Calcium Regulating Quorum Sensing in *Pseudomonas aeruginosa*

Thesis by

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In Collaboration with

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, potent, opportunistic pathogen that can cause a variety of infections in humans. While there is a large number of infectious pathogens that cause mortality in our population, *P. aeruginosa* stands out because of the combination of multifactorial virulence and resistance to antibiotics and host defenses.

P. aeruginosa infections can lead to the development of malignant external otitis, endophthalmitis, meningitis, and pneumonia, which is especially common in cystic fibrosis patients (3). Cystic fibrosis patients are most frequently infected by the bacterium in hospital settings since they spend a lot of time under clinical care, and the bacterium inhabits respiratory equipment, sinks, mops and countless other surfaces (1). Being exposed to an infection induced by *P. aeruginosa* almost doubles the risk of death in those suffering with cystic fibrosis, making it a critical pathogen to study in these patients (4). Cystic fibrosis is a disease that can affect multiple bodily systems in varying levels. The lungs, upper airways, liver, intestines and pancreas can all be impacted drastically by mutations occurring in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (2).

The *P. aeruginosa* bacterium piqued the interest of research labs in the world, in recent years, specifically due to its ability to develop resistance against a large number of antibiotics, in addition to the lethal infections it ensues on cystic fibrosis patients. *P. aeruginosa* is capable of rapidly developing resistance to antibiotics that are used during treatment, even if it is being exposed to them for the first time (1). Current research on the pathogen is focused on the regulation of its virulence and resistance to better understand its cellular mechanisms and develop effective therapeutic strategies to fight against the infections.

In order to cause infections in humans, *P. aeruginosa* expresses a number of virulence factors that include LasA and LasB elastases, the Type III secretion system, pyocyanin, phospholipases, and alkaline proteases (5). Virulence factors act collectively to enable pathogens to invade the host and bypass the innate and adaptive immune response that the host has in place. The LasA and LasB elastases, for example, are able to disrupt cytoskeletal structure of host tissues, leading to their degradation (6). These potent proteases are two of the main virulence factors that are produced in *P. aeruginosa*, and understanding the regulation of these factors will allow for the proper development of therapeutic strategies against the pathogen.

The ability for *P. aeruginosa* to sense and respond to their surroundings is critically important for its patho-evolution and long-term survival in the host. Quorum sensing molecules are used for cell-to-cell communication in *P. aeruginosa* and are encoded by the synthases *lasI, rhlI* and *pqsA,* which are under the transcriptional regulation of the corresponding regulators, *lasR, rhlR,* and *pqsR* (12).

These molecules control the cell density-dependent mechanisms that *P. aeruginosa* uses to respond to its community, which include the production of virulence factors allowing for the invasion of the pathogen into the host (5). The quorum sensing systems allow the bacterium to sense the density of its own cells, and respond by adjusting the expression of certain genes, including the genes of the quorum sensing systems that together strengthen *P. aeruginosa* pathogenicity in its host (5).

P. aeruginosa produces at least three different signals that drive quorum sensing: the LasI:LasR (LasIR), the RhlI:RhlR (RhlIR), and the *Pseudomonas* quinolone signal (PQS) systems. LasI and RhlI are both acyl-homoserine lactone synthases, while PqsA is the *Pseudomonas* quinolone synthase, characterized as a 3-hydroxy-4 quinolone (7,8). The LasI synthase produces N-3-oso-dodecanoyl-homoserine lactone (3OC12-HSL), and its production is increased as cell density increases (7). As 3OC12-HSL is produced by LasI, it binds to a transcription factor, LasR, and activates the promoters of target virulence genes. The other signal synthase, RhlI, produces N-3-oxo-dodoecanoyl-homoserine lactone (C4-HSL), which binds to RhlR in order to activate the expression of similar target genes. PqsA is a unique quorum sensing synthase, and has been shown to control the production of some of the main virulence factors in *P. aeruginosa*, including LasB elastase, pyocyanin, and LecA (11). It was also characterized to depend on the production of the LasIR and RhlIR quorum sensing systems (9,12).

Calcium ions play a critical role in the human body, and have major regulatory effects in eukaryotic cells. *P. aeruginosa* infections are extremely potent in cystic fibrosis patients, and since these patients have an abnormally high concentration of calcium in their nasal and pulmonary fluids (10, 15), calcium ions were hypothesized to play a critical role in regulating the virulence of *P. aeruginosa*. Our lab has generated experimental evidence confirming that calcium affects the growth of biofilms in *P. aeruginosa*. We showed that with the addition of calcium, the resulting biofilms were 10- to 20- fold thicker than those formed without calcium (10). We also showed that calcium regulates the production of virulence factors in the forms of exotoxins, lipases, secreted proteases, pyocyanin, siderophores, and rhamnolipids (10). Based on these data, we have hypothesized that calcium regulates at least one of the QS systems. The goal of my work was to characterize the regulatory impact of calcium on the three QS systems in *P. aeruginosa*.

Previously, we have determined that the calcium signaling system in *P. aeruginosa* is composed of several key components. These include the calcium channel (CalC), the EF-hand domain (EfhP), and the two-component regulatory system (CarRS) (10, 17, 18). My study aimed to characterize the role of these components in calcium regulation of the expression of the three QS systems. For this, I used promoter activity assays for the genes encoding the synthases *lasI, rhlI*, and *pqsA*. To evaluate the impact of calcium, I tested the promoter activities in the wild type strain, PAO1. To determine the regulatory role of CalC and EfhP, a similar analysis was

performed in the deletion mutants, each lacking one of the calcium signaling components. These experiments allowed me to pin-point which specific protein in the calcium signaling network is responsible for the regulation of QS in *P. aeruginosa*. This is important because if we understand the mechanisms of regulation, therapeutic strategies can be instrumented to target the specific protein, and control the virulence of *P. aeruginosa* in patients suffering from its infections.

METHODS AND MATERIALS

Bacterial Cultures and Growth

Table 1 lists bacterial plasmids and strains that were used in this series of experiments.

A biofilm minimal medium (BMM) was used to grow the bacterial cultures. The BMM was made by combining 9.0 mM sodium glutamate, 50 mM glycerol, 0.02 mM $MgSO_4$, 0.15 mM NaH2PO4, 0.34 mM K2HPO4, 145 mM NaCl, 200 µL trace metals and 1 mLvitamin solution for every liter of solution. The trace metals solution contained 5.0 g CuSO4.5H2O, 5.0 g ZnSO4.7H2O, 5.0 g FeSO4/7H2O, and 2.0 g MnCl2.4H2O for every liter of 0.83 M HCl. The vitamin solution consisted of 0.5 g thiamine and 1 mg biotin per liter of solution. Once the ingredients were put together, the BMM's pH was adjusted to 7.0 using HCl.

Lysogeny broth (LB) agar plates were used to grow the bacterial strains of interest to then inoculate pre-cultures for a promoter activity assay. To make the LB agar solution, 15 g agarose, 10 g tryptone, 10 g NaCl, and 5 g yeast extract were combined with 1 liter of nanopure water. The solution was then autoclaved and cooled down to about 55℃. When needed, a total concentration of 50 µg/mL Kanamycin (Kan) or 1.5 mg/mL Trimethoprim (Tmp) antibiotic was added into the solution before pouring it into sterilized petri dishes.

Table 1:

P. aeruginosa Strains	Description	Source
PAO1	Wild-Type strain	(13)
ΔcalC	PAO1 cell lacking *calC* gene	(15)
ΔefhP	PAO1 cell lacking *efhP* gene	(16)

Plasmids	Description	Source
pMS402	Promoter-less *luxCDABE*	(14)
pKD201	pMS402 with *lasI* promoter	This study
pKD202	pMS402 with *rhlI* promoter	This study
pKDpqsA	pMS402 with *pqsA* promoter	This study

Making Competent Cells for PAO1, ΔcalC, ΔefhP Mutants

The PAO1 competent cells were prepared by first taking the PAO1 frozen stock and streaking it onto an LB agar plate. A small amount of bacteria was then inoculated into a test tube containing 4 mL of LB medium and placed in a 37℃ incubator. After 12-16 hours of incubation, 1 mL of this culture was transferred into a microcentrifuge tube and centrifuged for 10 minutes, at 6.0 speed and 4℃. The supernatant was removed and 1 mL of electrophoresis buffer (10.3 g of 300 mM sucrose, 0.68 g of 5 mM KH_2PO_4, 100 mL H_2O) was added to wash cells, and centrifuged at the same conditions for 5 min, and the supernatant was once again removed. This was repeated with 700 µL of the buffer and then again with 500 µL of the buffer. After the final round of centrifugation, the supernatant was removed and 100 µL of electrophoresis buffer was added before putting the tube into the -80℃ fridge. This same procedure was repeated for the *calC* and *efhP* deletion mutants.

Transformation of Plasmids into Competent Cells

A vial with PAO1 competent cells was taken out of the -80℃ fridge and put on ice for ~20 minutes to thaw. Once thawed, 30 µL of the PAO1 cells was allocated into three microcentrifuge tubes and 2 µL of an empty plasmid (used as a vector control) was added into one of the tubes, while a plasmid containing the promoter region *lasI* was added to the second tube, one containing *rhlI* promoter region was added the third, and one containing the *pqsA* promoter region was added to the last tube. These samples were placed back into the ice for another ~20 minutes. While the samples were on ice, an electroporator was set up and 4 new microcentrifuge tubes were labeled with either the VC (vector control), *lasI, rhlI*, or *pqsA* plasmid names. Each tube contained 500 µL of SOC (5 mL of 1M $MgSO_4$, 10 mL of 20% glucose, 10 mL of 500mM $MgCl_2$) medium. The electroporator was set to the EC1 setting since 1 cm cuvettes were being used. The competent cell and plasmid sample were mixed, put into the cuvette, and shocked with a voltage of 1.8 kV for a time of 2.50 msec.

The SOC medium was poured into the cuvette immediately to make sure that the cells are able to find nutrients to survive after the shock. The cuvette's contents were transferred into the plasmid's labeled microcentrifuge tubes. This was repeated with each of the plasmids and then all of the tubes were incubated at 37℃ for 1 hour.

After incubation, 30 µL of the microcentrifuge tubes' contents were added onto LB-Tmp agar plates and spread across the plate. The plates were then incubated until isolated colonies were visible. In a test tube containing 3 mL of LB medium and 90 µL of 50 mg/mL Trimethoprim stock (for a concentration of 1.5 mg/mL in LB medium), one isolated colony was inoculated and incubated at 37℃ for

approximately 16 hours. To make a frozen stock, 750 µL of the incubated culture and 750 µL of skim milk were combined in a special frozen stock tube, the tube was labeled and placed in the -80℃ fridge.

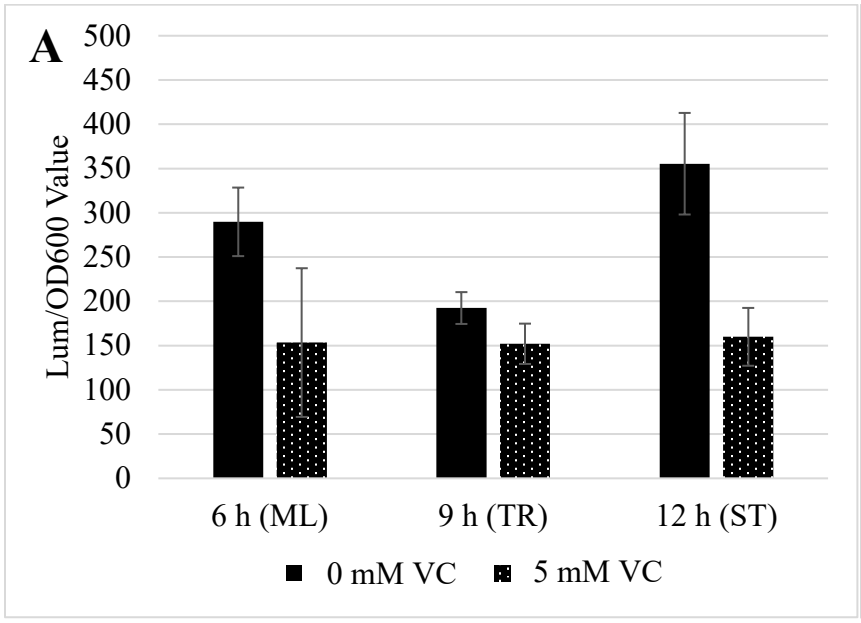
Promoter Activity Assay

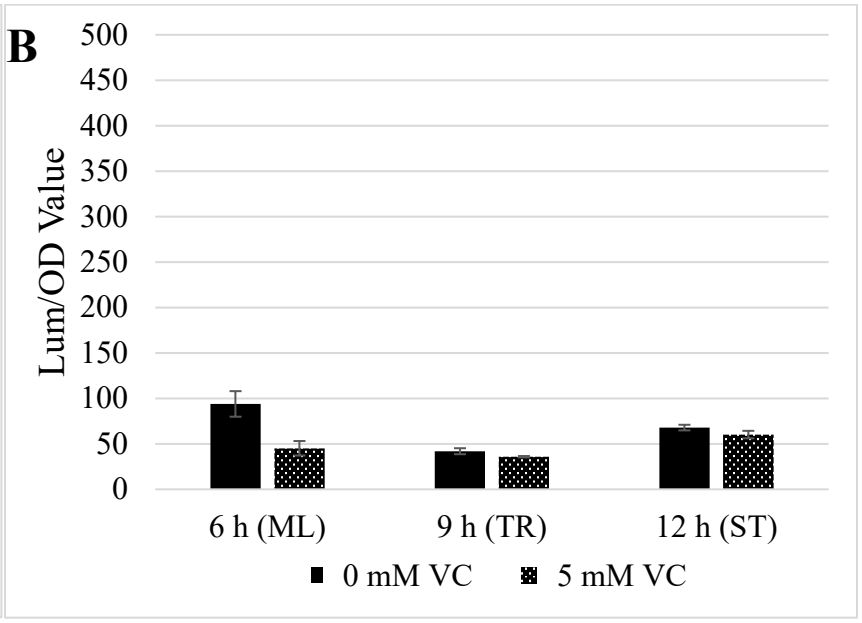
In order to evaluate the activity of the promoter regions of interest, a promoter activity assay was utilized that is based on the luminescence produced by the *lux* system cloned downstream of the promoter of interest. For the preparation of the cultures, the strain of interest was first streaked onto an LB agar plate and incubated at 37℃ overnight or until good growth was observable. A streak of the grown culture was then inoculated into test tubes filled with 3 mL of BMM and incubated at 37℃ for 12 hours exactly. Four biological replicates were made for each experiment. Once the test tubes were incubated for 12 hours, OD_{600} of the cultures was measured and used to normalize the cultures to 0.300 OD. If the preculture's OD was less than 0.300 or more than 0.800, the replicate was not used. After normalization of the cultures, 10 uL of the culture were added to 990 µL BMM that had either 0 mM or 5 mM $CaCl_2$ added in microcentrifuge tubes. The Biotek spectrometer was monitoring the luminescence produced by these cultures and cell density (OD_{600}) for 14 hours every 30 minutes. Once completed, I exported the data into an excel spreadsheet and plotted the averaged luminescence values of all the replicates at every time point. The same process was applied to the OD values that were measured throughout the 14 hours and plotted onto a line graph with standard error. Finally, each time point's luminescence value was divided by its respective OD_{600} value. This is done to ensure that the luminescence data are normalized by the number of cells measured by OD_{600}. The Lum/OD data was plotted and standard errors between biological replicates were estimated. The statistical significance was evaluated by Student T-test.

RESULTS

In order to determine how calcium affects the regulation of the genes encoding the QS synthases LasI, RhlI, and PqsA in *P. aeruginosa*, we used a promoter activity assay and measured the luminescence produced by a *lux* system cloned downstream of the corresponding promoters in PAO1 cells exposed to either 0 or 5 mM of calcium.

Data resulting from promoter activity assays for the *lasI*, *rhlI*, or *pqsA* promoter was plotted as the luminescence values normalized by the optical density (OD_{600}). As a control, I used a promoter-less vector construct pMS420 transformed into PAO1 and the deletion mutants lacking *calC* and *efhP*. Figure 1 illustrates the amount of normalized luminescence produced, here referred to as promoter activity, at various time points, including the mid-log (ML) growth phase (~6 hours), the transitional (TR) phase (~9 hours), and the stationary (ST) phase (~12 hours). These time points were determined based on analyzed growth curves for each experiment, as shown in Figure 2. The same procedure was done for the three main quorum sensing genes, *lasI*, *rhlI*, and *pqsA,* which are represented by the pKD201 strain, the pKD202 strain, and the pKDpqsA strain, respectively. The luminescence normalized by OD_{600} was below 350 establishing the "no activity" level for all the comparisons. Standard error bars were included in the graphs, and a student t-test was performed when applicable. The time points were selected to represent the three phases of growth, mid-log, transitional, and stationary (Fig. 2).





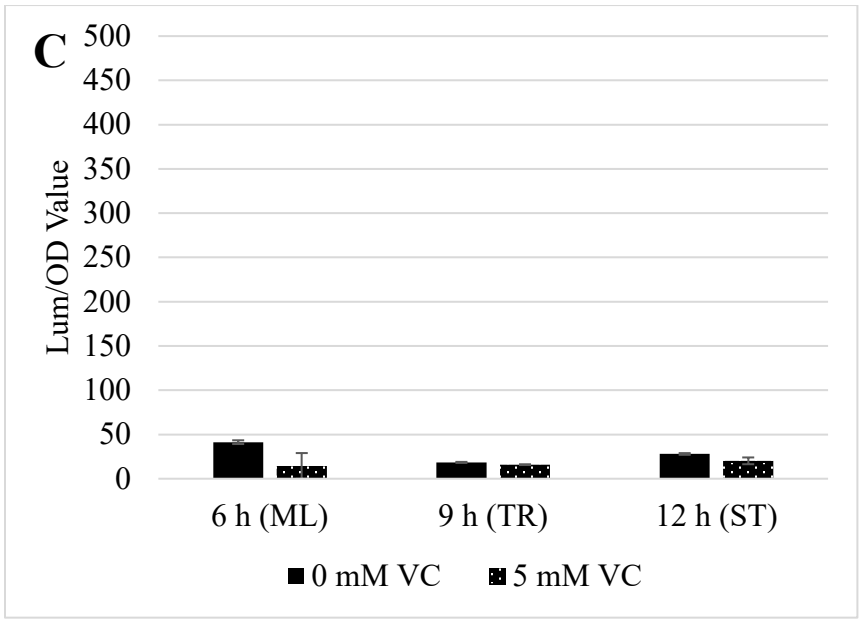


Figure 1. Promoter-less vector control (VC). The vector pMS420 was transformed into PAO1 (A), *Δ calC* (B), and *Δ efhP* (C). The cultures were grown in BMM in 96 well plate and their luminescence and OD_{600} were monitored by Biotek. The averaged levels are plotted with standard deviation.

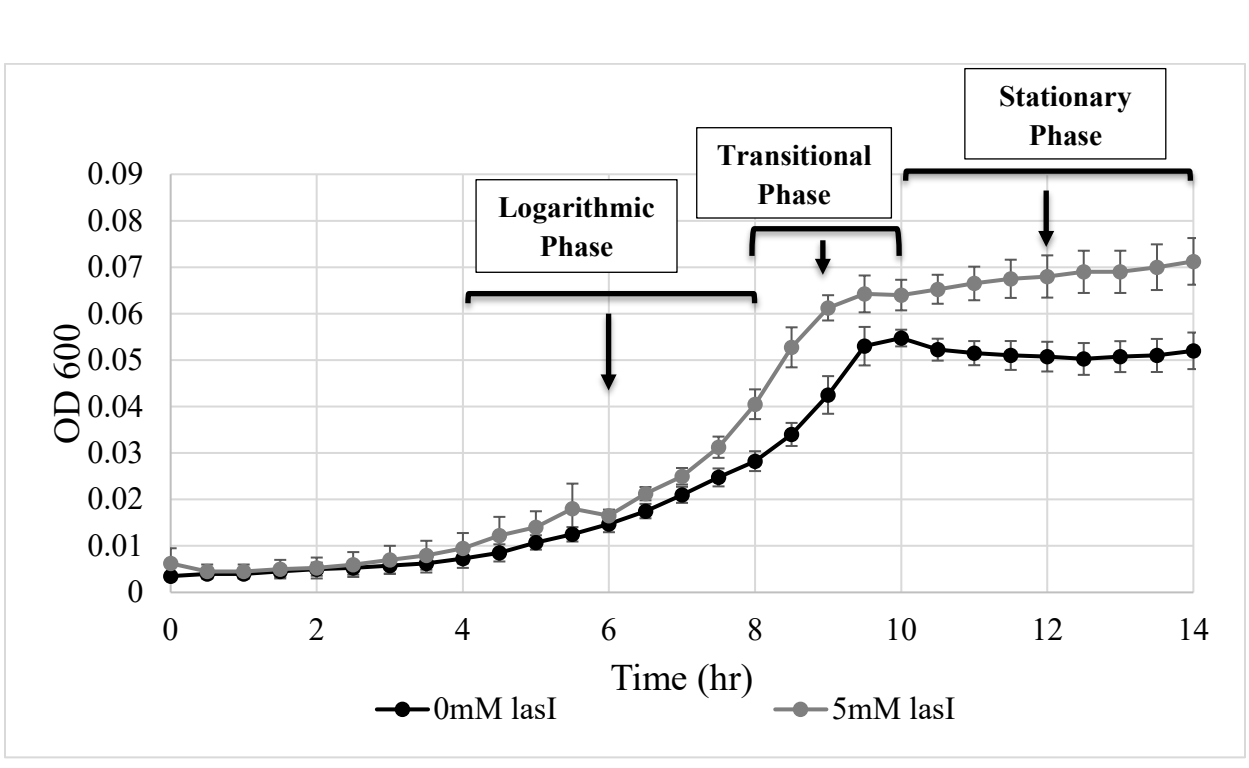
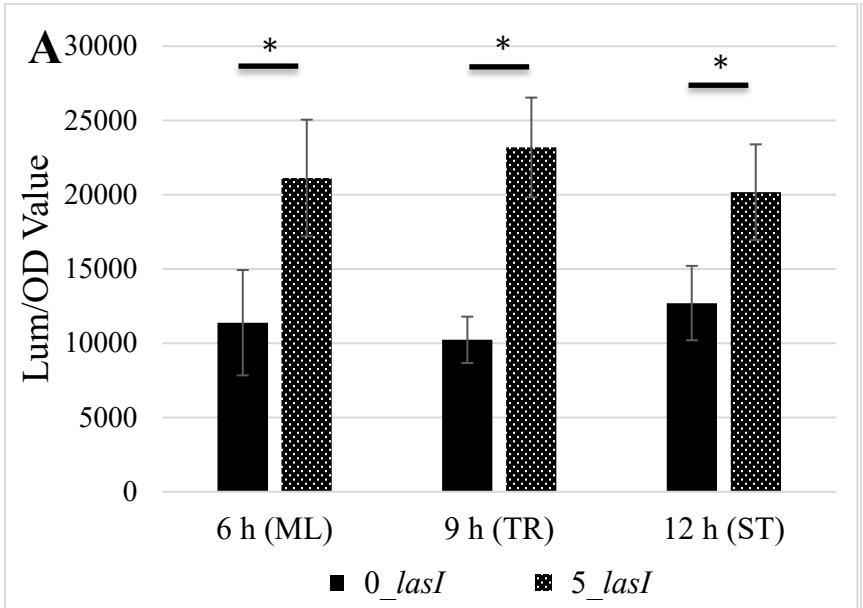
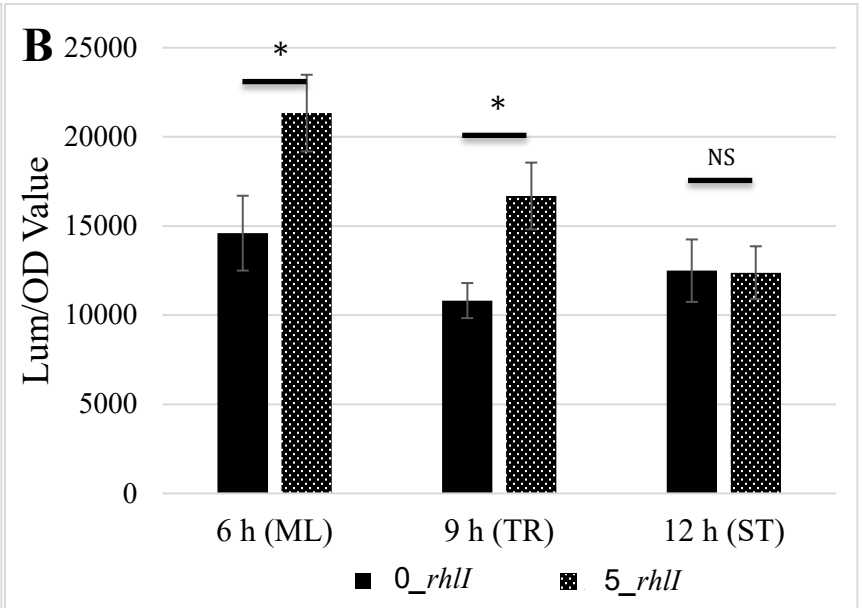


Figure 2*:* Depicted is an example of a growth curve with its growth phases, logarithmic (ML), transitional (TR) and, stationary (ST), labeled. These are the phases focused on in the study, and the arrows are pointing at the time points used to represent the ML, TR, and ST phases (6 h, 9 h, and 12 h, respectively). The time points can be slightly altered based on the growth patterns of different samples, therefore each experiment's data went through its own analysis.

Calcium regulates the expression of genes lasI, rhlI, and pqsA in P. aeruginosa

We observed a significant increase in the PAO1(*PlasI-lux*) promoter activity in the presence of 5 mM calcium at all three time points (Fig 3A). The PAO1(*PrhlI-lux*) cultures, when exposed to a higher concentration of calcium, experienced an increase in the promoter activity during the ML and TR phases, however, did not show a significant difference at the designated ST time point (Fig 3B). The PAO1(*PpqsA-lux*) samples showed an increase in promoter activity with the exposure to added calcium during ML, but there was no significant difference between the promoter activities of the two conditions at the TR phase (Fig 3C). Additionally, the PAO1(*PpqsA-lux*) sample showed a significantly lower promoter activity level at a higher calcium concentration at ST phase (Fig 3C).





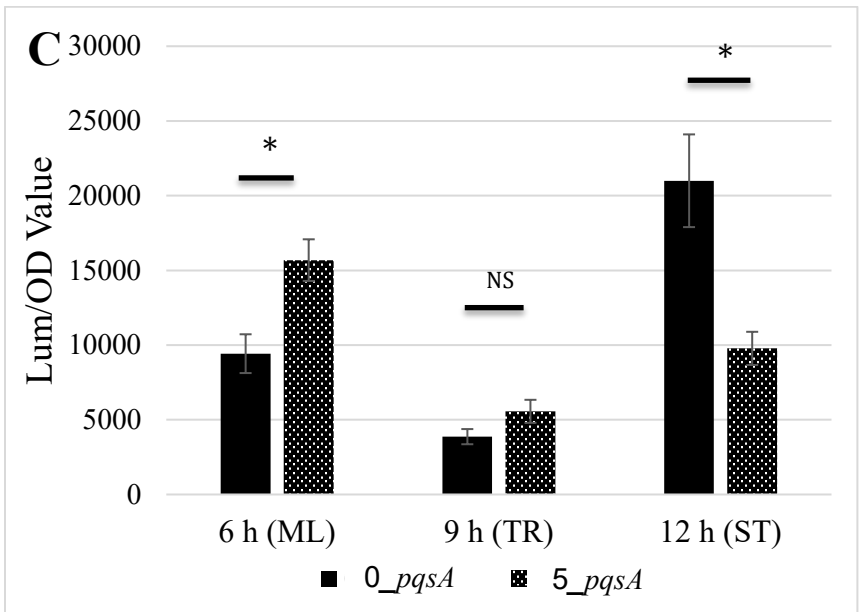
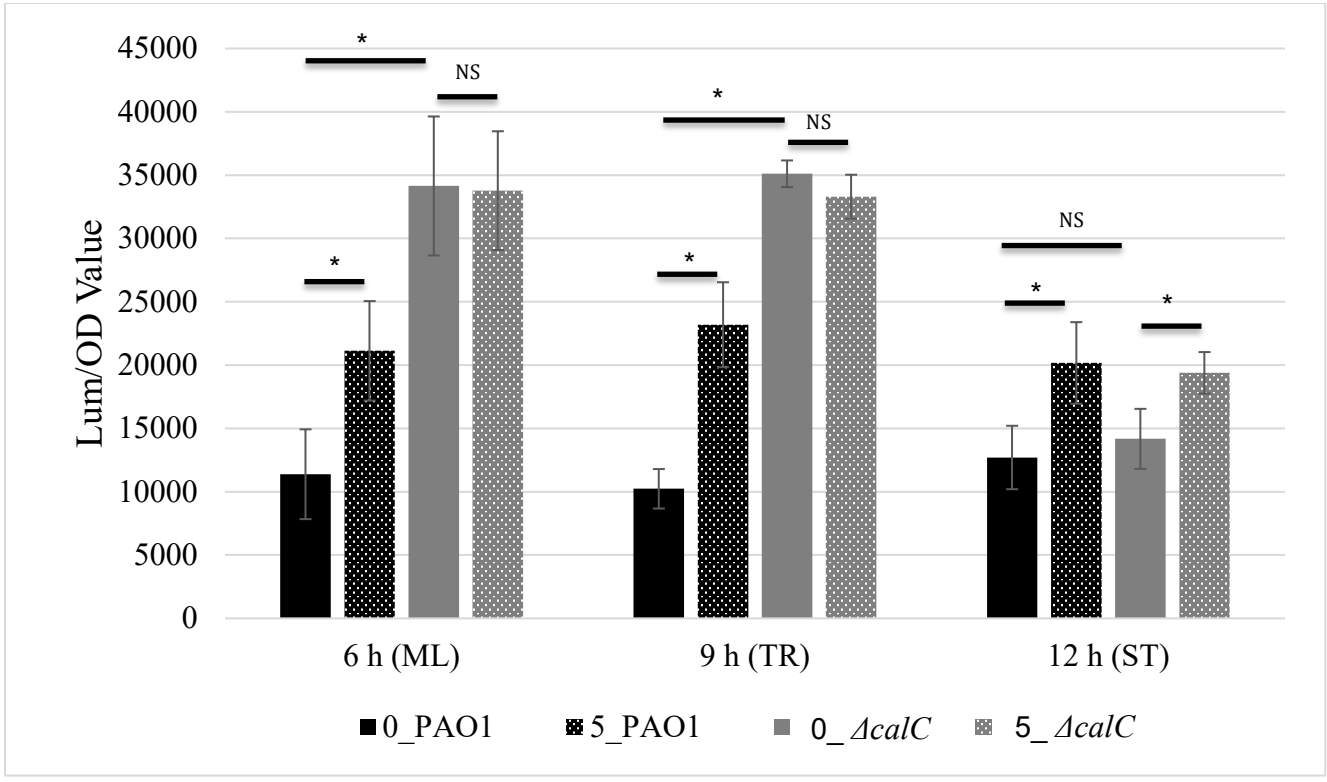


Figure 3: Promoter activity assays of the PAO1 samples. PAO1(*PlasI-lux*), PAO1(*PrhlI-lux*), and PAO1(*PpqsA-lux*) were grown in either 0- or 5-mM calcium condition at 37 for 14 hours while the Biotek machine measured their optical density (at 600 nm) and luminescent count every 30 minutes. Lum/OD values are plotted to show the amount of luminescence produced relative to how many cells are present at each time point. 3 biological replicates were used and standard error is depicted by error bars. Student t-tests were performed between calcium condition groups.

LasI gene loses its calcium sensitivity in the absence of the calcium channel CalC in P. aeruginosa

The deletion mutant, *ΔcalC,* is lacking the calcium channel, which is a part of the calcium signaling system in *P. aeruginosa*. We have used the mutant to study the role of CalC in calcium regulation of the QS synthases' promoter activities. The *ΔcalC*(*PlasI-lux*) cultures did not show a significant difference between the amount of luminescence produced at 0 mM and 5 mM calcium (Fig 4). This was observed at each of the time points, except for the ST phase, where there was a slightly higher promoter activity level recorded at 5 mM calcium condition. Compared to the samples in the wild type PAO1, the deletion mutant caused a relief from the effects that calcium had on the *lasI* promoter activity (Fig 4). On the other hand, there was an overall significant increase in the production of luminescence in the *ΔcalC*(*PlasI-lux*) samples, when compared to the wild type samples, independent of calcium condition. This implies that the calcium channel, CalC, has a negative impact on the activation of the *lasI* promoter region during *P. aeruginosa* infection.



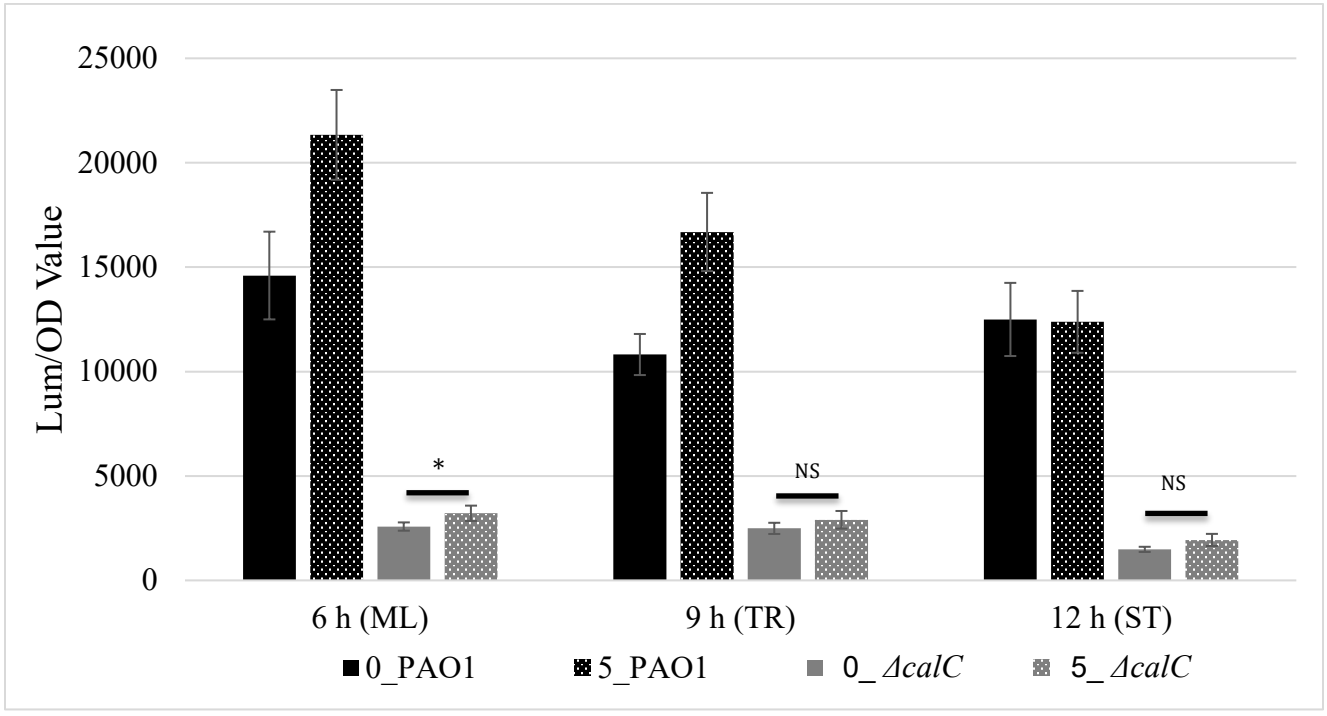
* = *significant by student t-test* *NS = not significant by student t-test*

Figure 4: PAO1(*PlasI-lux*), and *ΔcalC*(*PlasI-lux*) samples were grown in either 0- or 5-mM calcium condition at 37°C for 14 hours while the Biotek machine measured their optical density (at 600 nm) and luminescent count every 30 minutes. Lum/OD values are plotted to show the amount of luminescence produced relative to how many cells are present at each time point. 6, 9, and 12 hours are plotted to represent three different growth phases: Mid-Log (ML), Transition (TR), and Stationary (ST) phases. Each data point represents three biological replicates, and standard error was calculated and is depicted by error bars. Student t-test was also performed to indicate significant results between certain groups, where a value <0.05 was deemed significant. As labeled on the graph, the solid black bars correspond to PAO1(*PlasI-lux*) grown without any added calcium, while the patterned black bars correspond to the strain grown under 5 mM of added calcium. The solid gray bars correspond to *ΔcalC* (*PlasI-lux*) grown without any added calcium, while the patterned gray bars correspond to the strain grown under 5 mM of added calcium.

RhlI gene expression is dependent on the presence of the calcium channel protein in P. aeruginosa

Compared to the wild type samples (PAO1(*PrhlI-lux*)), *ΔcalC*(*PrhlI-lux*) showed roughly a 5-fold decrease in the luminescence produced by the 0 mM calcium condition group, and around a 6-fold decrease for those under the 5 mM calcium condition (Fig 5). *ΔcalC*(*PrhlI-lux*) displayed no significant differences between the promoter activities in the samples at 0 mM and 5

mM calcium, at the TR and ST phases. There was, however, a slight, significant increase in the promoter activity of *rhlI*, when the samples were exposed to added calcium at ML (Fig 5).

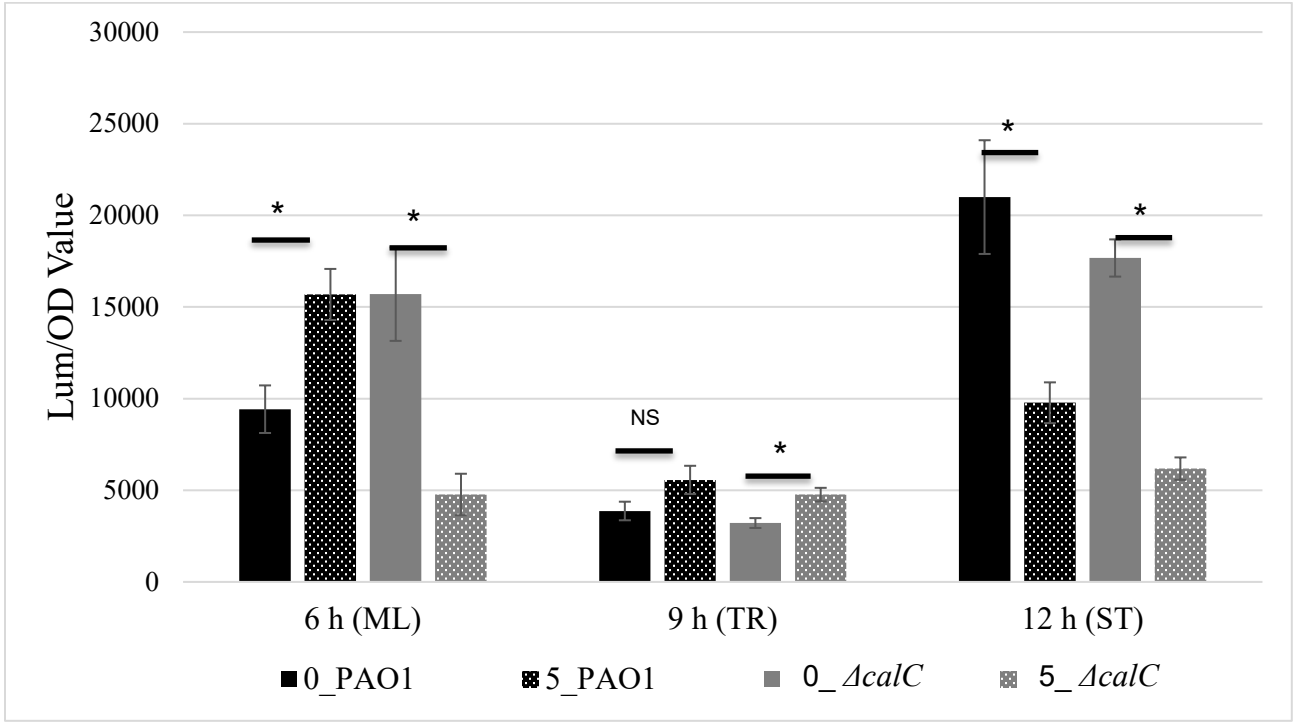


** = significant by student t-test* *NS = not significant by student t-test*

Figure 5*:* PAO1(*PrhlI-lux*), and *ΔcalC*(*PrhlI-lux*) samples were grown in either 0- or 5-mM calcium condition at 37℃ for 14 hours while the Biotek machine measured their optical density (at 600 nm) and luminescent count every 30 minutes, separately. Lum/OD values are plotted to show the amount of luminescence produced relative to how many cells are present at each time point. 6, 9, and 12 hours are plotted to represent three different growth phases: Mid-Log (ML), Transition (TR), and Stationary (ST) phases. Each data point represents three biological replicates, and standard error was calculated and is depicted by error bars. Student t-test was also performed to indicate significant results between certain groups, where a value <0.05 was deemed significant. As labeled on the graph, the solid black bars correspond to PAO1(*PrhlI-lux*) grown without any added calcium, while the patterned black bars correspond to the strain grown under 5 mM of added calcium. The solid gray bars correspond to *ΔcalC* (*PrhlI-lux*) grown without any added calcium, while the patterned gray bars correspond to the strain grown under 5 mM of added calcium.

Calcium regulation of pqsA gene is reversed in the mid-log phase of growth when the calcium channel is removed from cells

The data from *ΔcalC*(*PpqsA-lux*) samples showed no significant difference in the TR and ST phases, when compared to the Wild-Type samples, however a reverse in the effect of added calcium was seen in ML (Fig 6). At ML, *ΔcalC*(*PpqsA-lux*) samples saw a significant decrease in its promoter activity level when exposed to added calcium. However, the samples still saw very little change in the TR with added calcium, and a significant decrease in the promoter's activity with added calcium in ST (Fig 6).



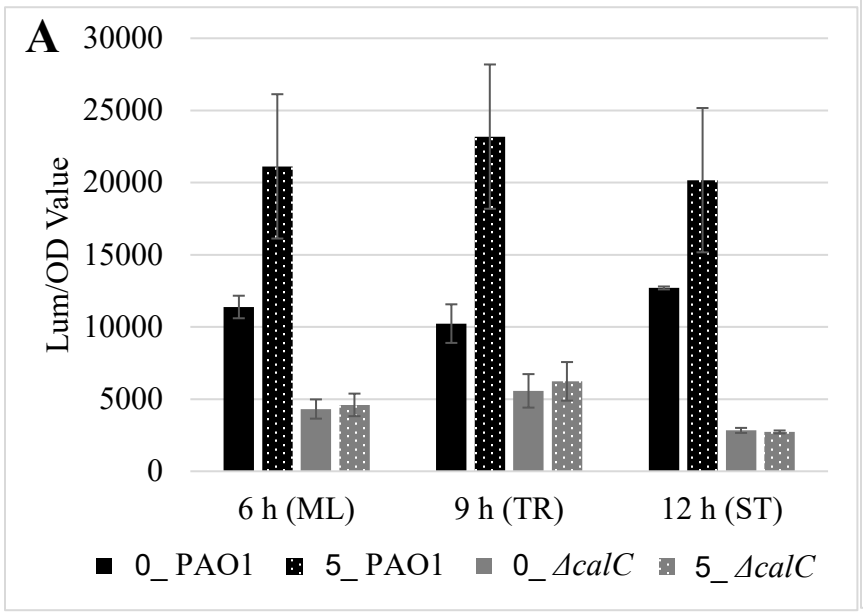
* = *significant by student t-test* *NS = not significant by student t-test*

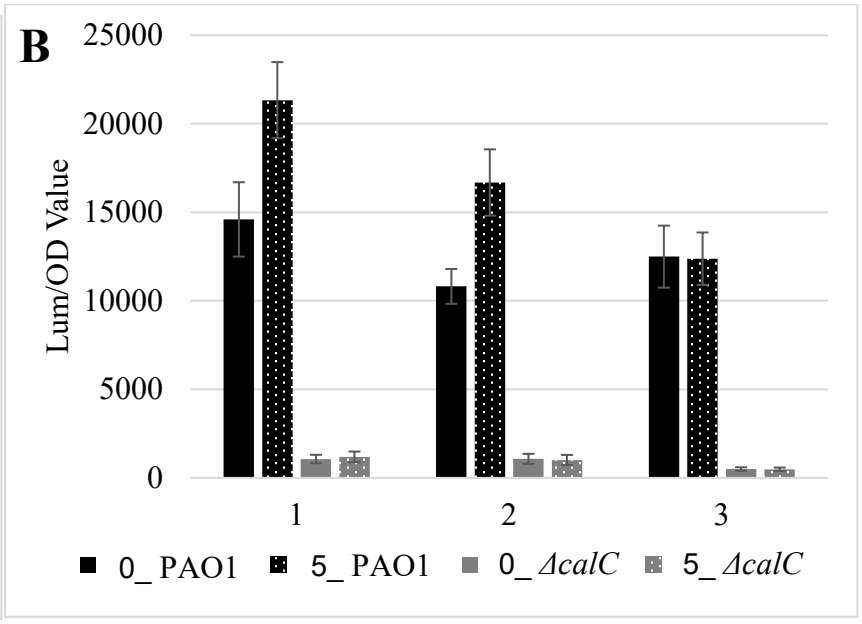
Figure 6: PAO1(*PpqsA-lux*), and *ΔcalC*(*PpqsA-lux*) samples were grown in either 0- or 5-mM calcium condition at 37°C for 14 hours while the Biotek machine measured their optical density (at 600 nm) and luminescence count every 30 minutes, separately. Lum/OD values are plotted to show the amount of luminescence produced relative to how many cells are present at each time point. 6, 9, and 12 hours are plotted to represent three different growth phases: Mid-Log (ML), Transition (TR), and Stationary (ST) phases. Each data point represents three biological replicates, and standard error was calculated and is depicted by error bars. Student t-test was also performed to indicate significant results between certain groups where a value <0.05 was deemed significant. As labeled on the graph, the solid black bars correspond to PAO1(*PpqsA-lux*) grown under no added calcium, while the patterned black bars correspond to the

strain grown under 5 mM of added calcium. The solid gray bars correspond to *ΔcalC*(*PpqsA-lux*) grown under no added calcium, while the patterned gray bars correspond to the strain grown under 5 mM of added calcium.

Calcium sensor, EfhP, has a positive impact on the activation of the three promoter regions, lasI, rhlI, and pqsA

To study the role of calcium sensor EfhP in the regulation of QS synthases' promoter activities in response to calcium, we used a deletion mutant lacking the encoding gene *efhP.* SO far, I have run the experiment once and we consider the results as preliminary. To validate the results, I plan to repeat the experiment two more times. The promoter activity assays of *ΔefhP*(*PlasI-lux*), *ΔefhP*(*PrhlI-lux*), and *ΔefhP*(*PpqsA-lux*) cultures showed a significant decrease in the activations of their promoter regions in comparison to the level of promoter activity seen in the wild type PAO1 samples (Fig 7). The PAO1(*PlasI-lux*) 0 mM calcium data was plotted along with the *ΔefhP* data to serve as a scale, in order to demonstrate this significant decrease.





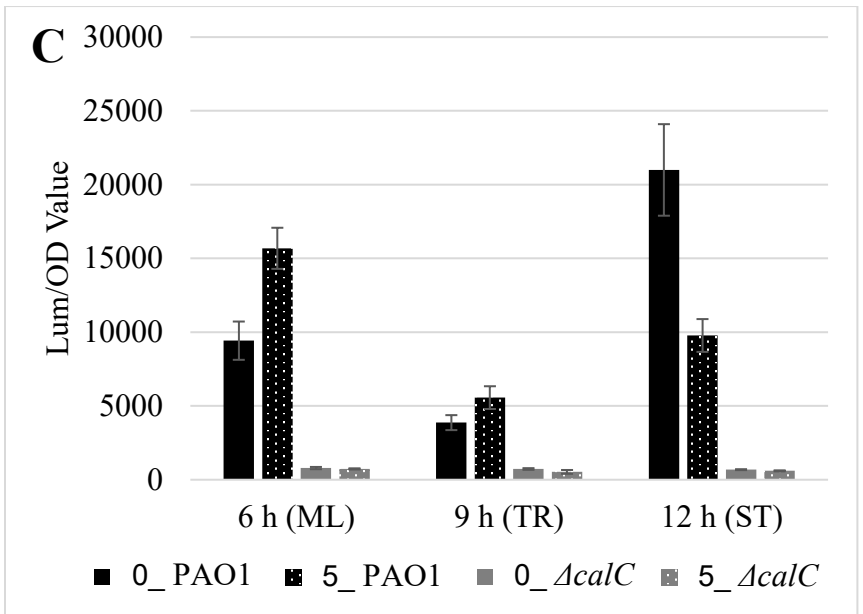


Figure 7: *efhP* mutant data compared to wild type. PAO1(*PlasI-lux*) and *ΔefhP(PlasI-lux*) plotted by their Lum/OD values (A). PAO1(*PrhlI-lux)* and *ΔefhP(PrhlI-lux)* plotted by their Lum/OD values (B). PAO1(*PpqsA-lux*) and *ΔefhP(PpqsA-lux)* plotted by their Lum/OD values (C). Standard error was estimated for the three replicates used in each sample.

DISCUSSION

The virulence of *P. aeruginosa* infections is characterized by the release of virulence factors, which can cause extensive damage to host cells, or perform mechanisms that further the life span of pathogenic cells, such as evading the immune system, and inhibiting phagocytosis of the bacterium (6). The production of these virulence factors is mainly regulated by *P. aeruginosa's* quorum sensing system, which is a cell-density dependent communication system (5). This system relies on molecules that exit the cell to provide the signal to the neighboring cells. The *lasI, rhlI*, and *pqsA* genes encode for enzymes that catalyze the production of the corresponding quorum sensing molecules, therefore, the regulation of these genes tends to impact the overall virulence of the bacterium. Calcium ions play a big role in many pathways in the human body, and this stays true in the pathogenicity of *P. aeruginosa* (10). Therefore, we hypothesized that the bacterium's calcium signaling system contributes to the regulation of these quorum sensing molecules by calcium. Our group has determined that the calcium signaling system include three main components: the calcium channel (CalC), the two-component calcium regulatory system (CarRS), and the EF-hand calcium sensor (EfhP) (10, 17, 18). Determining the role that each of these proteins have in the regulation of the genes *lasI, rhlI*, and *pqsA* will allow for a better understanding of the mechanisms that *P. aeruginosa* undergoes during infection in cystic fibrosis patients, and the production of effective therapeutic strategies.

This study characterized the calcium regulation that occurs in the gene expression of *lasI, rhlI*, and *pqsA*. An increase in calcium concentration caused an induction in the activation of the *lasI* promoter region. When grown in 5 mM of calcium, we saw a 2-fold increase in the promoter activity by the samples in the wild type PAO1 cells. This activation was consistently seen across the three time points (mid-log, transition, and stationary). The *rhlI* promoter region was also activated by added calcium, to produce nearly a 2-fold increase in the promoter's activity. This was observed only in the mid-log and stationary phases, however, there was no significant difference in the activation of the promoter in the stationary phase. In regards to the *pqsA* promoter activity, there was a 2-fold increase in the activation of the promoter region in the mid-log phase, however, there was no significant difference between the luminescence produced between the two calcium conditions in the transition phase. Furthermore, there was a significant reduction in the activity of the *pqsA* promoter region when grown in the 5 mM calcium condition at the stationary phase, which implies that the calcium regulation appearing in the mid-log time point did not persist throughout the cell's growth curve. The quorum sensing system are self-regulated in cell-density dependent manner. Therefore, when the cell density reaches a certain point, the activation of the

system reaches its maximum, which may explain why the *rhlI* and *pqsA* promoter regions did not see a calcium induction in their activation at the stationary phase. Based on these data, the quorum sensing genes *lasI*, *rhlI*, and *pqsA* contain promoters that are activated by an increase in concentration of calcium, however, this induction is mostly observed in their mid-log phase of growth.

The promoter activity data measured in the *calC*-deletion mutant revealed that the calcium channel (CalC) plays role in calcium regulation of the QS systems. Thus, the *lasI* promoter region lost its calcium responsiveness in the *calC*-deletion mutant. This tells us that without this channel, when calcium ions were not able to enter the bacterial cell and generate the intracellular calcium, *lasI* is not regulated by calcium. In addition, we observed an overall increase in the level of the promoter's activity, independent of calcium concentration, in the deletion mutant. This unforeseen result shows that there may be an upper level of regulation occurring with the *lasI* gene, such as the activation of gene repressors with an increase in calcium ion entry. The *rhlI* promoter, inserted into the *calC*-deletion mutant, had a promoter activity that also lacked calcium responsiveness, when compared to the wild type samples. Additionally, the luminescence produced by these samples were drastically lower than those in the wild type. This defined the importance of CalC-dependent changes in the intracellular calcium in regulating the expression of the *rhlI* gene. Repressors may be inhibited by calcium ions, which would explain why the gene is more highly expressed in the presence of the calcium channel. The *pqsA* promoter's activation was, interestingly, reduced by calcium when the construct was introduced into the *calC*-deletion mutant. This was the only difference observed between the wild-type and deletion mutant data for *pqsA*, implying that CalC-dependent regulation only occurs in the mid-log phase, but is lost at the later phases of growth.

The first round of experiments in the deletion mutant lacking the EF-hand calcium sensor, EfhP, showed that the *lasI*, *rhlI* and *pqsA* promoter regions produced much lower levels of luminescence than in the wild type, while simultaneously losing their calcium responsiveness. This implies that the EF-hand calcium sensor is needed in the cell for an impactful activation of the *lasI, rhlI*, and *pqsA* genes.

Future direction for this project would be to replicate the *ΔefhP* promoter activity assays, in order to confirm my preliminary results. I would also like to validate my data using RNA sequencing and quantitative RT-PCR testing. The *ΔcarR* promoter activity assays on the expression of *lasI, rhlI*, and *pqsA* will be completed in our next steps as well, so that its role in calcium regulation, and in the expression of these genes, can also be characterized in *P. aeruginosa*. An analysis of repressors related to the three quorum sensing genes will also be performed. This should be able to help discover the explanation behind the regulation that the calcium channel and EF-hand domain have on the gene expression of the *lasI*, *rhlI*, and *pqsA* genes.

CONCLUSIONS

This study found that calcium regulates the expression of the genes *lasI, rhlI*, and *pqsA* in *P. aeruginosa.* The *lasI* gene expression lost its calcium responsiveness upon deletion of *calC* encoding a calcium channel protein that allows for the cellular uptake of calcium. The *rhlI* gene expression is dependent on the presence of CalC in *P. aeruginosa,* which is shown through the drastic decrease in the *rhlI* promoter activity in the deletion mutant. There was also a lack in calcium regulation of the *rhlI* gene in the *calC*-deletion mutant. The *efhP*-deletion mutant data has described the necessity of the calcium sensor in the cell for the expression of these genes, because in its absence, the promoters were not nearly as activated as in the wild type cells.

Our data suggest that all three QS respond to calcium and that both signaling components CalC and EfhP play a major role in the regulation of *P. aeruginosa's* QS synthases genes, and inhibiting the synthesis of any of these proteins could potentially resolve the increased virulence of the bacterium found in patients with cystic fibrosis, as well as any individual with abnormally high levels of calcium found in their bodies.

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