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The AlgZ/R Two-Component System Is Responsible for Attenuation of Virulence in Pseudomonas aeruginosa

Danielle A. Williams
East Tennessee State University

The AlgZ/R Two-Component System Is Responsible for Attenuation of Virulence in *Pseudomonas aeruginosa*

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Danielle Williams

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Dr. Christopher Pritchett, Chair

Dr. Bert Lampson

Dr. Laraine Powers

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ABSTRACT

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Pseudomonas aeruginosa is an important opportunistic pathogen. Many *P. aeruginosa* virulence factors are regulated by the AlgZ/R two component system. AlgZ is the sensor histidine kinase which phosphorylates AlgR, the response regulator. AlgR activates transcription of different gene targets based upon its phosphorylation state. The genes that encode AlgZ and AlgR are transcribed in an operon. While regulation of *algR* expression has been well studied, regulation of *algZ* expression has not. Using a *pilW* mutant in concert with *algZ*TF-*lacZ* transcriptional fusion, we conducted a transposon mutagenesis to identify *algZ* regulators. We identified an unknown autoregulatory loop. The type IV pilus minor pilins prevent the phosphorylation of AlgR by AlgZ . This inhibition of the AlgZ/R system subsequently down-regulates both the expression of the *fimU* operon and the *algZ/R* operon. Because AlgR regulates virulence, it is possible that virulence can also be reduced by targeting activation of the AlgZ/R system.

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CHAPTER 1

INTRODUCTION

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative bacillus. It is metabolically versatile and is ubiquitous in the environment, capable of surviving in inhospitable settings such as chlorinated water (1, 13). Because of this hardiness and versatility, *P. aeruginosa* can colonize any tissue in the human body, however, it most frequently affects individuals with compromised immune systems. This is of special concern for burn victims, those who have recently undergone surgery, and individuals with pre-existing conditions such as diabetes and cystic fibrosis. In the United States alone, there are over 51,000 health-care associated *P. aeruginosa* infections each year. Over 6,500 of these infections are multi-drug resistant, resulting in approximately 440 deaths annually (4). Moreover, such cases are likely under-reported, and these statistics only take into account nosocomial infections, not infections acquired outside of a health-care establishment. Because of the intrinsic and accruing antibiotic resistance of *P. aeruginosa*, investigating alternative therapies for treatment is of particular significance. One such alternative option is the use of anti-virulence drugs, which target certain virulence factors of the bacteria and prevent their expression or activity (29). The goal of such treatment is to allow for easier clearance of the infection and less damage to human tissues. Understanding virulence factor regulation is important to target regulators that impact several virulence factors at once.

Virulence Factors

Virulence factors are defined as compounds produced by bacterial pathogens that allow for improved colonization of host tissues, damage to host tissues, or avoidance of host immune responses, permitting the bacteria to more efficiently utilize host resources and cause disease (5).

In the case of *P. aeruginosa*, the bacteria have myriad virulence factors available for use in a variety of infection types from acute to chronic (9, 30). These factors include a single polar flagellum, type IV pili (TFP), rhamnolipid production, as well as production of multiple exopolysaccharides involved in biofilm synthesis, quorum sensing, and numerous toxins (1, 9, 39). Acute infecting strains are motile and express the Type III Secretion System (T3SS), while chronic infecting strains tend to be nonmotile, due in part to the accumulation of exopolysaccharide leading to biofilm formation (6). In chronic infecting strains, alginate (an exopolysaccharide important in the formation of biofilm) is upregulated (12). This is due to activity of AlgU, an alternative sigma factor that is normally sequestered but is freed when there is membrane stress or when there is a mutation in the *mucA* gene, which encodes the MucA protein that sequesters AlgU at the cell membrane (12, 28). AlgR, the response regulator for the AlgZ/R two-component system (TCS) is also required for the increase in alginate found in chronic infecting strains, as it works with AlgU to promote expression of *algD*, which results in increased alginate synthesis (8, 12, 25). The presence of alginate in chronic infecting strains makes them exceptionally difficult to clear and also conveys resistance to many antibiotics, leading to damaging inflammation of tissues despite rigorous treatment (12).

Initial colonization requires several virulence factors. The T3SS present in acute infecting strains allows injection of effector proteins directly into eukaryotic target cells, often resulting in the death of these cells. This aids in bacterial infiltration from superficial surfaces into deeper tissues, as is the case in acute pneumonia when the bacteria move from endotracheal tubes deeper into the lungs (14, 30). Motility, especially as supplied by the presence and activity of Type IV Pili (TFP), also improves the ability of the bacteria to infiltrate and disseminate during the course of acute infections (38). Type IV Pili are small, hair-like appendages that are present

on the poles of the bacteria (33). These polar appendages allow for a type of motility referred to as “twitching” and, as previously stated, are important in virulence in acute infections. Due to their sensory properties, TFP are also important in the coordination of other virulence genes to respond appropriately to the environment sensed (34).

Virulence factors must be carefully regulated to allow for persistence of the bacteria, whether in the environment or causing disease within a host. Based upon environmental cues, the bacteria control which genes are transcribed at higher levels and which are not (6). Quorum sensing, in which the bacteria communicate in a cell-to-cell fashion through the use of diffusible molecules, is also important in surface sensing (34). The bacteria produce these molecules, but unless a certain threshold amount of bacteria is reached (a quorum), the quantity will not be sufficient for detection and/or to produce a response. Once the density of these quorum sensing molecules reaches a high enough concentration, the bacteria are able to change their regulation to accommodate the fact that they are in a group as opposed to a single cell (15). In *P. aeruginosa*, quorum sensing is key in the formation of biofilm (7). Once they have reached a quorum, it is possible for the bacteria to establish an efficient biofilm, but not before. Hence, quorum sensing regulates genes necessary in the production of exopolysaccharides, as well as genes required for the transition from a planktonic to a sessile lifestyle (7). As stated above, biofilms are a virulence factor of vast importance in chronic infections. In summary, it is plain to see that virulence factors in *P. aeruginosa* are tightly regulated by a wide variety of signals stemming from the environment. Because of this, signal transduction is of extreme importance in *Pseudomonas aeruginosa*, as it is in all bacterial species.

Two-Component Systems

Whether a bacterium is existing on a solid surface or in a liquid, whether it is surrounded

by plentiful nutrients or in a nutrient-depleted area, even whether it is within a quorum of other bacteria or a single cell on its own, these are all conditions that require particular actions from the bacterial cell in order to optimize its chances of survival and persistence. Such actions are usually carried out via regulating expression of certain genes in order to promote a certain response. In order to regulate gene expression, bacteria must be able to integrate information they receive from their environment (6, 24). This integration is most commonly found in the form of bacterial two-component systems (24, 32). In a bacterial two-component system, there is generally a sensor and a response regulator. The sensor is frequently a histidine kinase located within the cell membrane that senses a signal from outside the cell and then relays this information into the cell via phosphorylation of the response regulator (11, 17, 24, 32). The response regulator then brings about the necessary response via regulation of transcription due to DNA binding [Figure 1] (11). To prevent continuous transcription of certain genes (or blocking thereof), response regulators have both an active and inactive form. This is based upon phosphorylation of the response regulator by the sensor histidine kinase. Typically, the response regulator is only in its active form when it is phosphorylated, and can be unphosphorylated to "turn off" its activity (11, 17). Moreover, phosphorylation of the response regulator can be influenced by other gene products besides the histidine kinase (24). Thus, based upon signals from outside of the cell, the cell can efficiently produce the required gene products to respond appropriately to such signals, and can also regulate the necessity for such gene products based upon signals from within the cell.

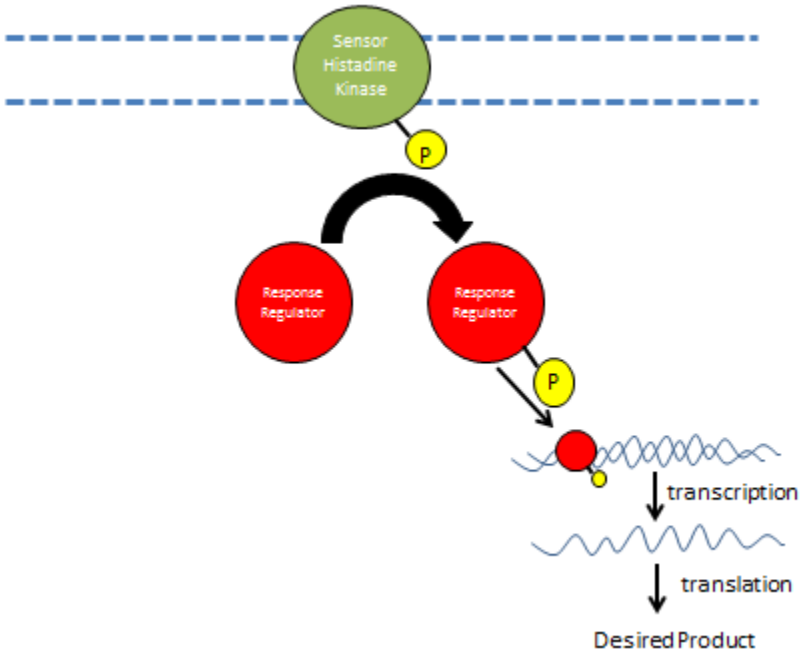


Figure 1. Diagram of a generic bacterial two-component system

AlgZ/R Two-Component System

In *Pseudomonas aeruginosa*, one two-component system of great importance is the AlgZ/R two-component system. In this case, AlgZ is the sensor histidine kinase, and AlgR is the response regulator. The signal that AlgZ senses is as yet unknown, though other studies from our lab do strongly indicate that it is unlikely that the signal is alginate or extracellular DNA (unpublished data). AlgR, the response regulator, behaves in a fashion slightly different from that of other response regulators. Most response regulators in bacteria are only active in their phosphorylated form (11, 17). However, in the case of AlgR, it is active in both its phosphorylated and unphosphorylated forms, but what it regulates varies depending upon its phosphorylation state (25). In its phosphorylated form, AlgR is capable of regulating twitching motility by inducing the expression of TFP (20). In its unphosphorylated form, AlgR works with the alternative sigma factor, AlgU, to regulate expression of *algD* and induce production of alginate (36). Unphosphorylated AlgR and AlgU are also able to regulate expression of *rsmA* in

mucoid backgrounds (31). RsmA, a post-transcriptional regulator, is itself a global regulator of *Pseudomonas aeruginosa* virulence, and influences the expression of the Type VI Secretion system, pyocyanin, hydrogen cyanide, and other virulence factors (26). It is because of this vast regulatory capability of AlgR that the AlgZ/R two-component system is so important in regards to *P. aeruginosa* virulence. Sensing the environment through AlgZ and relaying this information through the phosphorylation, or lack thereof, of AlgR allows the bacteria to modulate virulence appropriately to respond to their situation. This is of particular importance in an infection, allowing for avoidance of host immune responses and aiding in persistence of the bacteria. Previous studies have shown that in an *algR* mutant background, virulence of *P. aeruginosa* is decreased, verifying the importance of AlgR in regulation of virulence factors (19). Thus, understanding the regulation of the AlgZ/R two component system could provide potential avenues by which the system could be disrupted, leaving the bacteria essentially "blind" and thus easier to clear during an infection.

Regulation of AlgZ/R

The genes which encode AlgZ and AlgR are transcribed in an operon. Despite this fact, however, *algZ* and *algR* are not necessarily regulated identically. This is due to the presence of independent promoters for *algR* found within the *algZ* coding region [Figure 2].

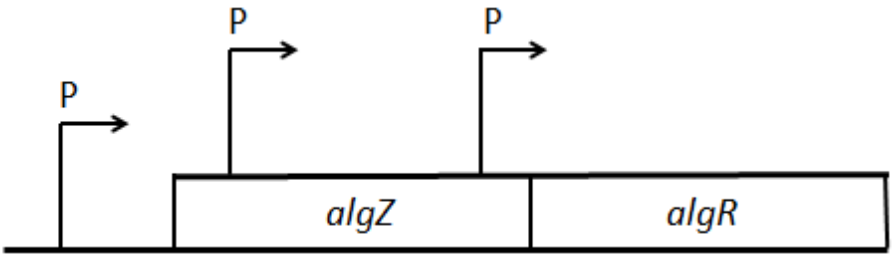


Figure 2. AlgZ/R operon. Promoters denoted with a "P" above a bent arrow.

While the *algZ* and *algR* are sometimes co-transcribed, the promoters within the *algZ* coding region allow for *algR* transcription independent of *algZ*. This means expression of *algZ* and *algR* can be regulated independently of one another. Regulation of *algR* expression has been well studied. Transcription of *algR* is known to be controlled by AlgU as well as RpoS (27). Regulators of *algZ* expression, however, have proven more elusive. One known regulator of *algZ* expression is Vfr, a homologue of *E. coli* Catabolite Activator Protein (CAP), which binds upstream of *algZ* and activates its transcription (18). Previous studies have shown that at least one of the minor pilin proteins, which are components of the *fimU* operon, have a negative regulatory effect on *algZ* expression (21). However, the exact mechanism of this regulation has yet to be elucidated. It was for this reason that we began to study *algZ* expression in other minor pilin mutants, as well as mutants for other components of the TFP and signal transduction proteins related in proximity to the TFP and the AlgZ/R TCS. Our investigation revealed that other proteins encoded in the *fimU* operon have the same negative regulatory effect on *algZ* as those previously studied, and that this regulation was not entirely due to activity through Vfr. Thus, this opened up the possibility for the discovery of as-yet unknown regulators of *algZ* expression. Moreover, because it is known that the minor pilin proteins are involved in signal transduction and mechanosensing, this connection expands upon the possibility of not only finding new regulators of *algZ* expression, but also identifying the role of AlgZ and AlgR in mechanosensing. Previous studies have shown that minor pilin protein mutants are also attenuated for virulence in a *Dictyostelium* model (34). This could also indicate a role for the AlgZ/R TCS in virulence regulation due to mechanosensory signals.

Identifying Potential *algZ* Regulators through Transposon Mutagenesis

To further investigate the connection between minor pilin proteins and regulation of *algZ* expression, the Δ*pilW* mutant strain in which there is an increase in *algZ* expression via an *algZ* transcriptional fusion was utilized in a transposon mutagenesis screen (21). In this screen, the Δ*pilW algZ*TF-*lacZ* strain was conjugated with an *E. coli* strain containing the pBT20 plasmid. The mariner transposon is engineered in this plasmid, such that when conjugated with a *Pseudomonas* strain, the mariner transposon will insert at random sites in the *Pseudomonas* chromosome and interrupt genes. For our purposes, when a transposon mutant was found with either an increase or decrease in reporter activity (the *algZ* transcriptional fusion) that was statistically significant, the gene interrupted was assumed to be a potential regulator of *algZ* expression. In the course of screening approximately 13,000 transposon mutants, 16 were found to have differences in *algZ* expression that could indicate genes for potential regulators being interrupted in these mutants.

Characterizing Potential Regulators of *algZ* Expression

To control for confounding effects that can result from the insertion of the transposon, isogenic mutants were made for several of the identified genes from the transposon mutagenesis screen. Among these were *pslA*, the product of which is involved in the production of the exopolysaccharide Psl (23). In this background, *algZ* expression was actually increased versus the Δ*pilW* background, but only when it was accompanied by the *pilW* mutation. This suggests that PilW and PslA (or the Psl exopolysaccharide itself) work in tandem to negatively regulate *algZ* expression. Another gene of interest identified was PA5567. The product of this gene is a conserved hypothetical protein that has yet to be fully characterized in *Pseudomonas aeruginosa*. However, its homologue, *trmE*, has been studied in *Pseudomonas fluourescens* and is shown to

be involved in the synthesis of a polyketide in this strain, though this polyketide is not synthesized in *P. aeruginosa* (37). In both the Δ*pilW* background, as well as a single PA5567 mutant background, *algZ* expression was reduced, indicating that PA5567 may have some positive regulatory effect on *algZ* expression. However, when a control fusion (*trc-lacZ*) was assayed in the PA5567 mutant, it showed decreased activity as compared to the wild-type, indicating that the reason for decreased *algZ* expression may be due to an overall transcriptional defect. The gene encoding for PilJ, a protein involved in chemotaxis that is located in close proximity to the TFP and shown to interact with AlgZ (21), was also identified in the transposon mutagenesis screen. Much like *pslA*, a single mutant of *pilJ* did not show any difference in *algZ* expression from wild-type, but in a Δ*pilW*Δ*pilJ* strain, *algZ* expression was increased. Again, this suggests a potential for these proteins to work in tandem in their negative regulation of *algZ* expression. However, by far the most interesting gene discovered in the transposon mutagenesis screen was *algR*. In a double mutant of *pilW* and *algR*, *algZ* expression is decreased substantially. The same can be said in a single mutant of *algR*. This mutant strongly suggests autoregulation of the *algZ/R* operon by AlgR.

Downstream Effects

As discussed previously, *algZ* expression is not the only thing that AlgR is capable of influencing. It is also capable of regulating expression of the minor pilin (*fimU*) operon by binding upstream and activating transcription, especially when AlgR is phosphorylated. After phosphorylation, many response regulators are more easily able to dimerize, the state in which they more readily activate transcription (32). This may also be the case for AlgR. Thus, expression of the *fimU* operon provides a good marker for the phosphorylation state of AlgR, and thus also for the activity of AlgZ. In all *algR* mutants, including double mutants of *pilW* and

algR, expression of the *fimU* operon was drastically decreased, verifying the influence of AlgR on *algZ* as well as on *fimU* operon expression. This indicates that the influence of the minor pilins may be transduced through AlgR. This strongly suggests that minor pilins signal through the AlgZ/R TCS. While components of the TFP have already been implicated in coordination of virulence gene expression, this alludes to a more significant role than previously indicated. Moreover, as this study and previous studies have shown, the components of the *fimU* operon, i.e. the minor pilins, also have a regulatory effect on *algZ* expression (21). This suggests a complex regulatory pathway for *algZ/R* expression that includes autoregulation as well as mechanosensing, and which has not yet been elucidated fully. To look at this regulation of expression, as well as regulation of phosphorylation, variants of *algR* were constructed to mimic the different phosphorylation states of AlgR. AlgR is phosphorylated at an aspartic acid residue in position 54 (35). In an *algR*D54A strain, the aspartic acid is mutagenized to an alanine. This mutant behaves as though AlgR is never phosphorylated. In an algRD54E strain, the aspartic acid is mutagenized to glutamic acid, and this strain behaves as though AlgR is always phosphorylated and acts as a phosphomimetic. Using these, it was determined in this study that the minor pilins influence the ability of AlgZ to phosphorylate AlgR, and this in turn regulates the expression of the minor pilins themselves, as well as the *algZ/R* operon. This information is a small but significant step toward fully elucidating the autoregulatory pathways of *algZ/R* expression and how this connects to mechanosensory input via the minor pilin proteins. In addition, this work suggests that the minor pilins must signal the AlgZ/R TCS in order to correctly coordinate virulence gene expression and allow expression of other virulence genes.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Pseudomonas aeruginosa strain PAO1 was used as the wild-type reference strain in all cases. All *Pseudomonas* strains (wild-type and mutant) were grown on *Pseudomonas* Isolation Agar (PIA) supplemented with antibiotics when appropriate. *E. coli* strain NEB5α (containing various plasmids) was conjugated with PAO1 to produce *Pseudomonas* mutants and *Pseudomonas* strains containing transcriptional fusions. *E. coli* strain pRK2013 was used as a helper strain in triparental mating in these cases (10). *E. coli* strains were grown on Luria Burtani (LB) agar supplemented with antibiotics when appropriate. In all cases, strains were grown at 37°C, excluding certain steps of the conjugation procedure, which will be discussed later. For certain assays, *Pseudomonas* strains were grown in LB broth at 37° with shaking, between eight and 16 hours. Glycerol freezer stocks were made of each strain by mixing 1mL of 30% glycerol with scrapings of the strains taken from solid media via a sterile applicator stick. Plasmids used in this study are listed in Appendix C. Growth media, with antibiotic concentrations, are listed in Appendix A.

Isolation of Genomic DNA

A single colony of the desired strain was inoculated into 5mL LB broth, supplemented with antibiotics as needed, and grown overnight at 37°C with shaking. The next day, 1mL of culture was spun down at 8000G for 5 minutes. The supernatant was decanted and the pellet resuspended in 400uL TNE. To this, 17uL of 30% sarkosyl and 2.5uL 20mg/mL proteinase K were added. The solution was then incubated at 37°C with occasional inversion for 30-60

minutes (or until solution cleared). After incubation, 400uL 4M NH_4OAc was added, and the solution mixed by inversion, vortexing is not recommended. Then, the solution was extracted twice with 600uL 25:24:1 CPI (chloroform: phenol: isoamyl alcohol). After vortexing, solution was centrifuged at 14000G for 10 minutes, and the supernatant transferred to a new tube. The solution was then extracted once with 24:1 CI (chloroform: isoamyl alcohol) to remove phenol contamination, and the supernatant transferred to a new tube. This was precipitated with 2.5 times volume cold 95% ethanol for 10 minutes at -20°C, then centrifuged at 10000G for 15 minutes to pellet the DNA. The pellet was then washed with 1mL 70% ethanol and centrifuged again. Ethanol was decanted, and pellet dried at room temperature for 10-20 minutes before being resuspended in 50uL of ddH_2O.

Triparental Mating

To construct non-polar mutants of *P. aeruginosa*, or to insert a transcription fusion into a *P. aeruginosa* strain, triparental mating was used. The desired *Pseudomonas* strain was grown on PIA at 42° overnight to prevent rejection of foreign DNA, *E. coli* NEB5α containing either a deletion construct or a fusion construct and an *E. coli* helper strain, pRK2013, were grown on LB with antibiotics at 37° overnight. All three strains were scraped from their plates using a sterile applicator stick and resuspended together in 500uL of sterile 0.85% saline. This solution was then plated in a spot on LB agar and incubated at 30°C overnight for optimal DNA transfer. After overnight incubation, the spot was then scraped up and again resuspended in 500uL 0.85% saline. The solution was then vortexed and 100uL spread plated on PIA supplemented with appropriate antibiotics. For insertion of fusion, the media was supplemented with tetracycline. For deletion constructs, the media was supplement with either tetracycline or gentamicin,

depending upon the suicide vector used for the deletion construct. The plate was incubated at 37°C overnight or until colonies appeared.

For Deletion Mutation

After the appearance of colonies, one colony was picked and recovered in 2mL LB with no antibiotics for 2-6 hours. During conjugation, homologous recombination occurred, leaving the bacteria with two alleles for the same gene: one wild-type and one mutant, a condition referred to as merodiploidy. The recovery allows the merodiploids to discard one of the alleles, either mutant or wild-type. This culture was then streaked for isolation on YT 10% sucrose media to kill off any remaining merodiploids, which retain the *sacB* gene from the suicide vector. If the *sacB* gene is retained, levansucrase is produced, which cleaves the sucrose present in the media into products which are lethal to the cell. These plates were then grown at 30°C overnight to prevent overgrowth of the remaining haploid bacteria. Resulting colonies were replica plated on PIA and PIA supplemented with appropriate antibiotics. Colonies that were sensitive to the antibiotic were then screened via PCR for the mutation.

For Insertion of Transcriptional Fusion

After appearance of colonies, one colony was picked and streaked on YT tetracycline^{50}irgasan^{25}. Irgasan is an antimicrobial to which *P. aeruginosa* has resistance. This was grown up at 42°C overnight. This plate was then scraped and conjugated with an *E. coli* strain containing the $PFLP_2$ to remove the excess miniCTX transcriptional fusion vector and leave only the required promoter region and *lacZ* gene inserted into a neutral site in the chromosome. This conjugation was done via biparental mating (similar to tri-parental mating, but limited to only two strains as opposed to three) at 30°C overnight. The spot was then scraped

and resuspended in 500uL 0.85% saline, and this was streaked out on VBMM carb^{300}, a minimal media supplemented with carbenicillin. This serves to kill remaining *E. coli*, while *P. aeruginosa* is able to grow. This plate was then grown overnight at 37°C. Resulting colonies were patched on PIA and YT tet^{50}irg^{25}. Colonies sensitive to the antibiotic were then screened via PCR for presence of the fusion.

Colony PCR

To screen for presence of fusions or mutations, colony PCR was performed. A single colony was transferred from a plate via a sterile applicator stick to 50uL of sterile water in a 1.5mL tube. This was then heated at 70°C for 10 minutes. During the heating process, the PCR master mix was assembled on ice.

Table 1. Colony PCR

Master Mix	PCR Program
Per sample: 18uL sterile ddH_20 2.5uL Thermopol Buffer 1uL DMSO 0.5uL 10mM dNTPs 0.5uL 10uM forward primer 0.5uL 10uM reverse primer 0.1uL Taq DNA polymerase	1. 95°C – 5 minutes 2. 95°C – 30 seconds 3. *53-68°C – 30 seconds 4. 72°C – 1 minute per kb to be amplified 5. Repeat steps 2-4 30 times 6. 72°C – 10 minutes *Temperature of step 3 was adjusted based upon calculated annealing temperatures of primers utilized

To a thin-walled PCR tube, 23uL of master mix was added along with 2uL of the heated sample. The mixture was topped with mineral oil to prevent boiling off of the reaction during thermal cycling. Tubes were placed in a thermal cycler and run with the appropriate setting.

β-Galactosidase Assay

A single colony of the desired *Pseudomonas* strain was inoculated into 3mL of LB broth and grown at 37°C with shaking for 12-16. These were then subcultured into 3mL fresh LB broth with 1% inoculation. The subcultures were grown for 8 hours at 37°C with shaking. For the assay, 500uL of culture were spun down at 8000G for 5 minutes. Supernatant was poured off and the pellet resuspended in 1mL Z-buffer plus 2.7% β-mercaptoethanol. Optical density of this solution was measured at 600nm and recorded. After, 100uL chloroform and 50uL 10% SDS were added to each tube and vortexed to permeabilize the cells. Tubes were allowed to settle for 10 minutes. Then, 50-500uL of the lysate was added to fresh Z-buffer plus BME to a volume of 1mL. To this, 200uL of 4mg/mL ONPG in Z-buffer (without BME) was added to each reaction tube. Reactions were stopped with 500uL of 1M Na_2HPO_4 when a yellow color developed. Time from addition of ONPG to cessation of reaction was noted for each tube. The optical density of each tube was then measured at 420nm and 550nm. Miller units were then calculated using the following formula:

$$((OD420 – (OD550*1.75))*1000) / (time * volume\ lysate * OD600)$$

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

Transposon Mutagenesis

The Δ*pilW Pseudomonas* mutant containing the *algZ* transcriptional fusion (denoted Δ*pilW algZ*TF-*lacZ*) was grown at 42°C overnight on PIA. *E. coli* strain SM10 λpir containing the transposon on plasmid pBT20 was grown on LB agar supplemented with 15ug/uL gentamicin. Both strains were scraped from their plates and resuspended together in 500uL

sterile 0.85% saline in a 1.5mL tube. The resuspended solution was plated in a patch on LB agar for 1 hour at 30°C. The LB plate was then rinsed with another 500uL sterile 0.85% saline, which was transferred to another 1.5mL tube. This tube was vortexed, and 100uL of the solution was spread plated on PIA supplemented with 150ug/uL gentamicin. Gentamicin present in the media killed any *P. aeruginosa* which had not taken up the transposon, while irgasan present in the media kill any remaining *E. coli*. These plates were allowed to grow up overnight at 37°C until isolated colonies appeared. These isolated colonies were then used for the high throughput β-Galactosidase assay

High Throughput β-Galactosidase Assay

Each gentamicin resistant *Pseudomonas* colony was inoculated into 120uL LB broth in a 96-well plate, with the reference strain Δ*pilW algZ*TF-*lacZ* inoculated in well A1. These were grown up overnight at 37°C with shaking. The next day, optical density of each well was measured at 600nm using a microtiter plate reader. Those cells with an OD600 significantly reduced from surrounding cells were considered growth defect mutants and were not used in the following screen. In a fresh 96-well plate, 100uL of Permeabilization Buffer (Appendix B) was added to each well. From the initial 96-well plate, 30uL of culture was transferred into the 100uL of Permeabilization Buffer and pipetted up and down several times. To each well, 30uL of 4mg/mL of ONPG in Z-buffer was added. The reaction was stopped with 70uL of 1M Na_2HPO_4 when a yellow color developed. Time from addition of ONPG to cessation of reaction was noted. Wells that appeared significantly more or less yellow were noted, and each mutant of interest was pulled from the initial 96-well plate and plated on PIA supplemented with 150ug/uL gentamicin. These were allowed to grow overnight at 37°C, then confirmed as transposon

mutants by screening via colony PCR for the gentamicin cassette present in the transposon. After this, differences in *algZ* fusion expression were confirmed via the standard β-Galactosidase Assay.

Arbitrary PCR

To amplify genes interrupted by transposon insertion, arbitrary PCR was performed. This is a two step PCR process, which requires two different master mixes and two different PCR programs.

A single colony from a plate of a mutant of interest was inoculated into 5mL LB broth supplemented with gentamicin and grown 16-24 hours at 37°C with shaking. For DNA template, 1mL of the cultures was spun down at 8000G for 5 minutes. The supernatant was then decanted, and the cell pellet resuspended in 1mL of Arbitrary PCR Lysis Buffer (Appendix B), the solution was then heated at 95°C for 10 minutes, and again spun at 8000G for 10 minutes. The supernatant was then used for template DNA in the arbitrary PCR reaction. During heating of the template samples, arbitrary PCR master mix 1 was assembled on ice.

Table 2. Arbitrary PCR #1

Master Mix (Arb1)	ARB1 PCR Program
Per reaction: 36.25uL ddH_2O 1uL DMSO 1uL 10mM dNTPs 5uL Thermopol buffer 2uL 10uM ARB1 primer (RdPa1, RdPa2, RdPa3, or combination of the three) 2uL 10uM internal specific primer (R1TnM) 0.25uL Taq DNA polymerase	1. 94^oC – 3 minutes 2. 94^oC – 30 seconds 3. 49 ^oC – 30 seconds (reduce temp by 1 ^oC for each subsequent round) 4. 72 ^oC – 3 minutes 5. Repeat steps 2-4 15 times 6. 94 ^oC – 3 minutes 7. 60 ^oC – 30 seconds 8. 72 ^oC – 3 minutes 9. Repeat steps 6-8 20 times 10. 12 ^oC Hold

For each reaction, 46uL of the master mix was aliquoted into thin-walled PCR tubes, along with 1.5uL of the supernatant of the lysed cultures. Samples were transferred to a thermal cycler and run using the above program, designated ARB1.

Immediately following the end of the ARB1 program, the master mix for arbitrary PCR 2 was assembled on ice.

Table 3. Arbitrary PCR #2

Master Mix (Arb2)	ARB2 PCR Program
Per reaction: 28.25uL ddH_2O 1uL DMSO 1uL 10mM dNTPs 5uL Thermopol buffer 5uL 10uM ARB2 primer (Rd2Pa) 5uL 10uM external specific primer (R2TnM) 0.25uL Taq DNA polymerase	1. 94^{o}C – 3 minutes 2. 94^{o}C – 30 seconds 3. 60 ^{o}C – 30 seconds 4. 72 ^{o}C – 2 minutes 5. Repeat steps 2-4 30 times 6. 72^{o}C – 5 minutes 7. 12 ^{o}C Hold

For each reaction, 43uL of the master mix was aliquoted into a thin-walled PCR reaction tube and 1.5uL of the ARB1 PCR product were used as template. The samples were then run in a thermal cycler under the program ARB2.

At conclusion of ARB2 program, samples were analyzed via agarose gel electrophoresis. If bands were present, the PCR product was cleaned up using the Monarch DNA Gel Extraction Kit (New England BioLabs®) as per the kit's instructions, and sent to the ETSU Molecular Biology Core for sequencing.

Rescue Cloning

In instances where arbitrary PCR could not yield viable bands, or to independently confirm identification of the gene interrupted by the transposon, rescue cloning was used. Isolated genomic DNA was cut using one restriction enzyme known to cut within the mariner transposon and one restriction enzyme known not to cut within the mariner transposon. The digestion mixture was assembled as follows:

Table 4. Restriction Enzyme Double Digest

Double Digest Mix
0.5ug DNA 2uL CutSmart Buffer (NEB) 1uL of Enzyme 1 1uL of Enzyme 2 QS to 20uL with ddH_2O

Samples were incubated at 37°C overnight. If applicable, restriction enzymes were then heat inactivated. Ligation was set up in a new tube with vector pUC19 cut with the same restriction enzymes as had been used on the genomic DNA.

Table 5. Ligation Reaction

Ligation Master Mix
100ng pUC19 100ng digested genomic DNA 2uL T4 DNA Ligase Buffer 1uL T4 DNA Ligase QS to 20uL with ddH_2O

Ligation was allowed to occur at 25°C for ~2 hours and was then transformed into chemically competent NEB5α *E. coli* cells

Transformation

Competent cells (100uL) were placed on ice in a 1.5mL tube. Approximately half (5uL) of the ligation mixture was added to the cells, and these were incubated on ice for 25 minutes. Flicking, vortexing, or pipetting up and down are not recommended as the competent cells are fragile. Cells were then heat shocked at 42°C for 45 seconds. For recovery, 950uL LB broth was added to each transformation and they were then incubated at 37°C with shaking for 45 minutes to an hour. Cells were then pelleted at 8000G for 5 minutes, and the pellet resuspended in 100uL LB broth. This was spread plated on LB agar supplemented with 15ug/uL gentamicin to select for only those clones which had ligated in a portion of the gentamicin cassette from the transposon. Resulting colonies were then patched onto a second LB gentamicin plate, as well as being inoculated into LB broth. Plasmid preps were performed using the Monarch Plasmid MiniPrep Kit (New England Biolabs®), and then a portion was digested using the same enzymes used for the cloning process. This digest was then visualized via gel electrophoresis. If an insert was dropped out, the remainder of the plasmid was sent for sequencing to the ETSU Molecular Biology Core Facility.

Mutant Construction

In order to control for confounding factors that can be caused due to transposon insertion, clean mutants were made of several genes revealed in the transposon mutant screen to confirm the data produced.

Splicing by Overlap Extension

Two sets of primers, F1 & SOER and SOEF & R1, are designed around the gene of interest (16). Primer F1 was designed approximately 1000 base pairs upstream of the gene of

interest, and included an appropriate restriction enzyme site that was not complementary to the DNA sequence of the region upstream from the gene. As well as this, a restriction enzyme "seat" consisting of four random nucleotides that were also not complementary to the DNA sequence, was placed in front of the restriction enzyme site. This same technique was used to design primer R1, with this primer being placed approximately 1000 base pairs downstream of the gene of interest. For primer SOER, design included a region just outside the transcriptional start site of the gene of interest, located on the 3' end of the primer. The 5' end of the primer consisted of a segment not complementary to the sequence just after the transcriptional start site of the primer, but rather a segment complementary to the sequence just downstream of the gene's terminus. The SOEF primer was designed complementary to the sequence just downstream of the gene's terminus on its 3' end, with its 5' end complementary to the sequence upstream of the transcriptional start site. The SOEF and SOER primers thus were reverse complements of each other, allowing for the overlap extension mentioned in the method's name. For individual PCRs, protocol was as is described above, with F1 and SOER used in one reaction and R1 and SOEF used in the other. A second PCR was performed using each PCR product from those first reactions as template, utilizing F1 and R2 as the primers. Once this "crossover" PCR product was obtained, it was digested along with the suicide vector (pEX18), and then cloned into the suicide vector via ligation [Figure 9]. This complete mutant construct plasmid was then transformed into NEB5a cells, which were plated on LB supplemented with appropriate antibiotics, and subsequently screened via PCR and sequencing to ensure the presence of the mutation. The mutant construct strain was then preserved via freezer stock for later use. To insert this mutation into the desired *Pseudomonas* strain, triparental mating was performed as described above, using PAO1, *E. coli* pRK2013, and the mutant construct strain.

Western Blot Analysis

AlgRHA

A strain containing the suicide vector pEx18gm with the *algR* gene tagged with hemagglutinin (HA) at the 3' end was conjugated via triparental mating with the desired *Pseudomonas* strains as described previously. PAO1 containing the AlgR epitope tagged with the HA allele was tested for twitching to confirm the activity of the mutant allele. Strains were screened for replacement of the wild-type *algR* with the HA-tagged *algR* using the primers algRintF and HAR in colony PCR as described previously. Those found to contain the appropriate *algR* allele were used for western blot analysis.

Strains were grown up in 5mL LB broth for 16 hours at 37°C with shaking. These were then sub-cultured into 25mL fresh LB with 1% inoculation. Sub-cultures were grown for 8 hours at 37°C with shaking. Total volume was centrifuged at 8000G for 10 minutes. Supernatant was decanted, and pellets frozen at -20°C for at least 4 hours. After freezing, pellets were allowed to thaw and then lysed using a modified B-PER protocol.

Table 6. Modified B-PER Protocol

B-PER Protocol
For each lysis: 2mL B-PER 10uL 10mg/mL lysozyme in TE 1uL DNase Resuspend the pellet in the B-PER solution and vortex for ~20 seconds. Let stand for 15 minutes at 25°C. Transfer to 2mL microfuge tube. Centrifuge at 15000G for 10 minutes. Supernatant is utilized for protein requirements, pellet is discarded.

To determine concentration of protein in each sample, BioRad® Bradford Protein Assay was used. In short, BioRad® Bradford Protein Assay reagent was diluted to 1X, and 3.5mL of reagent was used for each reaction. To this, 10uL of sample was added and allowed to sit at 25°C for 10 minutes. Absorbance of each sample was then measured at 595nm. A standard curve with an R^2 value = 0.98 had previously been generated using the BioRad® Bradford Protein Assay protocol and BSA standards with concentrations of 0.0625mg/ml, 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 1.0mg/ml, and 2.0mg/ml. Protein concentrations in the samples were determined using the trend line equation generated from the standard curve.

A total of 10ug protein for each sample was mixed with 4X SDS PAGE loading dye (Appendix B) to a total volume of 10uL. Samples with loading dye were heated at 95°C for 10 minutes, then flash centrifuged and placed on ice for 2 minutes. Samples were then run on a discontinuous (4% stacking, 15% resolving) SDS-PAGE gel at 170V for 45 minutes, or until dye front reached the bottom of the gel.

Table 7. Discontinuous Gel for Western Blot

15% Resolving	4% Stacking
3mL 40% Acrylamide/Bisacrylamide 2mL 1.5M Tris pH 8.8 2.88mL ddH_2O 80uL 10% SDS 8uL TEMED 80uL 10% Ammonium persulfate Thoroughly mix first four components. Add 80uL 10% APS and mix well. Using a pipette, swiftly pour gel into gel cassette until the cassette is ¾ full. Fill the gel cassette with 0.1% to the lip of the cassette to ensure even solidification at the top of the gel. Allow to solidify at room temperature for at least 1 hour.	500uL 40% Acrylamide/Bisacrylamide 1.25mL 0.5M Tris pH 6.8 3.1mL ddH_2O 50uL 10% SDS 5uL TEMED 25uL 10% Ammonium persulfate Thoroughly mix first four components. After 15% resolving gel has solidified in the gel cassette for at least 1 hour, pour 0.1% SDS off of the 15% gel. Add 25uL 10% APS to the 4% stacking gel. Mix thoroughly. Using a pipette, layer 4% stacking gel atop the 15% resolving gel within the gel cassette. Fill to the lip of the cassette. Before the 4% gel solidifies, insert comb with desired number of wells. Allow to solidify at room temperature for at least 1 hour.

The gel was run in 500mL 1X glycine running buffer, diluted from 10X glycine running buffer (Appendix B). While the gel was running, a section of polyvinyledine fluoride (PVDF) membrane, cut to a size slightly larger than that of the gel, was activated by soaking in methanol for at least 10 minutes. Transfer buffer was prepared, consisting of 1X Towbin buffer (Appendix B) and 10% methanol. Four sheets of blotting paper were cut to a size just larger than that of the PVDF membrane and placed in the Transfer buffer to soak. When the gel was finished running, the cassette was cracked and the gel placed into the semi-dry electroblotting apparatus in a "transfer sandwich." The transfer sandwich consisted of two sheets of blotting paper soaked in transfer buffer, the activated PVDF membrane, and the gel, and the sandwich was topped with the last two pieces of blotting paper soaked in transfer buffer. The transfer was run at 0.15 amps for 80 minutes.

After transfer, the membrane was removed from the transfer sandwich and placed in Blocking Buffer (Appendix B) for 1 hour at room temperature with agitation. Blocking buffer was then decanted off, and the Primary (1°) Antibody solution (Appendix B) poured on. The Primary Antibody solution is a 1:20000 dilution of mouse anti-HA antibody. The membrane was agitated in the Primary Antibody solution overnight at 4°C with agitation. After this, the membrane was washed with Wash Buffer (Appendix B) four times for 15 minutes each. Secondary (2°) Antibody solution (Appendix B) was then poured on and agitated for 2 hours at room temperature. The Secondary Antibody solution is a 1:20000 dilution of goat anti-mouse antibody conjugated with horseradish peroxidase. After incubation with the Secondary Antibody solution, the membrane was washed again with Wash Buffer four times for 15 minutes each. Finally, the Pierce™ ECL Western Blotting Substrate Kit was used to provide substrate for cleavage by the horseradish peroxidase conjugated with the 2° antibody, allowing for visualization of the protein bands. The Peroxide Solution (Detection Reagent 1) and Luminol Enhancer (Detection Reagent 2) were mixed in a 1:1 solution and decanted onto the membrane, which was allowed to sit for 5 minutes at room temperature before visualization in the ProteinSimple FluorChem M visualizer.

Growth Curves

To determine if a mutant had a growth defect that would thus result in its difference in expression of *algZ*, growth curves were performed. Both the mutant in question and wild-type PAO1 were inoculated into separate tubes of 5mL of LB broth. These were grown overnight at 37°C with shaking. The next day, these were subcultured into flasks of 250mL LB broth, using a 1% inoculation. These were also grown at 37°C with shaking. At 4, 6, 8, 10, 12, and 24 hours,

2mL of culture were removed from the flasks. One of these 1mL samples was measured for optical density at 600nm, the other was used to determine colony forming units per milliliter (CFU/mL). In brief, this milliliter was diluted 1:10 in sterile phosphate buffered saline (PBS) by adding 100uL of the undiluted culture to 900uL of PBS. This sample was then diluted again 1:10 in PBS. This was repeated seven times, resulting in seven dilutions along with the original undiluted culture. These dilutions were then spotted in 10uL volumes on LB agar plates, and plates were grown at 37°C until colonies formed. Upon growth, the colonies were counted. The number of colonies was multiplied by 10 raised to the initial dilution factor (0-7) and then multiplied again by 10^2 to account for the dilution of plating 10uL of 1mL of sample. For example, if the undiluted culture yielded 32 colonies in its 10uL patch on LB agar, the calculation would appear as follows:

$$32 \text{ colonies} \times 10^0 \times 10^2 = 32 \times 10^2 = 3200 \text{ CFU/mL}$$

Again, both CFU/mL and optical density at 600nm were determined at 6 different time points. These data were then charted and analyzed to determine any significant differences in growth between the mutant and wild-type *Pseudomonas*.

Twitching Assay

To determine if a mutant was deficient in twitching motility, twitching assay were performed. The *Pseudomonas* strain in question was streaked on PIA and grown at 37°C overnight. Isolated colonies were then stab inoculated into LB 1% agar plates (with reduced agar concentration from standard LB plates to allow for motility) using a sterile toothpick. These were incubated agar-side up at 30°C for 48 hours. The agar was then removed and discarded, and the twitching zones on the plate stained with crystal violet to allow for visualization.

Statistical Analysis

When possible and applicable, experiments were replicated multiple times. β-Galactosidase assays were performed in biological and technical triplicate, resulting in an n = 9 for each strain in each assay. Western blots were performed in biological replicate at least three times, if not more. All other assays were performed in biological triplicate. When applicable, values determined from assays were averaged and the standard deviation and standard error of the mean determined to produce error bars. Statistical significance was determined using Student's t-test, and reported in figure legends where appropriate.

CHAPTER 3

RESULTS

Expression of *algZ* is Increased in a *pilW* Mutant Strain, *algR* Expression is Not

Previous studies have shown that minor pilins play a role in mechanosensing, as well as in regulation of AlgZ (21). For this reason, mutants were constructed for several components of the TFP including PilW, a minor pilin protein, as well as PilA, the main structural component of the TFP. PilJ, a protein related spatially to the TFP that is involved in signal transduction and chemotaxis, was also deleted. Expression of *algZ* was assessed in these strains using the *algZ* transcriptional fusion.

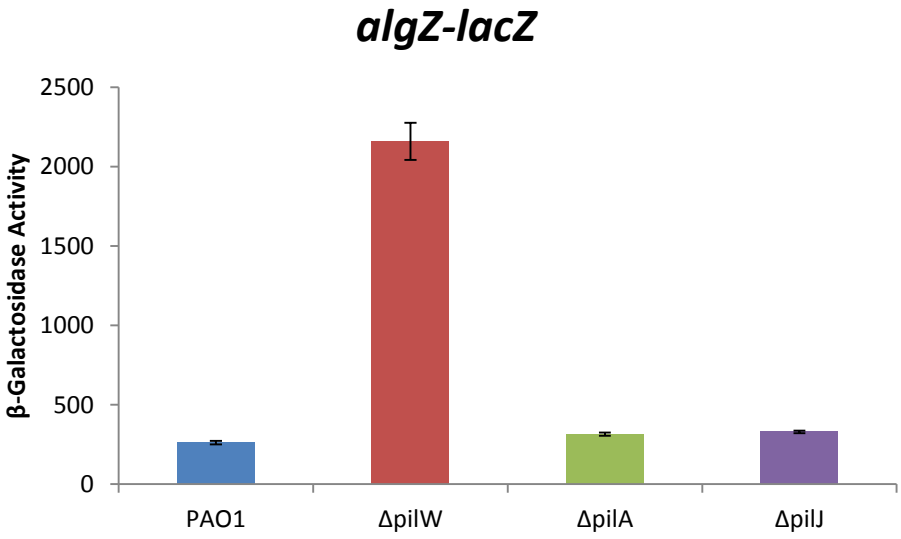


Figure 3. *algZ* expression is increased in a Δ*pilW* strain. PAO1, Δ*pilW*, Δ*pilA*, and Δ*pilJ* were assayed for β-Galactosidase activity. Assays performed in triplicate at 8 hours in LB broth. Statistical comparison of expression in PAO1 versus Δ*pilW* yields $p<0.001$.

In the *pilW* mutant background, *algZ* expression increases a significant amount, while this is not the case for the *pilA* or *pilJ* mutants. This suggests that *algZ* expression is controlled specifically by the minor pilins.

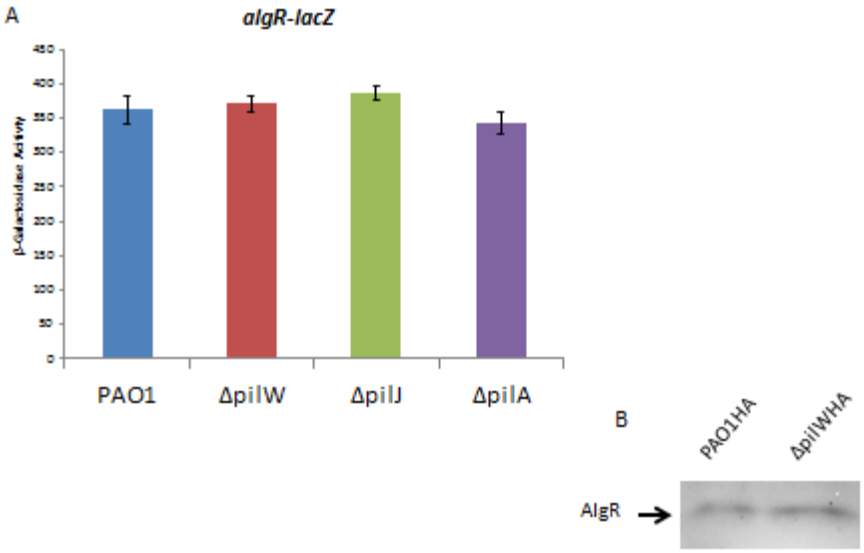


Figure 4. *algR* expression in the *pilW* mutant does not differ from wild-type. A) Transcriptional fusion analysis of *algR*. B) Western blot analysis of AlgR. β-Galactosidase assays performed in triplicate at 8 hours in LB.

Moreover, none of the mutants showed a change in *algR* expression as observed by the *algR-lacZ* transcriptional fusion. This was confirmed in the pilW mutant background via western blot, using an HA-allele tagged AlgR. Therefore, *algR* expression and AlgR levels are not affected by a *pilW* mutation.

This not only confirms what has been seen in minor pilin mutants in other studies, but it also provides an excellent basis for study of *algZ* expression, and how this relates to mechanosensing.

Increase in *algZ* Expression in Δ*pilW* Strain is Not due to Vfr

While *algZ* expression has not been extensively studied, one known regulator of *algZ* is Vfr, virulence factor regulator. It was possible that the upregulation in *algZ* expression in the *pilW* mutant was due to Vfr.

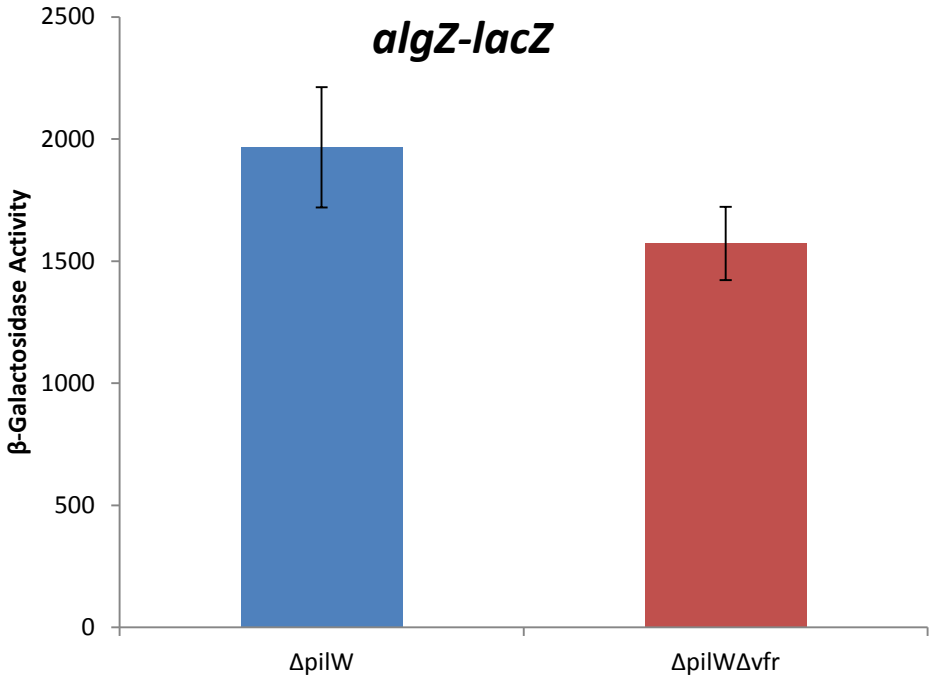


Figure 5. Vfr is not responsible for the increase in *algZ* expression in the *pilW* mutant background. β-Galactosidase assays performed in triplicate at 8 hours in LB broth.

However, in a double mutant of *pilW* and *vfr*, there was no significant decrease in *algZ* expression. This suggests that the increase in *algZ* expression is not due to Vfr, but due to another factor. It is possible that this is done through the activation of an activator of *algZ*, or repression of an *algZ* repressor.

Transposon Mutant Screen

To further investigate what was causing the increase in *algZ* expression in the *pilW* mutant strain, a transposon mutagenesis screen was initiated. The Δ*pilW algZ*TF-*lacZ* strain was

conjugated with an *E. coli* strain containing the plasmid pBT20. In this conjugation, the mariner transposon found within the pBT20 plasmid inserts randomly into the chromosome of the *Pseudomonas* strain, interrupting a gene at random that will hopefully produce a change in the *algZ* transcriptional fusion. The mariner transposon contains a gentamicin cassette that confers gentamicin resistance to the *Pseudomonas* strain. Gentamicin irreversibly binds to the 30s ribosomal subunits and prevents protein translation, leading to death of the cell that does not take up the transposon. The gene product of the gentamicin resistance cassette within the transposon prevents this binding. This allows for selection of transposon mutants which can then be screened for the desired changes. In this case, screening of the transposon mutants consisted of the high throughput β-galactosidase assay.

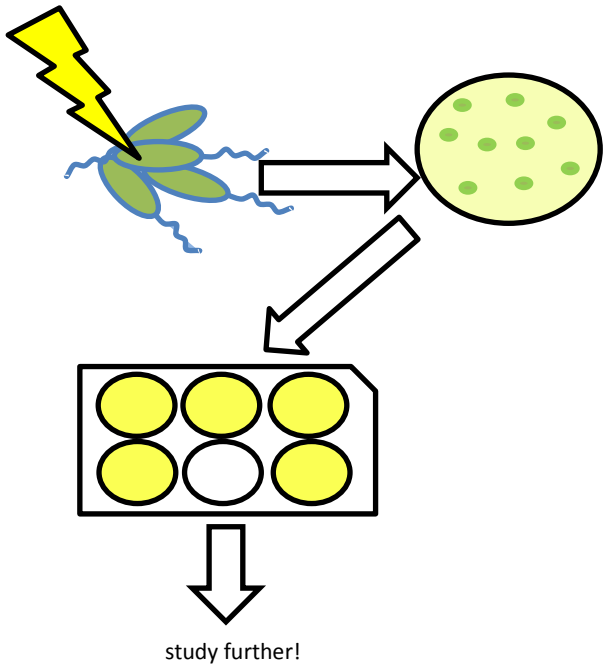


Figure 6. Representation of a transposon mutagenesis screen and high throughput β-Galactosidase assay. Lightning bolt represents mutagenesis of *Pseudomonas* cells by the mariner transposon. Mutants are then spread on PIA gentamicin, and then inoculated into 96-well plates to screen for differences in reporter activity. Those with changes were selected for further study.

Approximately 13000 transposon mutants were screened in this fashion, and 16 were found to be of particular interest because of their differences in *algZ* expression from the *pilW* mutant. While often the mutants tended to have a decrease in *algZ* expression according to the transcriptional fusion, there were a few that showed an increase in *algZ* expression from the *pilW* mutant. These were interesting due to the fact that the *pilW* mutant itself already had a drastic increase in *algZ* expression in comparison to the wild-type and suggested further negative regulatory activity on *algZ* expression.

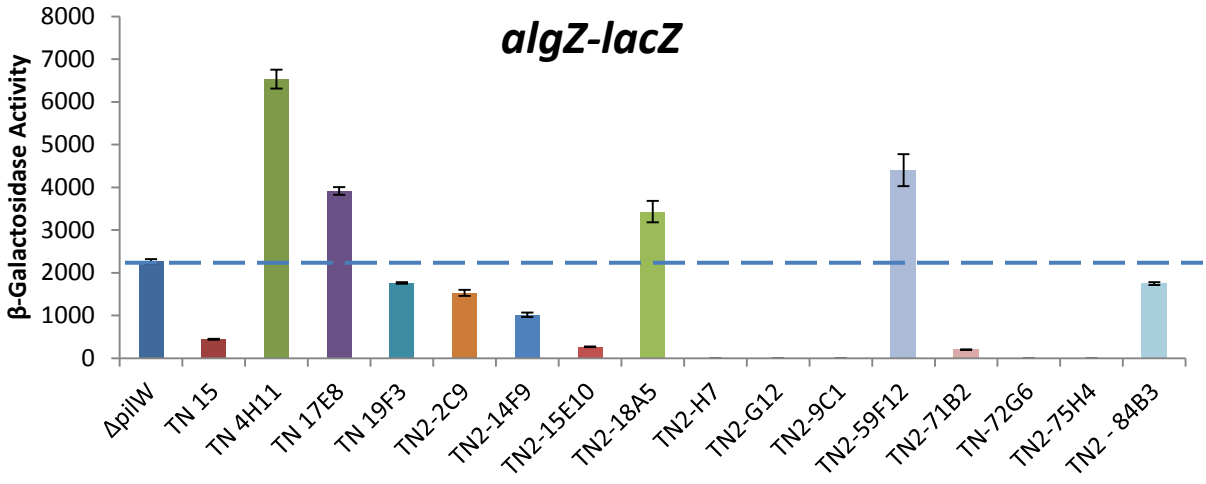


Figure 7. Transposon mutagenesis screen reveals mutants with both decreases and increases in *algZ* expression as compared to the *pilW* mutant reference strain. Β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of expression in Δ*pilW* versus transposon mutant yields $p<0.05$ in each case.

Clearly there were several mutants of interest. However, before further steps were taken to determine the genes interrupted in these mutants, we first had to determine that these expression differences were actually due to transposon insertion and not spontaneous mutation.

Gentamicin Resistance Screen

In order to select for colonies that have the transposon inserted into their chromosomes,

after conjugation with pBT20, the *Pseudomonas* strain was plated on PIA gent (Appendix A). Sometimes a *Pseudomonas* strain may be capable of growing on gentamicin-supplemented media due to a random mutation, or because of a reduction in gentamicin concentration in the media due to the presence of other gentamicin-resistant colonies. For this reason, it was important to screen for the presence of the gentamicin resistance gene conferred by the transposon, to ensure that the mutants selected for further study did, indeed, contain the transposon within their chromosomes. This screen was done via colony PCR. The primers utilized were gentF and gentR (Appendix C). The *E. coli* strain containing pBT20 was used as a positive control for the PCR. If colony PCR from the proposed transposon mutants yielded a strong band that was approximately 500bp, it was concluded the gentamicin cassette, and therefore the mariner transposon, had inserted into the chromosomes of the mutants. All mutants in the above figure were screened in this fashion and the transposon insertion confirmed.

Arbitrary PCR and Rescue Cloning

To identify the gene interrupted by the transposon, two methods were used. These were arbitrary PCR and, when this was unsuccessful, rescue cloning. For the arbitrary PCR protocol, three arbitrary primers were recommended for use in the initial PCR of the two step PCR protocol (3). However, in this instance it was found using the single Arb2 primer (RdPa2) produced stronger and cleaner bands than any other combination. Cloning the arbitrary PCR fragments into the pMiniT vector using the NEB© PCR Cloning kit was attempted at first, following the included instructions. However, this cloning technique proved too sensitive to provide product efficient for sequencing because all PCR products were cloned. For this reason, the PCR products were instead cleaned up using the Monarch DNA Gel Extraction Kit (New England BioLabs®) as per the kit's instructions.

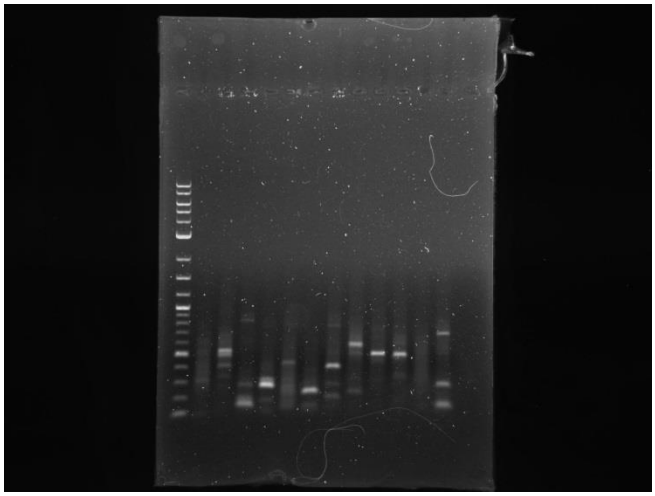


Figure 8. Gel electrophoresis of several successful arbitrary PCR products, amplified from transposon mutants.

After cleanup, samples were sent for sequencing to the ETSU Molecular Biology Core Facility, and yielded a success rate of approximately 70%. Rescue cloning proved to be less efficient than arbitrary PCR, but served in circumstances where arbitrary PCR failed or where a second method of confirmation was desired. For these instances, genomic DNA was digested and cloned into pUC19, plated for gentamicin resistance, and then these plasmids were sent for sequencing. For the sequencing, these samples had a 100% success rate, however, generating the plasmids was only successful in about half of all attempts. For this reason, arbitrary PCR was relied upon more extensively. Both methods combined yielded information on ten of the 16 transposon mutants.

Mutant	Identified Gene
TN 15	PA5567
TN4H11	*pslA*
TN17E8	PA4873
TN19F3	Intergenic region between PA2770 and PA2771
TN2 – 14F9	PA2771
TN2 – 15E10	*algR*
TN2 – 18A5	PA1181
TN2 – 59F12	*pilJ*
TN2 – 71B2	PA1487
TN2 – 84B3	*purD*

Table 8. Transposon mutants and their corresponding interrupted genes

Isogenic Mutants

While the results of the transposon mutagenesis screen are very suggestive, it is necessary to control for the effects the transposon may produce aside from the desired interruption of genes. The transposon has its own independent promoters and sometimes the effect seen in the transposon mutant may actually be from the activation of these promoters increasing the expression of downstream genes, as opposed to the interruption of the identified gene. Because of the method of insertion, it is also possible that the effect of the transposon may be due to the disruption of expression of multiple genes if the gene interrupted is within an operon. For this reason, it was important to confirm the results of the transposon mutagenesis screen by making isogenic, or "clean," mutants. It was not feasible to make isogenic mutants for every single identified gene, so only those of the greatest interest were selected. These included *pslA* and PA5567, for which deletion constructs had to be made for this study. Deletion constructs for both *pilJ* and *algR* were already available in the lab from previous studies.

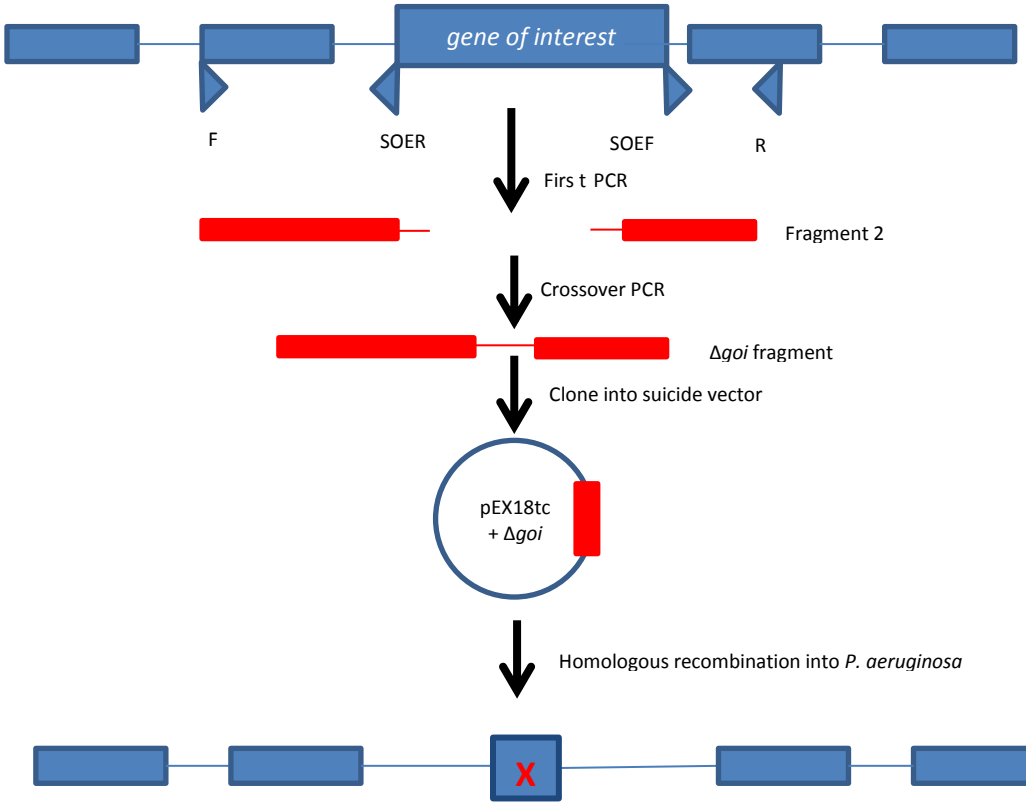


Figure 9. Diagram for construction of mutant strains. Blue triangles indicate primers utilized. Gene of interest also referred to as *goi*.

PslA

The *pslA* gene was selected first because the transposon mutant 4H11 had reporter activity more than twice that of the *pilW* mutant. This is of particular interest because *algZ* expression in wild-type *P. aeruginosa* is fairly low and the *pilW* mutant had already shown a significant increase from that. For *algZ* expression to increase even further indicates that in the wild-type there may be some strong negative regulation of *algZ* that has not yet been fully elucidated.

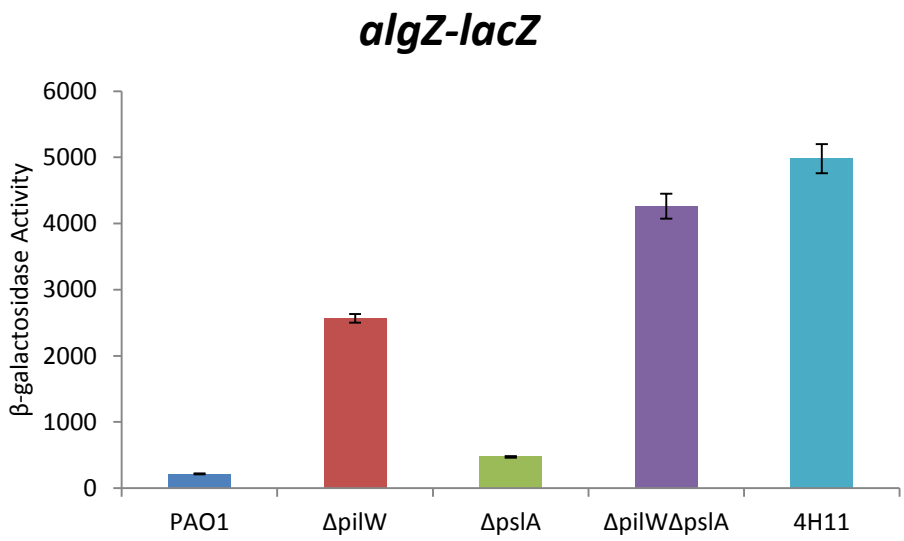


Figure 10. *pslA* alone is not responsible for regulation of *algZ* expression. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of Δ*pslA* to PAO1 is not significant, while comparison of PAO1 as well as *pilW* to the *pslApilW* double mutant and the transposon mutant yields $p<0.001$

Despite the increase in *algZ* expression shown by the transposon mutant, a single mutant of *pslA* produces no significant effects on *algZ* expression. However, a double mutant of *pslA* and *pilW* does significantly increase *algZ* expression in comparison to both wild-type and the single *pilW* mutant. This indicates that while PilW and PslA may both have a negative regulatory effect on *algZ* expression, PslA only expresses this negative regulation when PilW is not present. Because PslA is required for the production of the Psl exopolysaccharide (23), it is possible that psl is involved in the signal transduction through the minor pilins and the AlgZ/R TCS. It is also possible that both PilW and PslA act as negative regulators, with PslA in a supportive role to PilW, therefore its effects on *algZ* expression are only visible when PilW is absent.

PA5567

The second mutant of interest was TN15, which showed a significant decrease in *algZ* expression compared to the Δ*pilW* strain. The gene interrupted in this case was PA5567, a

conserved hypothetical protein. This gene has not been studied in *P. aeruginosa*, though its homologue, *trmE*, has been studied in *Pseudomonas fluorescens*. In *P. fluorescens*, *trmE* has been shown to be involved in the regulation of production of the polyketide 2,4-diacetylphloroglucinol (37). However, no such polyketide is produced by *P. aeruginosa*, indicating that PA5567 may have different regulatory capabilities in *P. aeruginosa* than *trmE* has in *P. fluorescens*.

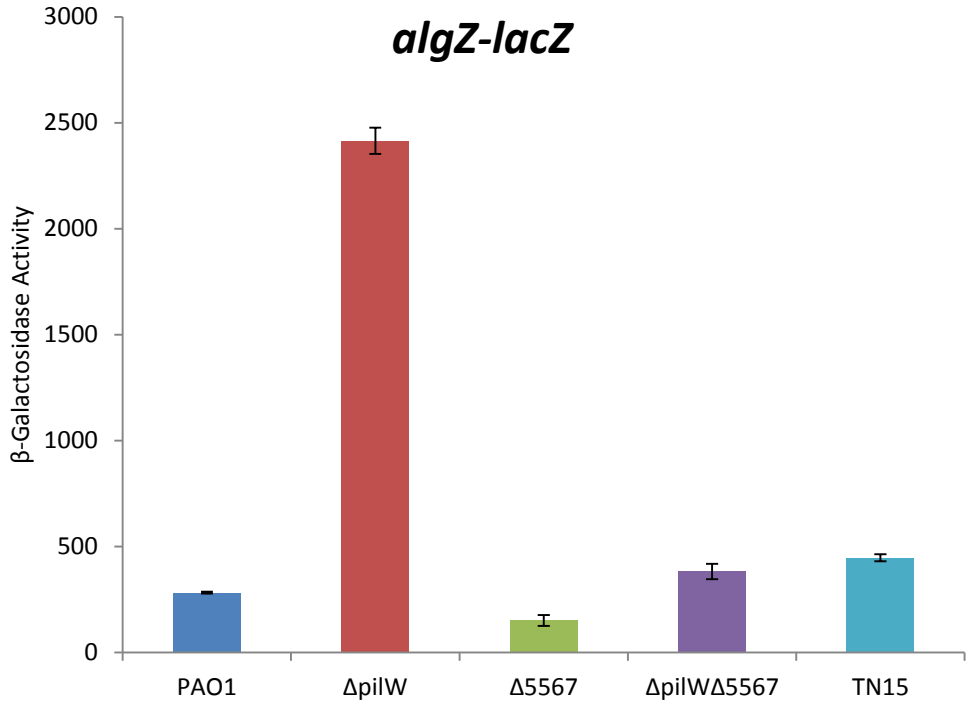


Figure 11. PA5567 regulates *algZ* expression independently of PilW. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAO1 and Δ5567 yields $p<0.001$, as does comparison of Δ*pilW* and the double mutant Δ*pilW*Δ5567.

Analysis of *algZ* expression in the PA5567 mutant strain revealed a marked decrease as compared to the wild-type. This indicated that the gene product of PA5567 may be a positive regulator of *algZ* expression that works independently of the effects of PilW. This is in contrast

to the *pslA* mutant, which required the absence of PilW for its effects to be seen.

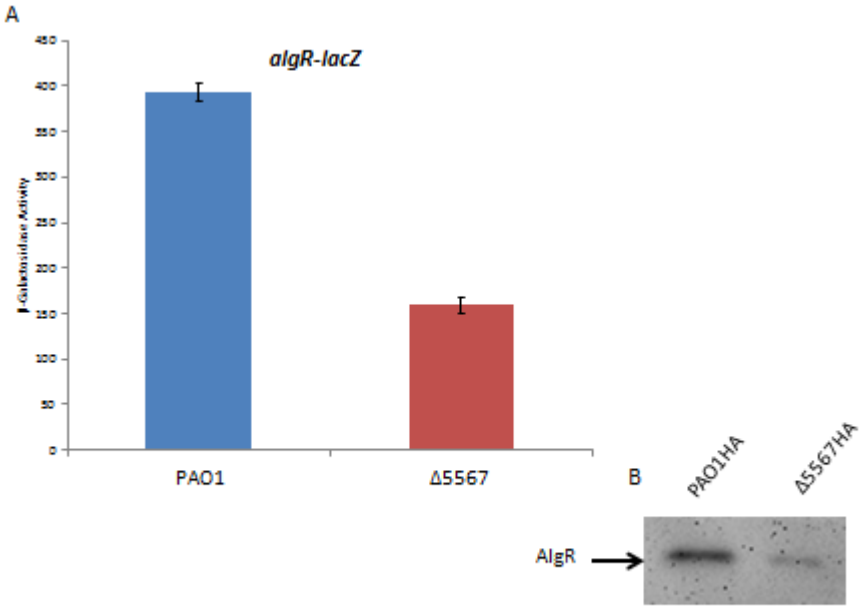


Figure 12. PA5567 influences *algR* expression. A) Transcriptional fusion analysis of *algR*. B) Western blot analysis of AlgR. Assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAO1 and Δ5567 yields $p<0.001$

Moreover, the gene product of PA5567 also appears to have an effect on *algR* expression. In the mutant, *algR* expression is decreased by approximately half as indicated by the transcriptional reporter. This combined with its influence on *algZ* expression indicates that PA5567 affects multiple promoters of the *algZ/R* operon. Western blot analysis also shows a decreased amount of AlgR protein present in the Δ5567 mutant strain. To further investigate this, a *fimU* fusion was assayed in the Δ5567 strain. The *fimU* operon is responsible for the production of the minor pilin proteins, and is thus involved in the production of TFP, a known virulence factor. This virulence factor is also known to be regulated by AlgR, which acts as a transcriptional activator for the *fimU* operon (2).

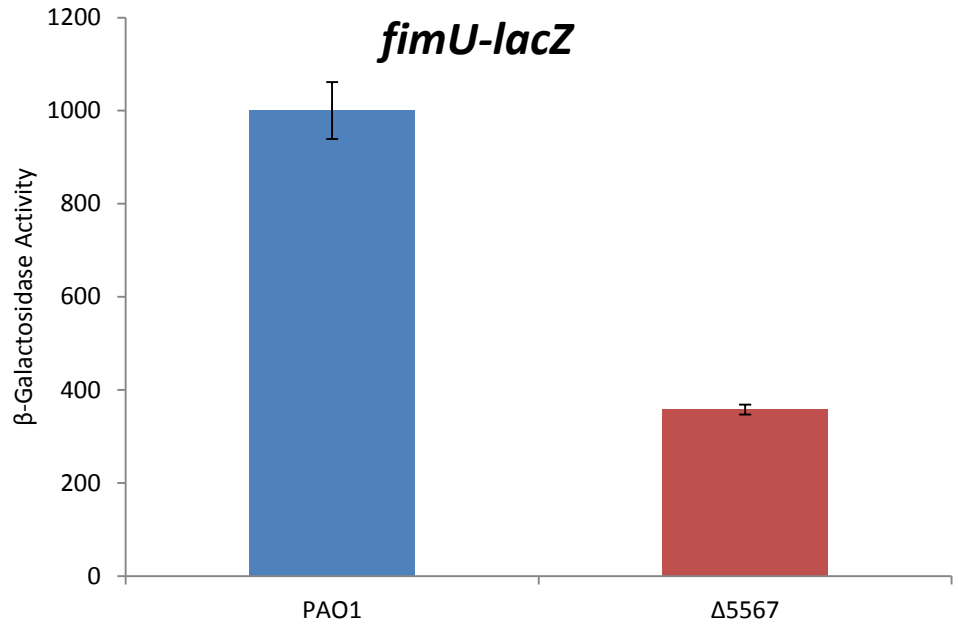
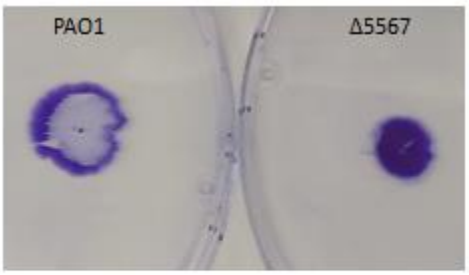


Figure 13. PA5567 regulates *fimU* expression through the activity of AlgR. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAO1 and Δ5567 yields $p<0.001$

Expression of the *fimU* operon is also decreased in the Δ5567 strain, indicating a downstream effect of the decrease in both *algZ* and *algR* expression to impact the virulence factors that they regulate. The impact of decreased expression of the *fimU* operon was elucidated via a twitching assay.



PAO1	Δ5567
9.4 ± 2.3mm	5.8 ± 1.6mm

Figure 14. Mutants of PA5567 have reduced twitching motility as compared to PAO1. Assays done in triplicate for 48 hours on twitching media at 30°C. Statistical analysis of comparison of PAO1 to Δ5567 yields p=0.001

The decreased expression of the *fimU* operon in this background does result in a decrease in twitching motility. However, in the course of performing β-Galactosidase assays on PA5567 mutants, a discrepancy was noted in the growth of the strains. PA5567 mutants did not appear to grow as robustly as their wild-type counterparts. While *trmE* mutants in *P. fluorescens* do not display any growth defects (37), it is possible due to the different roles of this gene in *P. aeruginosa* that a growth defect may be present, and this could account for the decreases in expression of the various genes assayed. To look further into this, growth curves were performed.

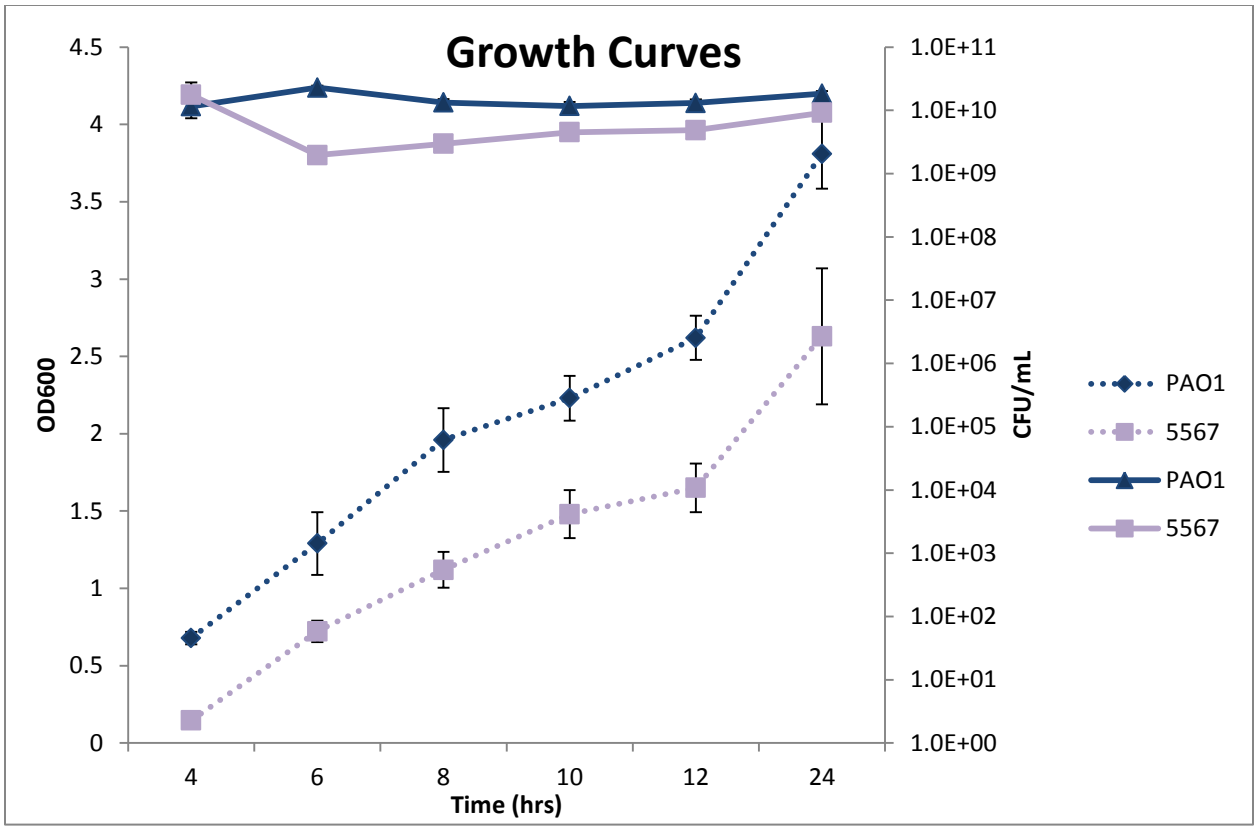


Figure 15. Growth curves of PAO1 and Δ5567. Solid lines indicate CFU/mL and correspond to the right axis of the figure. Dashed lines indicate OD600 and correspond to the left axis of the figure. Assays performed in triplicate. Statistical analysis for each time point comparing PAO1 to Δ5567 yields $p > 0.05$ at each time point, except for 6 hours for CFU/mL, which yields $p < 0.05$

Growth curves did not seem to indicate any significant growth defect in PA5567 mutants. However, due to the fact that no transcriptional fusion assayed had produced β-galactosidase activity similar to wild-type coupled with the mutant's strange appearance, the possibility of a defect still remained. For this reason, a *tcr* fusion was assayed in the mutant. The *tcr* gene encodes for tetracycline resistance, and its promoter is not native to *P. aeruginosa*. For this reason, all strains should activate this promoter at the same rate as wild-type, unless they suffer from a transcriptional, metabolic, or growth defect.

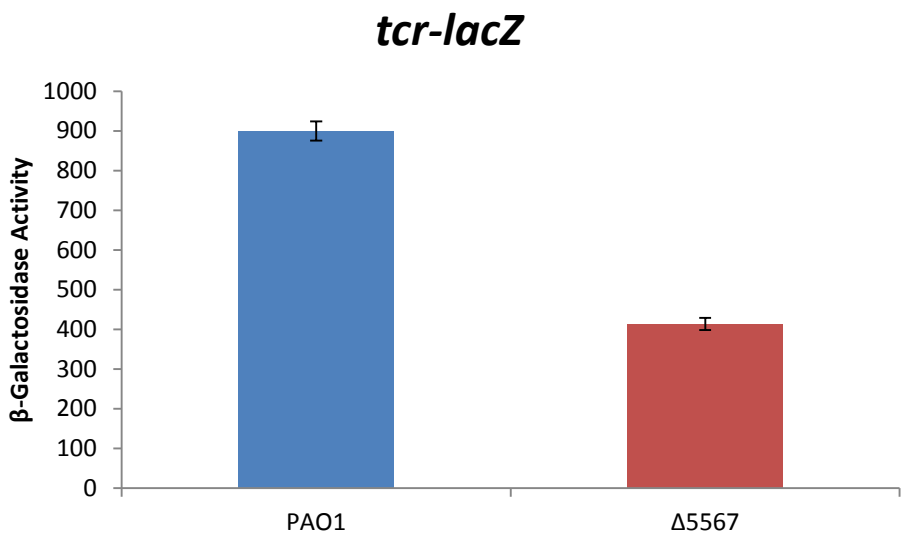


Figure 16. Activation of the *tcr* promoter is reduced in the PA5567 mutant, indicating a defect in this strain. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAO1 and Δ5567 yields $p<0.001$

Analysis of the *tcr* transcriptional fusion in the PA5567 mutant revealed a significant decrease in activation of transcription. This indicates that while the PA5567 mutant may not have a growth defect, it does appear to have general defects in transcriptional regulation. For this reason, further study on PA5567 as a potential regulator of *algZ* expression was halted.

AlgR

The discovery of *algR* as the interrupted gene in transposon mutant 2-15E10 was very suggestive of an autoregulatory system for the AlgZ/R TCS which had not been previously described.

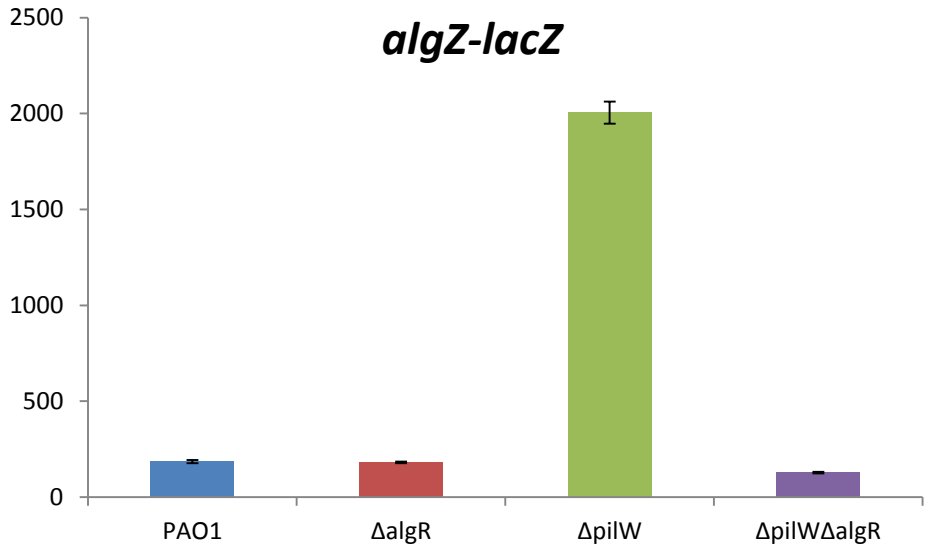


Figure 17. Expression of *algZ* is dependent on AlgR in the *pilW* mutant background. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of Δ*pilW* and Δ*pilW*Δ*algR* yields $p<0.001$

Deletion of *algR* in the *pilW* mutant background reduced *algZ* expression to wild-type levels. This strongly indicates that increased expression of *algZ* in the *pilW* mutant is caused via AlgR activity. Despite this, however, it was unclear which phosphorylation state of AlgR is responsible for the increased *algZ* promoter activation.

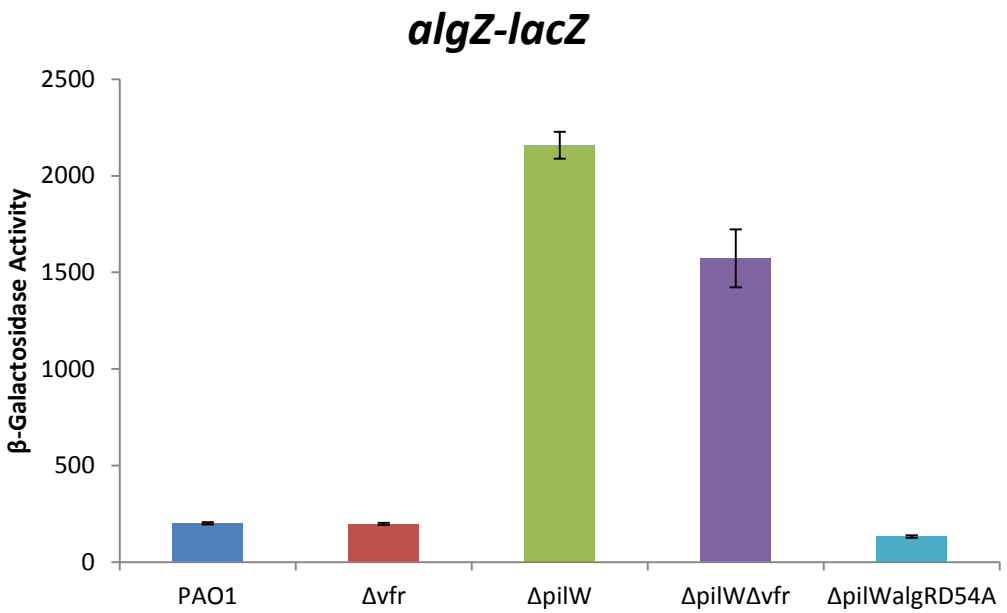


Figure 18. Increased *algZ* expression is due to activity of phosphorylated AlgR in the *pilW* mutant background. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of Δ*pilW* and Δ*pilWalgRD54A* yields $p<0.001$

The Δ*pilW*algRD54A strain produces an AlgR protein with an alanine at residue 54 as opposed to aspartic acid, effectively producing an AlgR that cannot be phosphorylated. In this strain, *algZ* expression is almost completely abrogated. This leads to several conclusions: 1) that the increased expression of *algZ* in the *pilW* mutant background is entirely due to phosphorylated AlgR and 2) *algZ* is a target of phosphorylated AlgR. Neither of these things have previously been discovered in other works, and *algZ* as a facet of the AlgR regulon is entirely novel information. Moreover, this data strongly indicates a novel autoregulatory pathway for the *algZ/R* operon by the AlgZ/R TCS. This also indicates a regulation of AlgZ phosphorylation activity by the minor pilin proteins, possibly due to their involvement in mechanosensory transduction. To investigate this potential further, assays of the *algZ* transcriptional fusion were performed in several strains in both liquid and on solid media.

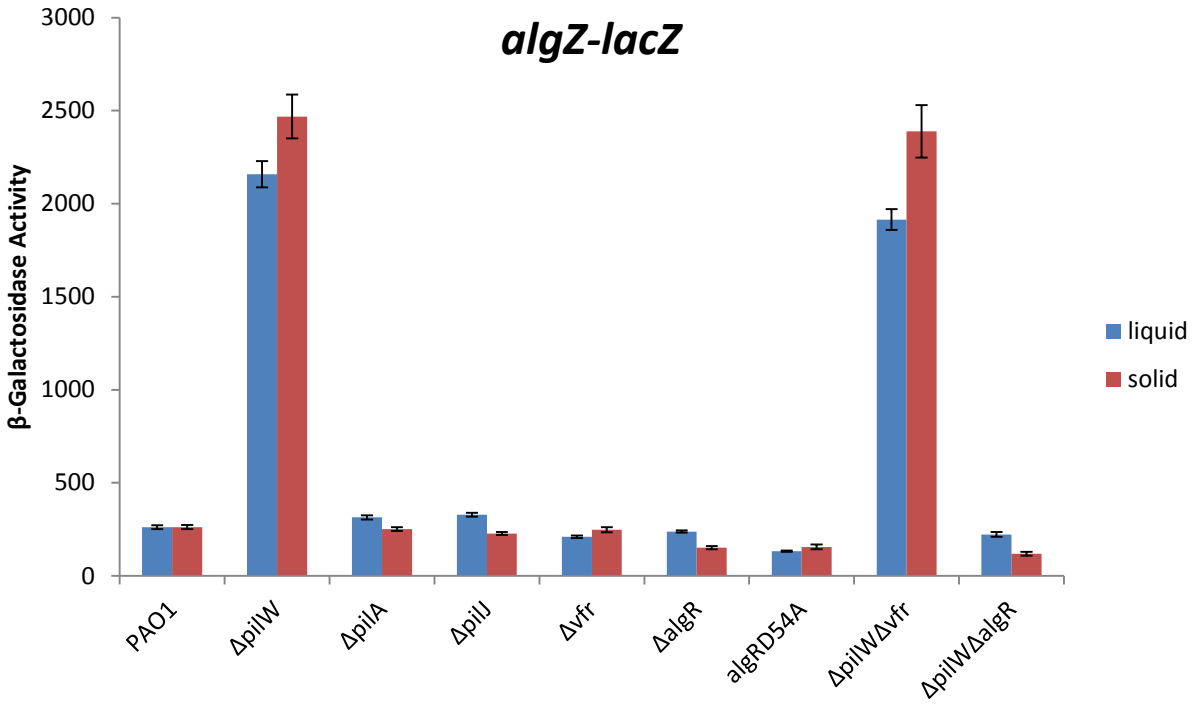


Figure 19. PilW is required for mechanosensory transduction on a solid surface. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAOI and ΔpilW and ΔpilWΔvfr yields $p<0.001$

In strains lacking the minor pilin mutation, *algZ* expression tends to decrease on a solid surface in comparison to expression within a liquid media. However, strains with the *pilW* mutation do not show this decrease and, indeed, show an increase in *algZ* expression on a solid surface. This indicates that a functional minor pilin complex is required for appropriate transduction of the mechanosensory signal. Moreover, in a *pilWalgR* double mutant, this increase in *algZ* expression on a solid surface is not seen. Just as the trend in other strains lacking a minor pilin mutation, in the *pilWalgR* double mutant, *algZ* expression decreases. This indicates that not only is a complete minor pilin complex required for the transduction of the mechanosensory signal, but that this signal is relayed into the cell via the activity of the AlgZ protein.

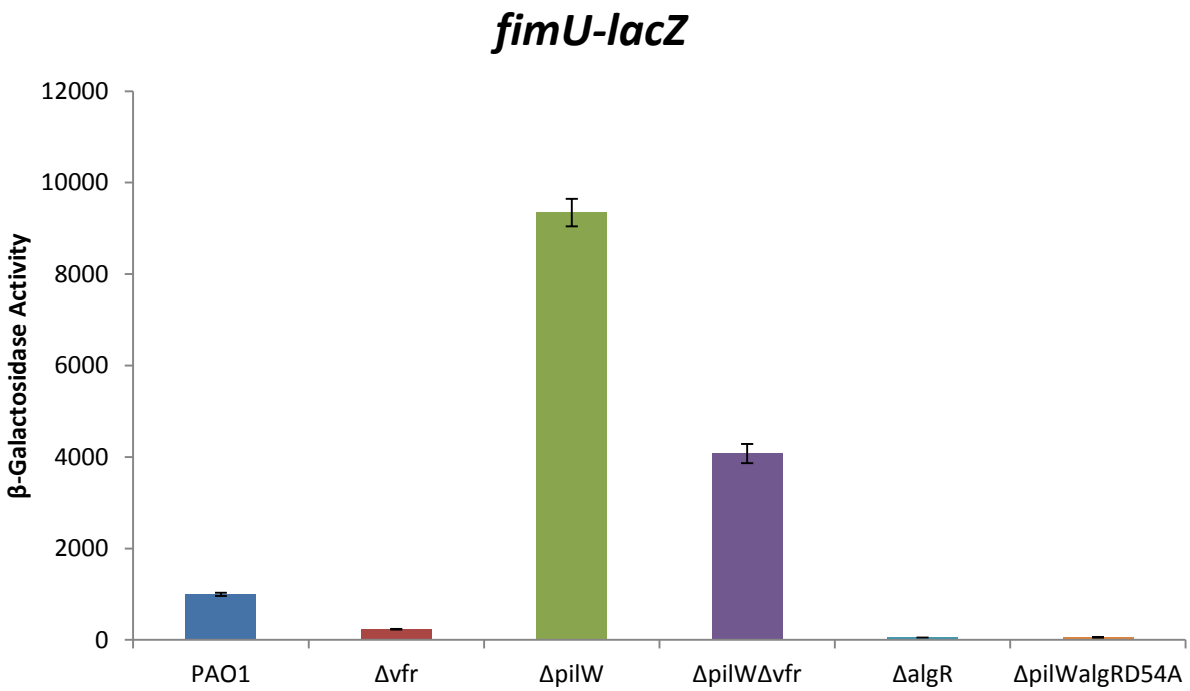


Figure 20. Phosphorylated AlgR is responsible for increase in *fimU* expression in the *pilW* mutant background. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of Δ*pilW* and Δ*pilW*Δ*vfr*, Δ*algR*, and Δ*pilWalgRD54A* yields $p<0.001$

To support the results shown from the *algZ* transcriptional fusion, a *fimU* transcriptional fusion was assayed in several strains. Phosphorylated AlgR is known to regulate expression of the *fimU* operon (2). This fusion follows the same trend as observed in the *algZ* fusion. In the *pilW* mutant strain, expression is significantly increased from wild-type, and this increase in expression is entirely dependent on phosphorylated AlgR. These data taken together would seem to indicate a signaling pathway through the minor pilins and the AlgZ/R TCS system, resulting in dysregulation of the AlgR regulon. When all minor pilins are present, they act in a manner that prevents the phosphorylation of AlgR. Previous studies have shown a direct interaction between AlgZ and several minor pilin components (22), which would seem to indicate the minor pilin interaction with AlgZ prevents its phosphorylation of AlgR.

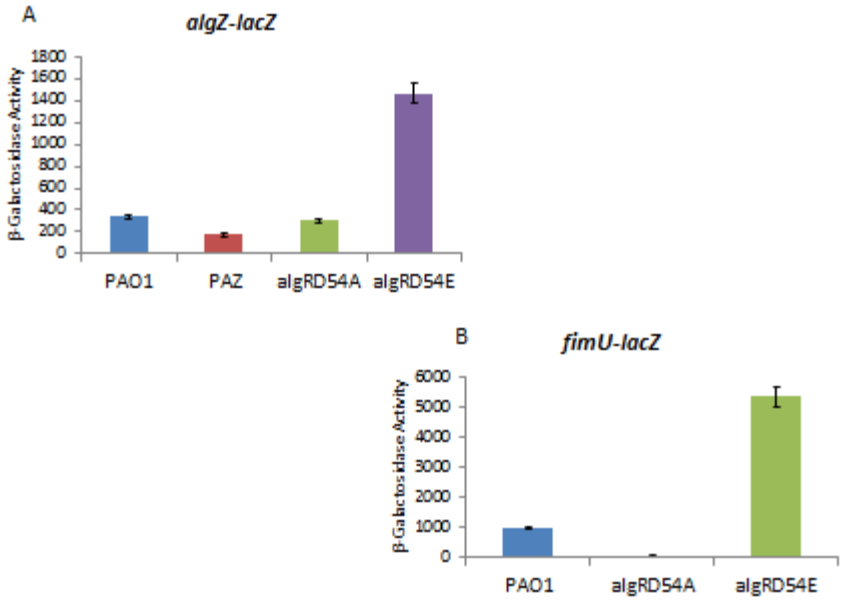


Figure 21. Phosphomimetic strains of AlgR do not influence expression of *algZ* or *fimU* to the same extent as a *pilW* mutant. A) Transcriptional fusion analysis of *algZ*. B) Transcriptional fusion analysis of *fimU*. β-Galactosidase assays performed in triplicate at 8 hours in LB broth. Statistical comparison of PAO1 versus algRD54E yields $p < 0.001$ in both cases, and comparison of PAO1 versus algRD54A yields $p < 0.001$ in the case of the *fimU* fusion

Curiously, in the phosphomimetic strain *algR*D54E, neither the *algZ* nor the *fimU* transcriptional fusions show an increase in expression equal to the *pilW* mutant strain. This discrepancy would seem to indicate not only an increase in phosphorylation of AlgR in the *pilW* background, but an increase in the amount of AlgR to be phosphorylated as compared to the wild-type. However, this is not actually the case, as evidenced by western blot analysis (Figure 4). Such a discrepancy may be due to a previously undiscovered defect in the AlgR phosphomimetic strain, or it may be the result of some aspect of the regulation of AlgR phosphorylation by the minor pilins that has yet to be elucidated. The potential also remains for post-transcriptional or post-translational regulation of AlgR. Further study is required to draw a firm conclusion.

CHAPTER 4

DISCUSSION

Pseudomonas aeruginosa is a highly adaptable opportunistic pathogen with a plethora of virulence factors at its disposal. It is extremely resistant to multiple forms of treatment, and is of particular concern for those with compromised immune systems. For this reason, understanding regulation of virulence factors is an important area of study to determine new potential targets for future therapeutics. In order to sense its environment, *P. aeruginosa* must make use of two-component systems, one of which is the AlgZ/R TCS, in order to properly regulate gene expression in response to environmental cues. The signal which AlgZ senses is not yet known, and regulation of *algZ* expression has not been extensively studied. It was for this reason that this study was undertaken. The potential for elucidating the regulation of *algZ* expression could potentially aid in both determining how to reduce virulence through dysregulation of *algZ* and also shed more light on what the sensor of the AlgZ/R TCS senses based on the work presented.

Regulators of *algZ* Identified via Transposon Mutagenesis

Despite the fact that transposon mutagenesis screens have fallen out of vogue in recent years due to the advent of more sophisticated technologies and techniques, they still remain a powerful tool for the identification of novel genes involved in a process of interest. This was shown in our screen for regulators of *algZ* expression. In a screen of approximately 13000 transposon mutants, many were found to have differences in *algZ* expression, ten of which the genes involved were identified. For *pslA*, the influence of this was only seen when coupled with a *pilW* mutation. PilW and PslA may work in tandem to down-regulate *algZ* expression or, alternatively, the Psl exopolysaccharide is involved in a form of autocrine signaling. PA5567

was another potential regulator that showed promise, yet proved to be involved either in metabolism or transcription in general, and while this could prove of interest as a target of therapeutics later on, it was not a specific *algZ* regulator as anticipated. The most interesting gene identified in the transposon mutagenesis screen, however, was *algR*.

AlgR

The identification of AlgR as a regulator of *algZ* expression in this study indicates an unknown autoregulatory loop. Moreover, we found that AlgR must be phosphorylated in order to activate transcription of *algZ/R*, thus requiring the activity of AlgZ for this transcriptional regulation. This indicates a complex autoregulatory loop in which AlgZ phosphorylates AlgR, which then acts to activate transcription of *algZ/R*. All of this, of course, must be dependent on the signal received by AlgZ. One can assume that the signal that AlgZ detects serves to regulate its phosphorylation activity. In conjunction with this, we know that the minor pilins serve to modulate the phosphorylation activity of AlgZ (21, 22, our unpublished results). This adds yet another layer to the story, as the minor pilin operon (*fimU*) is also part of AlgR's regulon (20). In a normally functioning bacterial cell, the minor pilin proteins act to repress the activity of AlgZ to phosphorylate AlgR. This reduction in phosphorylated AlgR thus results in a reduced expression of both the *fimU* operon and the *algZ/R* operon. Due to mechanosensing of the TFP, it is not unreasonable to assume this down-regulation results from the bacteria setting down on a solid surface and turning on other genes necessary for later stages of colonization. When the bacteria are engaged in a planktonic lifestyle, as they are when growing in a broth, there is the possibility that both *fimU* and *algZ/R* have increased expression as the bacteria are essentially "reaching out" to sense when they come into contact with a solid surface such as a tissue. When this contact signal is transduced into the cell via the TFP, phosphorylation of AlgR is down-

regulated, allowing for expression of genes more conducive to a sessile, or surface-associated, lifestyle. In a mutant that is lacking a minor pilin protein, a functional TFP cannot form, which leaves the bacterial cell without the capability to sense a solid surface. This could be the cause for the drastic increase in phosphorylated AlgR in these minor pilin mutant backgrounds. However, at this point, only genetic experiments have been performed and future biochemical studies examining AlgR phosphorylation state directly are necessary. The cell is upregulating *fimU* and *algZ/R* expression to attempt to "reach out" and sense that contact with a solid surface, but it is incapable. Therefore, the cell is constantly in a state of anticipation for contact and never down-regulates the phosphorylation of AlgR in preparation for a sessile lifestyle. This illustrates the importance of AlgZ/R in mechanosensing.

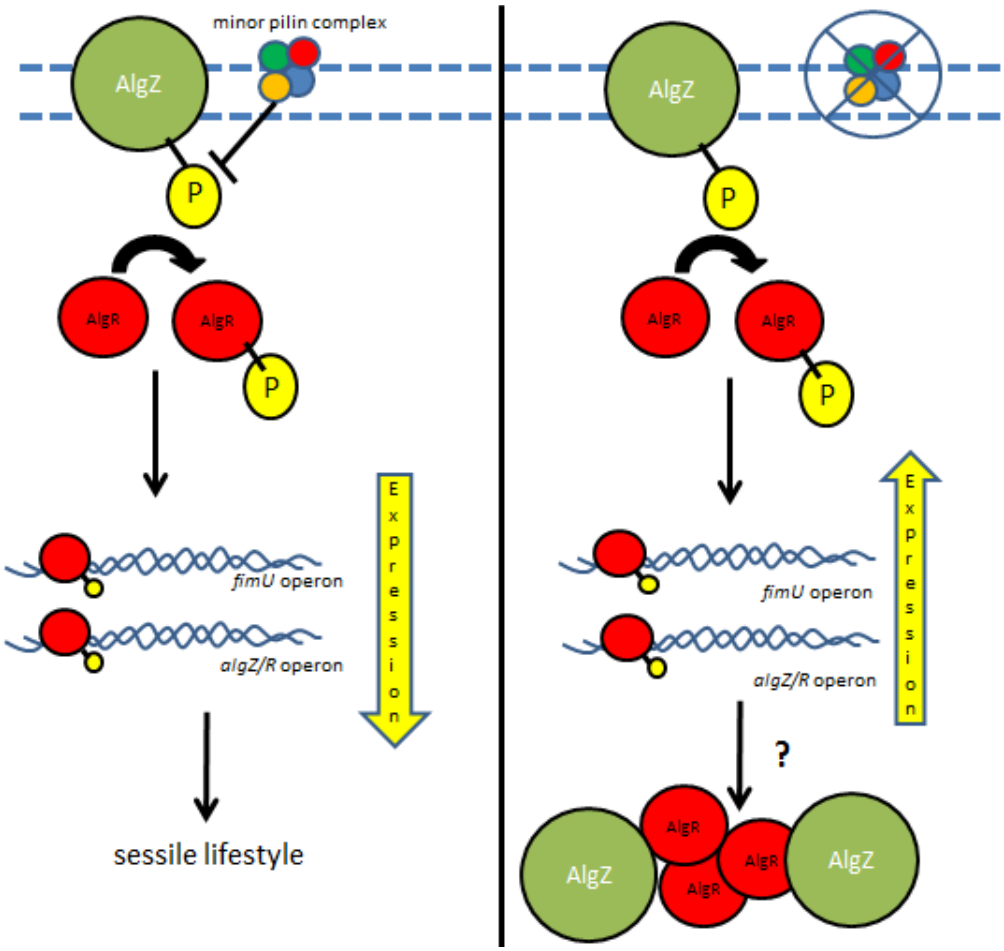


Figure 22. Proposed mechanism for the regulatory loop of AlgZ/R TCS and the minor pilin complex of TFP. In a piliated strain, bacteria can sense contact with a surface and decrease AlgR phosphorylation in preparation for surface-associated growth. In a minor pilin mutant, the signal cannot be transduced and so the bacteria continually upregulate both algZ/R and fimU in an attempt to sense their surroundings.

The proposed mechanism illustrates the result uncovered in this study that there is a way to turn off the AlgZ/R system using the minor pilins. Previous studies have shown that minor pilin protein mutants are attenuated for virulence in a *Dictyostelium* model (34). Because we have seen in this study that a functional minor pilin complex is required for mechanosensory signal transduction, and moreover that this signal appears to rely on AlgR for relay into the cell, this strongly indicates that the attenuation of the minor pilin mutants is due to the hyperphosphorylation of AlgR. While this attenuation is likely not due to the overexpression of the *fimU* operon and the *algZ/R* operon resulting from hyperphosphorylation of AlgR, AlgR is

known to have other virulence related targets. However, further support for the AlgR attenuating virulence was provided by complementation and overexpression studies that demonstrated attenuation in a mouse septicemia model (19). Dysregulation of these virulence targets resulting from the hyperphosphorylation of AlgR could be the mechanism by which the minor pilin mutants are attenuated. Further studies are required, but the data presented here is very suggestive of this theory.

Importance

Inappropriate activation of the AlgZ/R TCS leading to an attenuation of virulence could result in new possible therapeutic targets to treat *P. aeruginosa* infection. One of the greatest virulence factors, especially in terms of individuals with cystic fibrosis, is biofilm formation. Biofilms decrease the ability of the host immune response to clear the infection, while increasing subsequent damage from the host immune system due to an increased inflammatory response. Moreover, biofilms increase antibiotic resistance through the formation of thick physical barriers that antibiotics are rarely able to penetrate effectively (12). However, if treatment results in hyperphosphorylation of AlgR in the initial colonization phase, this might disrupt the events required for *P. aeruginosa* to colonize the CF lung. Moreover, if the AlgZ/R TCS can be continually activated as it is in a minor pilin mutant, this can also serve to attenuate virulence in the midst of either a chronic or an acute infection. While this would not be a perfect solution in terms of treatment, it could potentially allow for easier clearance of infection and combination treatment with antibiotics might allow eradication of *P. aeruginosa*. This study reveals a major facet of *P. aeruginosa* gene regulation that has not previously been investigated and suggests that the AlgZ/R system is critical for mechanosensing and virulence coordination.

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APPENDICES

Appendix A

Growth Media

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

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
10ug/mL tetracycline
100ug/mL ampicillin
35ug/mL kanamycin

YT (1 liter)

950mL ddH_2O
10g tryptone
5g yeast extract
15g agar

QS to 1000mL with ddH_2O
Autoclave

To make YT $tet^{50}irg^{25}$:
Add 25mg irgasan before autoclaving
50ug/mL tetracycline after autoclaving

To make YT 10% sucrose:
Add 200mL filter sterilized 50% sucrose after autoclaving

VBMM (500mL)

400mL ddH_2O
7.5g agar

Autoclave, then cool to 50℃

Add:
50mL 10X VBMM stock
500uL 1M magnesium sulfate
50uL 1M calcium chloride

Antibiotics:
300ug/mL carbenicillin

10X VBMM stock (500mL):
400 ml ddH2O
15g Trisodium Citrate
10g Citric Acid
50g K2HPO4
17.5g $NaNH4PO4 \cdot 4H2O$
pH to 7 and autoclave

Appendix B

Buffers

Agarose Gel (1%)
makes 2 gels

5mL 10X TBE
45mL ddH_2O

boil in microwave
cool to ~50°C

add 2.5uL EtBr
pour into gel casts, add comb

10X TBE (1 liter)

900mL ddH_2O
108g Tris Base
55g Boric Acid
9.3g Na_2EDTA
QS to 1000mL with ddH2O
Autoclave

Permeabilization Buffer

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

Arbitrary PCR Lysis Buffer

10mM Tris-HCl
0.05% SDS

for 4mLs:
40uL 1M Tris-HCl
20uL 10% SDS
3.94mL ddH_2O

1M TNE

50mM Tris-HCl
10mM NaCl
10mM EDTA

4x SDS Loading Dye

4.4 ml 0.5M Tris HCl pH6.8
4.4 ml Glycerol
2.2 ml 20% SDS
0.5 ml 1% Bromophenol Blue
0.5 ml β-mercaptoethanol
Store at -20ºC

10X Glycine Running Buffer

30.3g Tris Base
144.1g Glycine
10g SDS
QS to 1L with ddH_2O
store at 25°C

10X Towbin Buffer

30.3g Tris base
144g Glycine
QS to 1L with ddH_2O

Electroblotting Transfer Buffer

10mL 10X Towbin Buffer
10mL 100% methanol
80mL ddH_2O

10X TBS
12.1g Tris base
43.8g NaCl
QS to 500mL with ddH_2O
pH to 7.6 with HCL

Blocking Buffer (for western blots)

5mL 10X TBS

25uL Tween 20
2.5g skim milk powder
QS to 50mL with ddH_2O

Primary (1°) Antibody Solution

5mL 10X TBS
25uL Tween 20
2.5g skim milk powder
25uL anti-HA/anti-RpoB antibody
QS to 50mL with ddH_2O

Wash Buffer (for western blots)

5mL 10X TBS
25uL Tween 20
QS to 50mL with ddH_2O

Secondary (2°) Antibody Solution

5mL 10X TBS
25uL Tween 20
2.5g skim milk powder
25uL goat anti-mouse antibody
QS to 50mL with ddH_2O

Appendix C

Primers and Plasmids

Arbitrary PCR primers

RdPa1: GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG

RdPa2: GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC

RdPa3: GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT

R1TnM: TATAATGTGTGGAATTGTGAGCGG

Rd2Pa: GGCCACGCGTCGACTAGTAC

R2TnM: ACAGGAAACAGGACTCTAGAGG

TnMSq: CACCCAGCTTTCTTGTACAC

Gentamicin Screen primers

gentF: ATGTTACGCAGCAGCAACGATG

gentR: TTAGGTGGCGGTACTTGGGTC

PA5567 primers

PA5567EcoR1F: GCGCGAATTCCCTGGGTTCCGACCAAGGGC

PA5567XbaIR: GCGCTCTAGATGGAAAGGCGTGGGAAAGGC

PA5567 SOEF: GCTATCCGGAGATATCCACATGGGCAAGTAATGGGGCTCGGC

PA5567 SOER: GCCGAGCCCCATTACTTGCCCATGTGGATATCTCCGGATAGC

PA5567intF: CTTCTTCCTCTGGTTCCCAGC

PA5567intR: GATATCCATCCCATGCAGACCG

pslA primers

pslAEcoR1F: GCGCGAATTCGCCGCGCTACTACTTCCTCG

pslABamH1R: GCGCGGATCCTCGATGTAGCAGTTGCTGACG

pslA SOEF: CGGCAGAGCAAACAACATGCATTACGACCTCGAATACATCGCC

pslA SOER: GGCGATGTATTCGAGGTCGTAATGCATGTTGTTTGCTCTGCCG

pslAintF: CACTAAATTGACGCTTCAGCGC

pslAintR: GGCGCATGAAGGGTTTCGGC

algR Screen Primers

algRintF: GCAACTGGACTGGCAGGTGC

algRintR: CGCGACTGGTCATCGGCAG

algRRT1: GATATCGGGCTTGAGGCTGTC

HAR: GCGTAATCTGGAACATCGTATG

pilW Screen Primers

pilWintF: GATCGAAGTGCTGGTCGCCC

pilWintR: GCGACGCTATTGGTCTCGTAG

Fusion Screen Primers

algZF2EcoR1: GCGCGAATTCCTCTCGCTGCAACAAGAA

fimUHindIIIF: GCGCAAGCTTCTCAACATTCACAACGGGCGGT

lacUV5F: CTTTACACTTTATCGTTCCGGCTCGTATA

lacZR for TF: GATGTGCTGCAAGGCGATTAAG

VITA

DANIELLE WILLIAMS

Education:

M.S. Biology, Microbiology Concentration, East Tennessee State University, Johnson City, Tennessee 2017

B.S. Health Sciences, Microbiology Concentration, East Tennessee State University, Johnson City, Tennessee 2015

Science Hill High School, Johnson City, Tennessee 2011

Professional Experience:

Graduate Assistant, East Tennessee State University, College of Public Health, 2015-2017

Tutor, TRIO Student Support Services, East Tennessee State University, 2015

Publications:

Stacey SD, **Williams DA**, Pritchett CL. (2017). "The *Pseudomonas aeruginosa* two-component regulator AlgR directly activates rsmA expression in a phosphorylation independent manner." J. Bacteriol. 199: Article Number UNSP e00048-17

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Honors and Awards: Outstanding Microbiology Student and Outstanding Student in Health Sciences Awards, 2015

Boris Franzus Memorial Scholarship Award for Excellence in Undergraduate Organic Chemistry, 2013