regulator of *PA3327*, indicating an indirect regulatory mechanism between Vfr and *PA3327* expression via LasR. The non-ribosomal peptide synthesized by the NRPS PA3327 can be considered as a transcription enhancer, upregulating the expression of *algZ* gene. To elucidate the signaling pathway and understand the role of *PA3327* in *algZ* expression and virulence regulation, *PA3327* deletion construct expressing *algZ_lacZ* or *algZ_lux* reporter in different mutant backgrounds of the genes known to regulate *PA3327* expression should be made and *algZ* expression measured using beta-galactosidase assay. Since AlgZR system regulates biofilm formation, twitching motility, cyanide production, and rhamnolipids production, a *PA3327* mutant would be tested to determine which of these phenotypes it regulates by repressing the expression of *algZ*.

Proposed Model of *algZ* Activation



Importance

In bacteria pathogenesis, virulence genes regulation is essential for disease causation and survival of pathogens against host immune response and antibiotic treatment. Considering *P. aeruginosa* pathogenesis, the AlgZ/R TCS regulate a number virulence factors and inactivation of this system leading to virulence attenuation could be explored as possible therapeutic targets to treat and prevent spread of *P. aeruginosa* infection. This opportunistic pathogen exhibits acute phase virulence factors such as protease and exotoxins production, and chronic phase virulence

factors such as biofilm formation and alginate production. Therefore exploring how *PA3327* regulates *algZ* expression and virulence could be a great step towards the development of anti-virulence drugs for treating *Pseudomonas aeruginosa* infections

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APPENDICES

Appendix A: Growth Media

Pseudomonas Isolation Agar (PIA) (1 Liter)

950mL ddH_2O
20mL glycerol
25mg Irgasan

Bring to a boil, then add

20g peptone
10g potassium sulfate
1.4g magnesium chloride
15g agar

QS to 1000mL with ddH_2O
Autoclave

Antibiotics:
150ug/uL gentamicin

Luria-Bertani (LB) (1 liter)

950mL ddH_2O
10g tryptone
5g yeast extract
10g sodium chloride
10g-15g agar (depending on desired percentage)

QS to 1000mL with ddH_2O
Autoclave
Antibiotics:
15ug/mL gentamicin
100ug/mL ampicillin

Appendix B: Buffers

10X TAE Buffer (1Liter)

800mL ddH_2O
48.5g Tris
11.4mL glacial acetic acid
20mL 0.5M EDTA (pH 8.0)
QS to 1000mL to 1Liter with ddH_2O

Dilute 10X TAE 10:1 to make a 1X working solution

Permeabilization Buffer

1% v/v Triton X-100
1.9mM DTT
8mM EDTA
8mM Na_2HPO_4
24mM Tris-HCL

Z Buffer

900mL ddH_2O
16.1g $Na_2HPO_4 \cdot 7H_2O$
5.5g $NaH_2PO4 \cdot H_2O$
0.75g KCl
0.246g $MgSO_4 \cdot 7H_2O$
pH to 7 and autoclave
Add 2.7uL/mL of β-mercaptoethanol (BME) when ready to use

Appendix C: Primers and Plasmids

Arbitrary PCR primers

RdPa1: GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG
RdPa2: GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
RdPa3: GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
R1TnM: TATAATGTGTGGAATTGTGAGCGG
Rd2Pa: GGCCACGCGTCGACTCTAGTAC
R2TnM: ACAGGAAACAGGACTCTAGAGG
TnMSq: CACCCAGCTTTCTTGTACAC

Gentamicin Screen primers

GentF: ATGTTACGCAGCAGCAACGATG
GentR: TTAGGTGGCGGTACTTGGGTC

PA3327 primers

PA3327EcoR1F: GCCGGAATTCGCCTTGTTTGACTCGCCCTGA
PA3327XbalR: GGCATCTAGACTGACCTTCCCAGGAGTCGA
PA3327 SOEF: GTTGAAGAGGACGCAGGGGTATGCTGGTCGCAGACATCCCCTGA
PA3327 SOER: TCAGGGGATGTCTGCGACCAGCATACCCCTGCGTCCTCTTCAAC
PA3327intF: CGGCATATCGAAACCTTCCT
PA3327intR: TGTTCTGGTGGAGTTTCACC

Appendix D: Supplementary Figures

Transposon mutants and corresponding genes

Table 4. Transposon mutants and corresponding genes

MUTANT	GENE IDENTIFIED
TnM1	*PA3327*
TnM2	*algR*
TnM3	*fimS*
TnM4	*lacZ*
TnM5	*lacZ*

Site of transposon insertion in *P. aeruginosa* genome

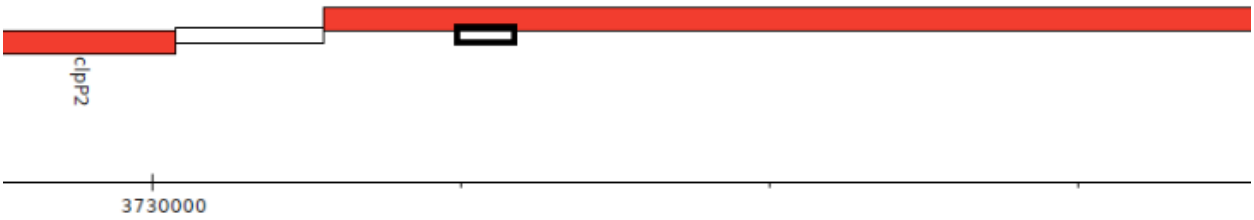

Figure 9. Genomic position of *PA3327* and the position of transposon insertion in *P. aeruginosa.* The transposon inserted close to the 5' end of the gene. Intergenic region between *clpP2* and *PA3327* indicate *PA3327* is the start of an eight member gene cluster that has the non-ribosomal peptide synthetase as a subunit.

Arbitrary PCR products were sent for sequencing at the ETSU Molecular Biology Core Facility.

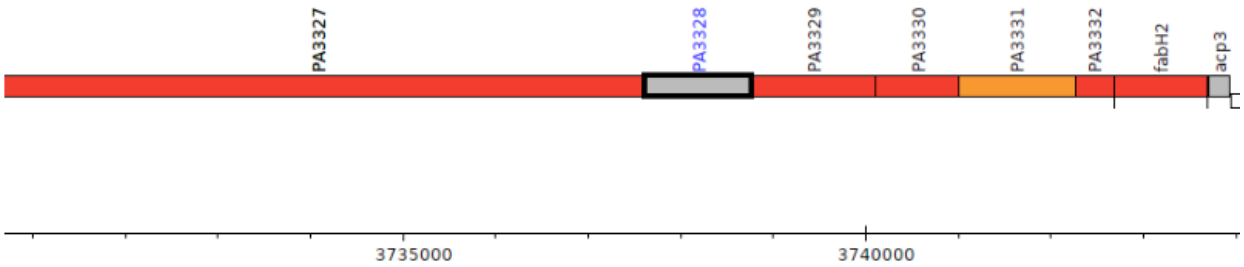

Figure 10. Eight member gene cluster containing the *PA337* encoding the putative non-ribosomal peptide synthetase, *fabH2* encoding an FAD-monooxygenase, and *acp3* encoding a protein that catalyze the orthophosphoric monoester to alcohol and orthophosphate.

Gel electrophoresis of Gent gene amplicon

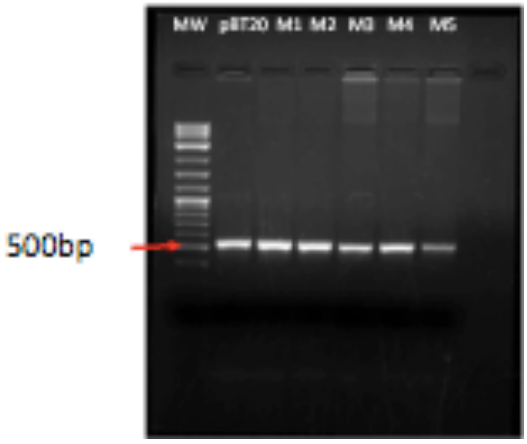

Figure 7. Gel electrophoresis of transposon mutants containing gentamicin gene. All mutants had the gent gene present in their genome. *E. coli* containing the pBT20 plasmid was used as a positive control for the gent gene. The size of the gent gene amplicon is around 500bp.

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