The role of calcium sensor EfhP in regulation of virulence in *Pseudomonas aeruginosa*

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Honors Thesis

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that causes severe chronic infections in the lungs of Cystic Fibrosis (CF) patients, where elevated levels of calcium (Ca^{2+}) are commonly detected. Our group has discovered that elevated Ca^{2+} increases antibiotic resistance, enhances biofilm formation, and induces the production of several virulence factors in *P. aeruginosa*. We also identified several components of Ca^{2+} regulatory network, which included a novel putative Ca^{2+} sensor, which we designated EfhP based on the encoded Ca^{2+}-binding EF hand domains. We hypothesized that EfhP mediates Ca^{2+} regulation of the pathogen ability to cause disease in an animal host as we earlier showed in plants. To test this hypothesis and define the role of EfhP in Ca^{2+}-dependent virulence of this pathogen, we used *Galleria mellonella* wax worm as an animal model. We injected the wax worms with the wild type and *efhP* deletion mutant that were grown either in the presence or absence of 5 mM Ca^{2+}. The results showed that growth in the presence of Ca^{2+} enhanced the ability of *P. aeruginosa* to kill in the animal model. We also showed that *efhP* plays in role in virulence, in that upon removal of the gene less worms were killed compared to the wildtype. In addition, we assessed the transcriptional regulation of *efhP* in response to Ca^{2+} and Fe^{2+} using *efhP* promoter construct. We determined that there is potential regulatory connection between Ca^{2+} and Fe^{2+} in the transcription of *efhP*. Additionally, we identified several proteins that have a role in regulating *efhP* transcription, specifically CarP, CarR and BfmS. These findings pertaining to transcriptional regulation of *efhP* will allow for a better understanding on how to inhibit EfhP functions. This knowledge is essential for future development of strategies and approaches for preventing or controlling *P. aeruginosa* infections.

INTRODUCTION

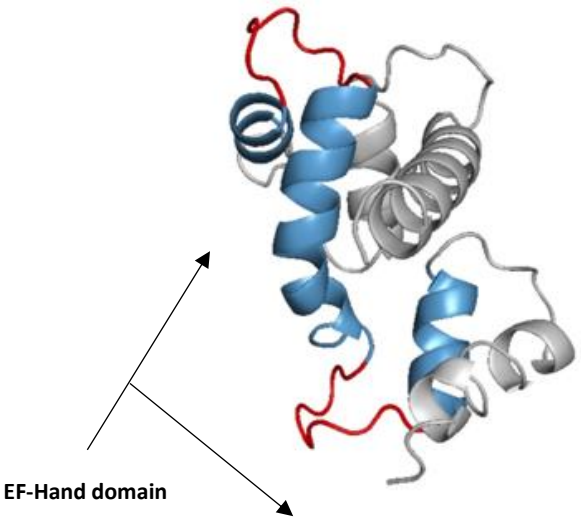
Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes severe acute and chronic infections in plants and animals. It is well known for infecting the lungs, urinary tract, open flesh or burn wounds, and surgical sites [15]. This pathogen can cause death in patients with Cystic Fibrosis (CF) by causing cellular damage in the lungs and airway blockage [10]. The airway blockage is attributed to the pathogen's ability to form biofilms in the respiratory systems of CF patients, which are difficult to eradicate with available antibiotics [11]. In fact, *P. aeruginosa* was recognized by the World Health Organization as a critical priority and the Center for Disease Control (CDC) as a serious public health threat that requires immediate and urgent research for discovering new antibiotics or other means of control [3].

The severity and resistivity of *P. aeruginosa* infections can be attributed to the production of numerous virulence factors and resistance of the pathogen. These virulence factors promote host invasion and enhance the development of chronic and acute infections within the host [4]. *P. aeruginosa* has Type I, II, III, V, and VI secretion systems that secrete exotoxins and extracellular proteases which aid in the damage of host tissue damage and enable bacterial reproduction and survival within the host. [2,7]. Adhesins and pili aid in pathogen attachment to host tissues while pyoverdine and pyochelin helps chelate iron allowing for bacteria to thrive in iron deficient environments within a host [4]. Alginate and pyocyanin have been shown to promote *P. aeruginosa* biofilm formation and maturation [14]. These are just a few examples of virulence factors. Others include quorum sensing, swarming motility, and lipopolysaccharides [9,10]. Although a lot is known about virulence factors and mechanisms of resistance in *P. aeruginosa*, the host factors triggering pathogen virulence are not completely understood. Of

these factors, calcium ions (Ca^{2+}) have been found to have a significant role in *P. aeruginosa* virulence.

Ca^{2+} is a signaling ion that has a regulatory role in many essential cellular processes, including innate immunity and hyperinflammatory response and host defense strategies like ion transport and mucin secretion [6,9]. Although the role of Ca^{2+} signaling in eukaryotes is well established, Ca^{2+} signaling in prokaryotes is not studied as well. The Patrauchan group has shown that *P. aeruginosa* actively maintains the free intracellular Ca^{2+} levels, which temporarily increase in response to external environmental and physiological factors [9]. The Patrauchan lab has also discovered that elevated levels of extracellular Ca^{2+}, that are commonly detected in CF lungs, enhance the virulence and the ability of *P. aeruginosa* to cause disease [9,13]. This was confirmed through a variety of experiments, specifically through the implementation of a leaf lettuce infection model and measuring the levels of pyocyanin, pyoverdine, biofilm and extracellular protease production [13,14]. Identifying the molecular mechanisms of such regulation is of high clinical significance. In attempts to identify the main components of Ca^{2+} signaling and regulation in *P. aeruginosa*, our lab has predicted several putative Ca^{2+}-binding proteins. One of them is the EF-hand protein, EfhP [13].

Figure 1: Predicted EfhP structure (B. Kayastha)



Earlier, our laboratory studied the role of EfhP in Ca^{2+}-regulation of *P. aeruginosa* virulence and resistance to various stresses. Based on sequence analysis and structure prediction (Figure 1), EfhP has two EF- hand domains, likely facing the periplasm [11]. These EF- hand domains are known to bind Ca^{2+} and are commonly present

in eukaryotic calcium sensors, such as calmodulin [13]. The role of calmodulin is to bind Ca^{2+}, which allows for structural conformation changes in this protein, leading to surface exposure of hydrophobic patches, allowing for binding to target proteins [13]. This binding alters the activities of the target proteins and defines the overall cellular response to Ca^{2+}. Based on similarity to calmodulin, we have predicted that EfhP also serves as a Ca^{2+} sensor and transduces Ca^{2+} signal towards regulation of *P. aeruginosa* virulence. Earlier, we deleted *efhP* from *P. aeruginosa* genome, and discovered that the mutant reduced production of pyocyanin and alginate. The mutant also showed decreased biofilms formation and resistance to oxidative stress [13]. However, further studies are needed to define the role of this protein in Ca^{2+} signaling and Ca^{2+}-regulated virulence of *P. aeruginosa*.

The main goal of this research was to generate new and more detailed knowledge about the regulation of *efhP* and its role in *P. aeruginosa* virulence. To achieve this goal, I 1) tested the role of EfhP in the ability of *P. aeruginosa* to cause a lethal infection using the *Galleria mellonella* animal model, 2) determined the transcriptional regulation of EfhP in response to Ca^{2+} and iron, and 3) determined the role of several proteins in regulation of *efhP* transcription by monitoring *efhP* promoter activity.

This research provides insight into the host factors modulating the pathogen's ability to cause a disease and the role of Ca^{2+} regulation in *P. aeruginosa* pathogenic interactions within a host. This knowledge is imperative for a better understanding of the regulatory systems controlling virulence and therefore is essential for future developments of novel medicinal approaches for preventing or controlling *P. aeruginosa* infections and reducing the number of fatalities caused by the pathogen.

METHODS AND MATERIALS

Virulence assay using *Galleria mellonella*.

Bacterial Culture Preparation. Two to three precultures were inoculated from a plate of the *efhP* deletion mutant, the quadruple mutant, complementation strain or wild type strains of *P. aeruginosa* in 5 mL of Luria Bertani (LB) or Biofilm minimal medium (BMM) [13]. The BMM contained (per liter): 9.0 mM sodium glutamate, 50 mM glycerol, 0.02 mM $MgSO_4$, 0.15 mM NaH_2PO_4, 0.34 mM K_2HPO_4, and 145 mM NaCl, 20 µl trace metals, 1 ml vitamin solution. Trace metal solution (per liter of 0.83 M HCl) contained 5.0 g $CuSO_4 \cdot 5H_2O$, 5.0 g $ZnSO_4 \cdot 7H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 2.0 g $MnCl_2 \cdot 4H_2O$. Vitamins solution (per liter) contained 0.5 g thiamine, 1 mg biotin. The pH of the medium was adjusted to 7.0. The level of Ca^{2+} in BMM was below the detection level when measured by QuantiChrom™ calcium assay kit. The precultures were grown for 12 h at 37°C then normalized to an Optical Density (OD_{600}) of 0.3 by using a spectrophotometer. From the normalized culture, 500 µL was added into 125 mL Erlenmeyer flasks containing 50 mL of BMM or LB no added Ca^{2+} or with added 5 mM Ca^{2+}. In order to achieve 5 mM final level of Ca^{2+}, we first measured the concentration of Ca^{2+} in freshly made LB or BMM by using a QuantiChrom™ calcium assay kit. Then, $CaCl_2 \cdot 2H_2O$ was added to the final to solutions to obtain a concentration of 5 mM. The cultures were grown for 16 h at 37°C. After 16 h, thus prepared main cultures were normalized to an OD_{600} of 0.1 and serial dilutions were performed by using sterile PBS containing 0 or 5 mM Ca^{2+} in a 96 well plate. These diluted cultures were used to inject the wax worms.

Determination of colony-forming units (CFU). To determine the CFU of the bacterial cultures injected into the worms, the prepared dilutions were plated on LB agar plates, followed by

incubation for 24 h at 37°C. After 24 h, colonies were counted and recorded. After the conclusion of the experiment, all solutions that the worms come in contact, such as 0 and 5mM Ca^{2+}PBS, and 10 mM $MgSO_4$, were plated on LB agar plates and incubate for 24 h at 37°C. This also allowed to ensure that contamination did not occur during preparation of cultures or during injections.

Worm Preparation and Injection. Worms were purchased from American Cricket Ranch and kept in at 4 ^{0}C for not longer than 24 h. Prior to injections, the worms were removed from the refrigerator and selected based on size and color, ensuring that they were in between 0.75-1 in in length for injection and free of dark pigmentation. All selected worms were placed in a foil-wrapped Tupperware container, which was then placed on ice to keep the worms inactive during the injection process. In groups of five, the selected worms were placed on filter paper and washed, first with ethanol then with rifampicin (1 mg/mL), excess of the solutions was removed with a Kimwipe. This was done by using a sterile syringe to drop a couple drops of each solution on each worm's right hind leg, to refrain from drowning the worm. Injections began after the completion of washing.

PBS was injected as a control first. For this, five worms were injected with 5 µL of PBS (no Ca^{2+} added), and then the other five worms with 5 µL of 5 mM Ca^{2+} PBS. After each worm injection, the syringe was sterilized by aspirating up and down in 70% ethanol, and then rinsed with 1mM $MgSO_4$**.** Following control injections, groups of ten worms were injected in the following order for each tested bacterial strain: 0mM Ca^{2+} 2.5 CFU; 0mM Ca^{2+} 10 CFU; 5mM Ca^{2+} 2.5 CFU; 5mM Ca^{2+} 10 CFU. As before, the needle was cleaned after each injection.

Worm Observation. All worms were incubated at 37º for 24 h. The worms were checked every 2 h for death, and time of death (TODs) was recorded. A pigmentation chart was created on the

scale from 1 to 5, and the amount of melanization was recorded at TOD, and averaged among worms for each condition (Figure 2). All worms selected at the start of the experiment were the same level of pigmentation.

Figure 2: Worm Pigmentation

1 3 5

Dead worms were placed in -20°C, for further analysis. At the 24th h of incubation, all worms were removed and placed in -20°C. This time interval was selected to prevent the larvae from cocooning. The TODs were considered to assess the median lethal dose (LD_{50}) and pathogenic potential of the different mutant strains at different Ca^{2+} levels. The pathogenic potential was calculated using: PP= (Fs/I)(10^{M}) where PP is the pathogenic potential, Fs is the fraction of symptomatic larvae, I is the amount of infecting inoculum and M is the mortality faction [1]. This was compared to the LD_{50} and pathogenic potential of the wildtype *P. aeruginosa*. To calculate the $LD_{50,}$ two infection doses were required: one to achieve below 50% death and the other to achieve above 50% death. LD_{50} was calculated using the following formulas (Mariette Barbier, personal communication):

1. $\%\ \boldsymbol{Mortality} = \frac{\boldsymbol{\#\ dead\ worms\ x\ 100}}{\boldsymbol{\#\ total\ worms}}$
2. $\boldsymbol{Fractional\ titer\ (f.t.)} = \frac{\boldsymbol{50 - (\%\ mortality < 50\%)}}{\boldsymbol{(\%\ mortality > 50\%) - (\%mortality < 50\%)}}$
3. $\boldsymbol{LD_{50}\ titer} = \boldsymbol{\log_{10}(dose\ causing < 50\%\ mortality) + f.t \times log_{10}\left(\frac{dose\ causing > 50\%\ mortality}{dose\ causing < 50\%\ mortality}\right)}$
4. $\boldsymbol{LD_{50}} = \boldsymbol{10^{LD_{50}\ titer}}$

Promoter Activity Assay.

Promoter activity assays were used to study the regulation of *efhP* transcription in wild type PAO1, and several deletion strains lacking one of each of the following genes: *carR, carP, calC,* and *bfmS*. We also tested the effects of extracellular Ca^{2+} and iron (Fe^{2+}) on the transcription of *efhP*. For this, we cloned the native promoter of *efhP* upstream of the *lux* operon. The construct was named pREN and allowed monitoring the activity of *efhP* promoter by measuring luminescence produced by the *lux* system upon activation.

To introduce the construct into *P. aeruginosa* mutant strains, these strains and the SM10 *E. coli* donor strain containing the pREN construct were first grown on an LB plate for 24 h. From the inoculated plate, the bacteria were inoculated in a culture of 5 mL of LB media and grown at 37°C at 200 rotations per minute (rpm) for 16 h. Afterwards, 1 mL of this culture was heated at 42°C for 2 h. While this culture was heating, 50 µL of the donor SM10 *E. coli* containing pREN was placed on the center of an LB plate. The plate was placed close to a flame and the lid was left half in to ensure a quick drying. After two hours, 50 µL of the heated recipient stain was added on top of the dried donor SM10 strain and dried next to the flame, on the LB plate. After the plate completely dried, the LB plate was placed in an incubator for 24 h at 37°C. Subsequently, the mating mixture was scraped with a sterile inoculating loop and mixed with 1 mL of PBS in a sterile test tube. From this solution, 10 µL was spread on a Pseudomonas Isolation Agar plate (IA) supplemented with 60 µg/mL of Tetracycline and allowed to grow at 37°C, until colonies appeared. Colonies from this plate were replicated on a LB plate supplemented with 60 µg/mL of Tetracycline and grown at 37°C until colonies appeared. These colonies were tested for luminesce to verify transformation. Once verified, the strains were grown to make a frozen stock.

For promoter activity assays, five 3 mL pre-cultures of wild type PAO1 or the previously mated mutant strains carrying pREN construct were grown in BMM until mid-Log phase (for 12 h). The collected cultures were normalized to an OD of 0.3 by using sterile BMM. Ten µL of the normalized culture from each sample was added into 990 µL of BMM with the corresponding level of Fe^{2+} or Ca^{2+}. After this addition, each of the 5 cultures were thoroughly mixed to ensure that the culture is evenly distributed in the medium. 200 µL of each culture was added into a well on a 96-well plate, to achieve five replicates for each condition to be analyzed. Additionally, two wells were filled with 200 µL of BMM with control medium to serve as a non-inoculated control.

These assays were conducted using a Biotek 96-well plate reader that enabled both incubation, shaking, and monitoring optical density and luminescence every 1 h. The plate was incubated in the Biotek at 37°C, shaking on the fast setting. Luminescence and absorbance at 600 nm were measured every hour for 12 h. Luminescence measurements were normalized by Ab_{600} measurements for the corresponding cultures following the subtraction of the corresponding non-inoculated controls measurements (luminescence and Ab_{600}). Lastly, averages of the normalized luminescence for at least 3 replicates were calculated and the ratios of promoter activities were determined.

RESULTS

The effect of calcium and iron on the *P. aeruginosa* ability to cause lethal infection in *Galleria mellonella*.

During the worm experiments, we observed that larvae injected with the *P. aeruginosa* cells grown in the presence of 5 mM Ca^{2+} died faster than those that were not (Figure 3). As hypothesized, worms that were injected with cells grown in 5mM Ca^{2+} had a threefold decrease in LD_{50} value than those that were not (Table 2). In other words, when cells were grown at elevated Ca^{2+}, a smaller number of them were required to cause infection/death in worms compared to the injection of cells grown at no added Ca^{2+}. These results showed that growth in the presence of Ca^{2+} enhances the ability of *P. aeruginosa* to kill larvae.

Additionally, all the worms that died in either conditions underwent pigmentation, i.e. they produced melanin and therefore their color went from a tan color to dark brown or black. Pigmentation due to melanization is a known visual indicator for the occurrence of *P. aeruginosa* infections. Worms injected with cells grown in calcium exhibited a darker pigmentation compared to worms injected with no calcium (Table 1).

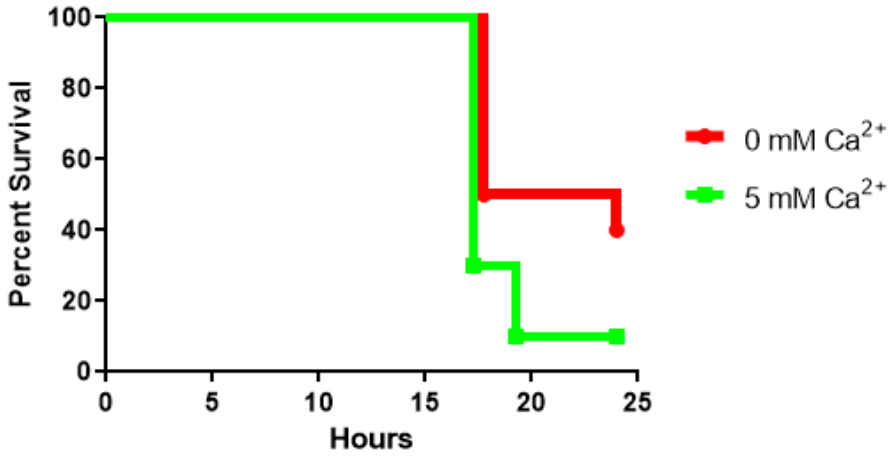


Figure 3: Death curves for *G. mellonella* injected with PAO1 grown at 0 mM and 5 mM Ca^{2+}. The infection doze was 16-17 CFU.

EfhP role in *P. aeruginosa* ability to cause lethal infection in *Galleria mellonella*.

When determining the role of efhP in *P. aeruginosa* virulence, we found that slightly fewer worms died if they were injected with the *efhP* gene deletion mutant (Δ*efhP*) compared to those injected with the wildtype (Figure 4). Additionally, worms injected with the *efhP* deletion mutant experienced less pigmentation than those injected with PAO1 (Table 1).

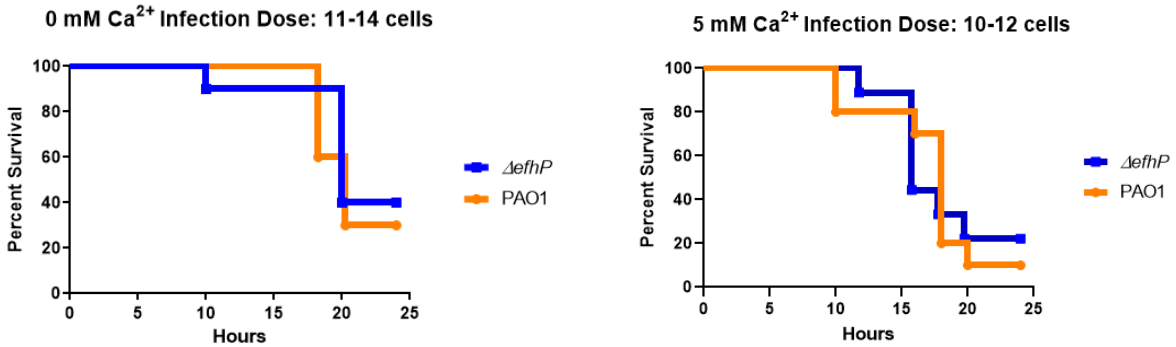


Figure 4: Death curves for *G. mellonella* injected with PAO1 and Δ*efhP* grown in 0 mM and 5 mM Ca^{2+}.

To confirm the difference observed between the *efhP* mutant and wildtype was due to mutation, worms were injected with an *efhP* complemented strain (Δ*efhP*::*efhP*). We observed that

more worms died if they were injected with the complemented *efhP* strain (Figure 5). This may reflect a higher expression of the gene in the complemented strain than in the wild type.

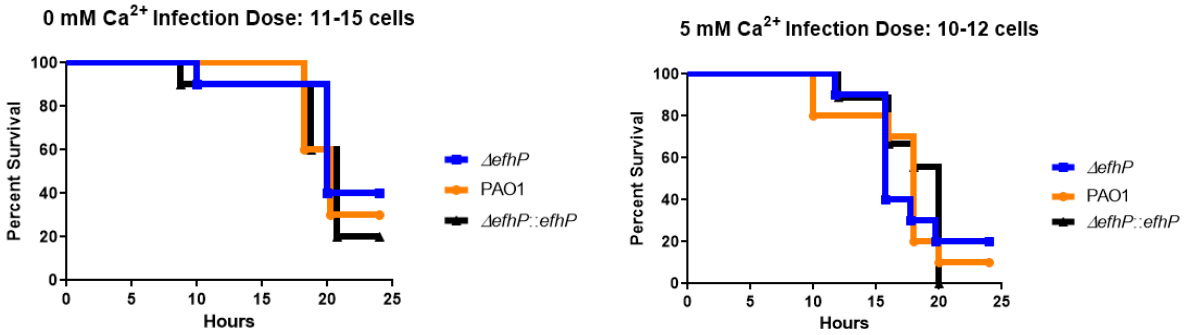


Figure 5: Death curves for *G. mellonella* injected with PAO1, Δ*efhP* and Δ*efhP*:: *efhP* grown in 0 mM and 5 mM Ca^{2+}.

Table 1: Calculated Pigmentation Factors for injected worms per each *P. aeruginosa* strain

Condition	Pigmentation Factor	Standard Deviation	Condition	Pigmentation Factor	Standard Deviation
0mM PBS	2.4	1.94	0mM PBS	2.4	1.94
0mM PAO1 14 CFU	2.5	1.35	0mM EfhP 10 CFU	2.2	1.22
5mM PBS	1	0	5mM PBS	1	0
5mM PAO1 10 CFU	3.4	1.34	5mM EfhP 12 CFU	1.3	1.31

When calculating LD_{50} for each condition (Table 2), we found the value increased for worms injected with the *efhP* deletion mutant strain as expected.

Table 2: LD_{50} values for *P. aeruginosa* wildtype and mutant

PAO1 5mM LD50	*ΔefhP* 5mM LD50	PAO1 0mM LD50	*ΔefhP* 0mM LD50
1 CFU	6.3 CFU	3.01 CFU	9.7 CFU

Overall, the mutant lacking *efhP* gene exhibited a lower virulence than the wild type when grown at elevated Ca^{2+}. An additional finding revealed that magnitude of worm deaths was amplified when the bacteria were grown in LB versus grown in BMM (data not shown). This finding provides insight into the role of EfhP contributing to Ca^{2+} regulation when the pathogen was grown in a rich LB medium, but the effect was reduced for cells grown in mineral BMM medium. All of these findings allow for the conclusion that this research supports the mediatory role of EfhP in Ca^{2+} regulation of virulence in an important human pathogen, *P. aeruginosa.*

Transcriptional regulation of *efhP* in response to iron and calcium

The luminescence measured in the promoter assays shows the transcriptional expression of *efhP* in the presence Ca^{2+} and Fe^{2+} in *P. aeruginosa*. Upon the addition of 5 mM extracellular Ca^{2+}, we saw am increase in *efhP* promoter activity in the wild type during log phase (Figure 6). In contrast, we observed a slight reduction in the response in the presence of 4 µM extracellular Fe^{2+} (Figure 7).

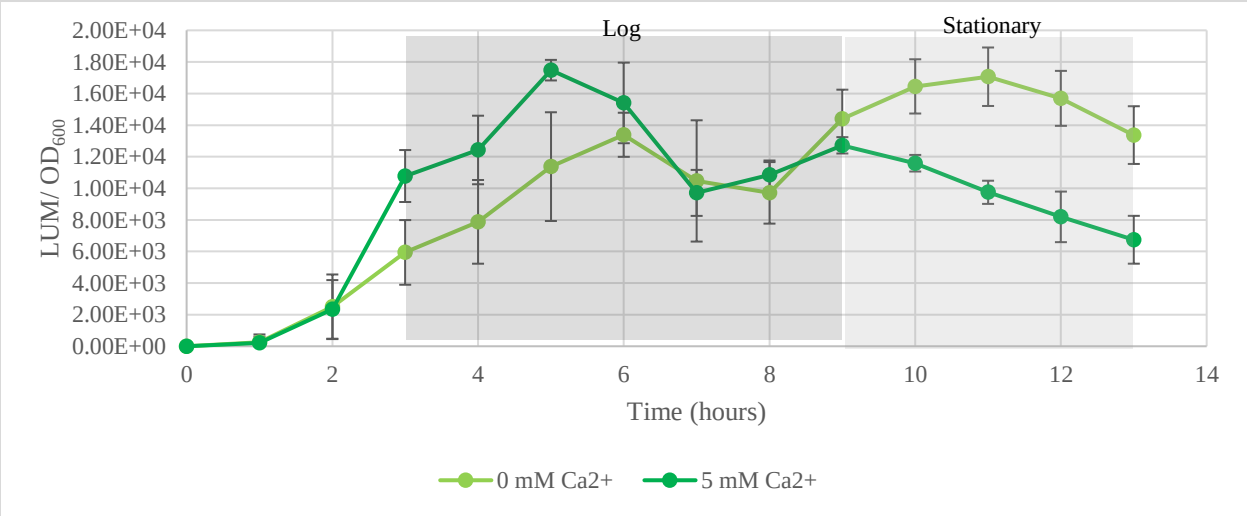


Figure 6: Normalized Luminescence of WT promoter construct in the presence and absence of Ca^{2+}

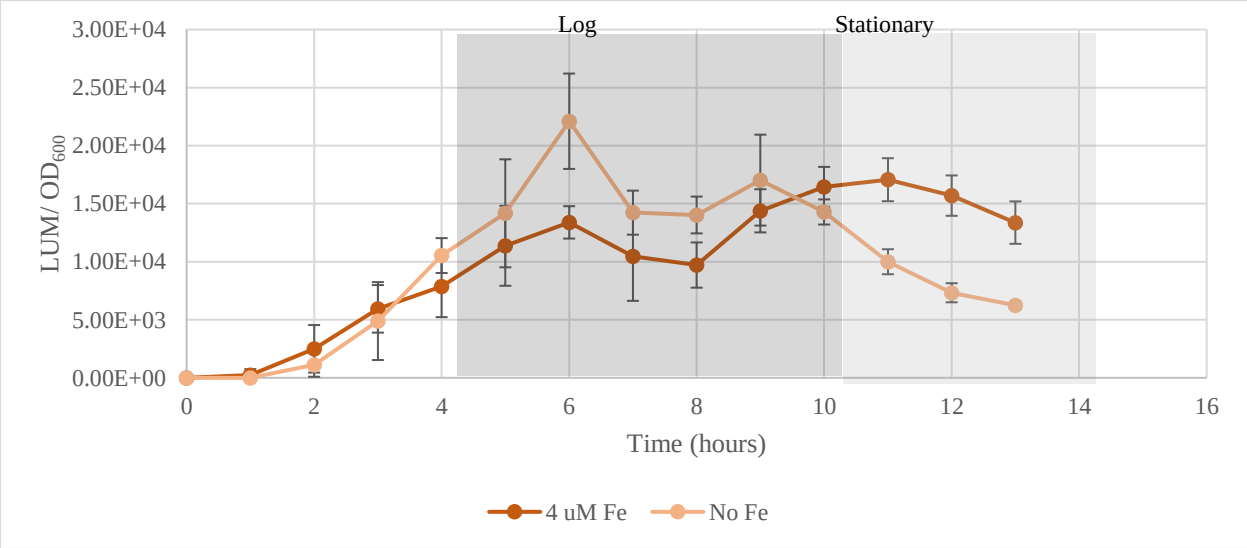
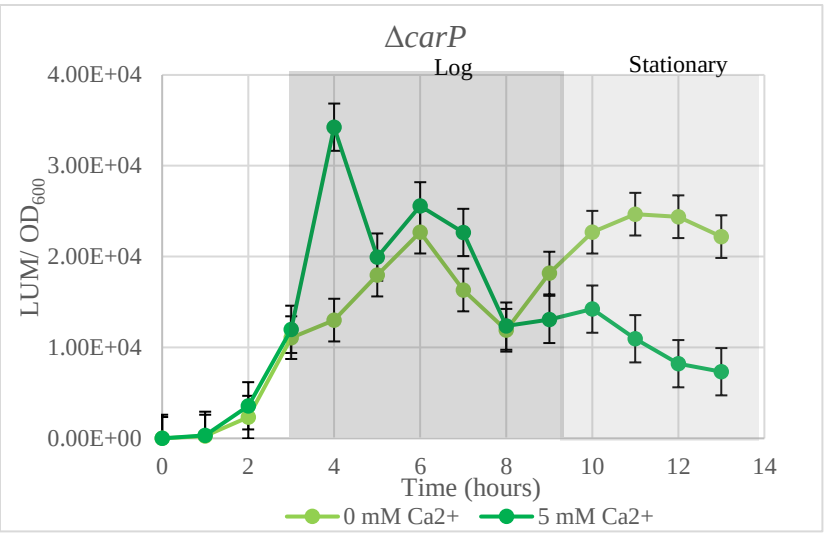


Figure 7: Normalized Luminescence of WT promoter constructs in the presence and absence of Fe^{2+}

The role of transcriptional regulators of *efhP*

We selected four proteins potentially regulating the transcription of *efhP* and tested their effect by monitoring *efhP* promoter activity in the mutants individually lacking the corresponding genes. The first protein we tested was calcium-regulated β-propeller protein CarP, which is a putative phytase that is involved in regulation of the intracellular calcium levels in *P. aeruginosa* [8]. Our lab has also found that CarP is required for *P. aeruginosa* growth at high calcium concentrations and moderates swarming motility and pyocyanin production which are two virulence factors of *P. aeruginosa* [8]. We hypothesized that since this protein has a significant role in the maintenance of the intracellular Ca^{2+}, it may affect the transcriptional regulation of *efhP*. We found that in the absence of *carP*, the level of *efhP* promoter activity increased at both Ca^{2+} conditions (Fig.8). This suggest potential negative regulation of *efhP* transcription by CarP, likely due to its role in the maintenance of the intracellular Ca^{2+}.



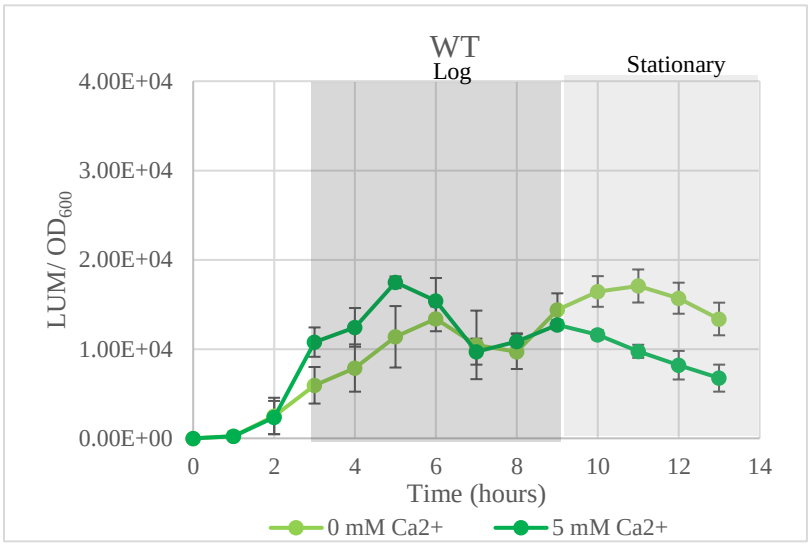
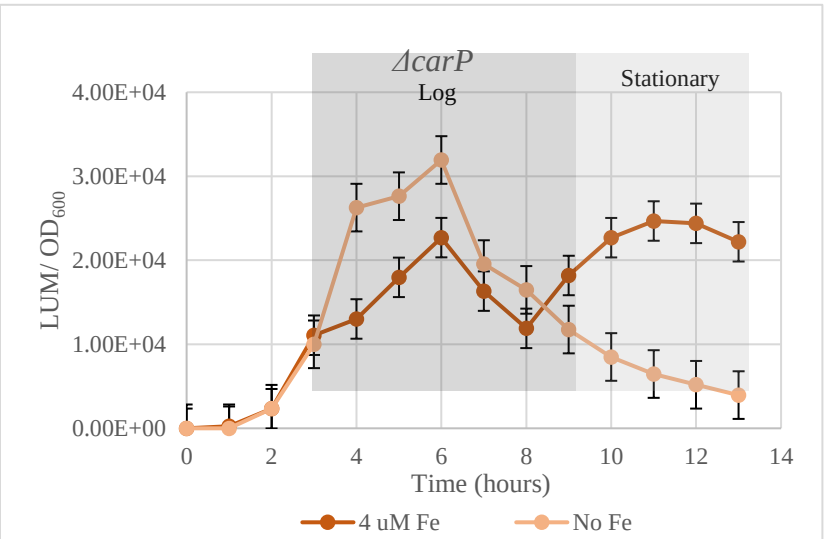


Figure 8: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*carP* in the presence and absence of Ca^{2+}

Regarding the effects of Fe^{2+} on the transcriptional regulation of *efhP*, when *carP* was removed, we found a similar effect (Figure 9). This suggest a potential regulatory connection between Ca^{2+} and Fe^{2+}, but more testing will need to be done to confirm.



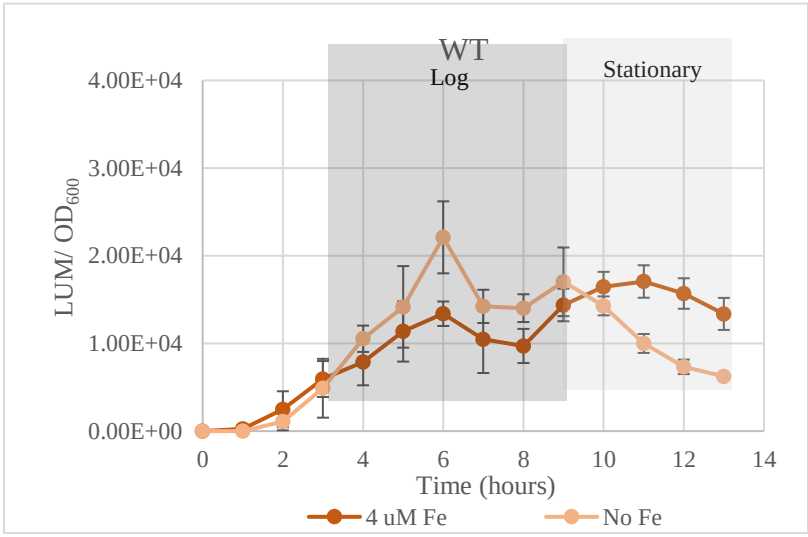


Figure 9: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*carP* in the presence and absence of Fe^{2+}

The second protein we tested was CalC, a putative calcium channel that regulates uptake of Ca^{2+} in *P. aeruginosa*. Interestingly, the mutant showed no effect of Ca^{2+} on *efhP* transcription, supporting the idea that the intracellular Ca^{2+} is involved in regulating the transcription of *efhP* (Figure 10). We also observed a decrease in the *efhP* promoter activity in the mutant, suggesting that CalC has a positive impact on *efhP* promoter activity.

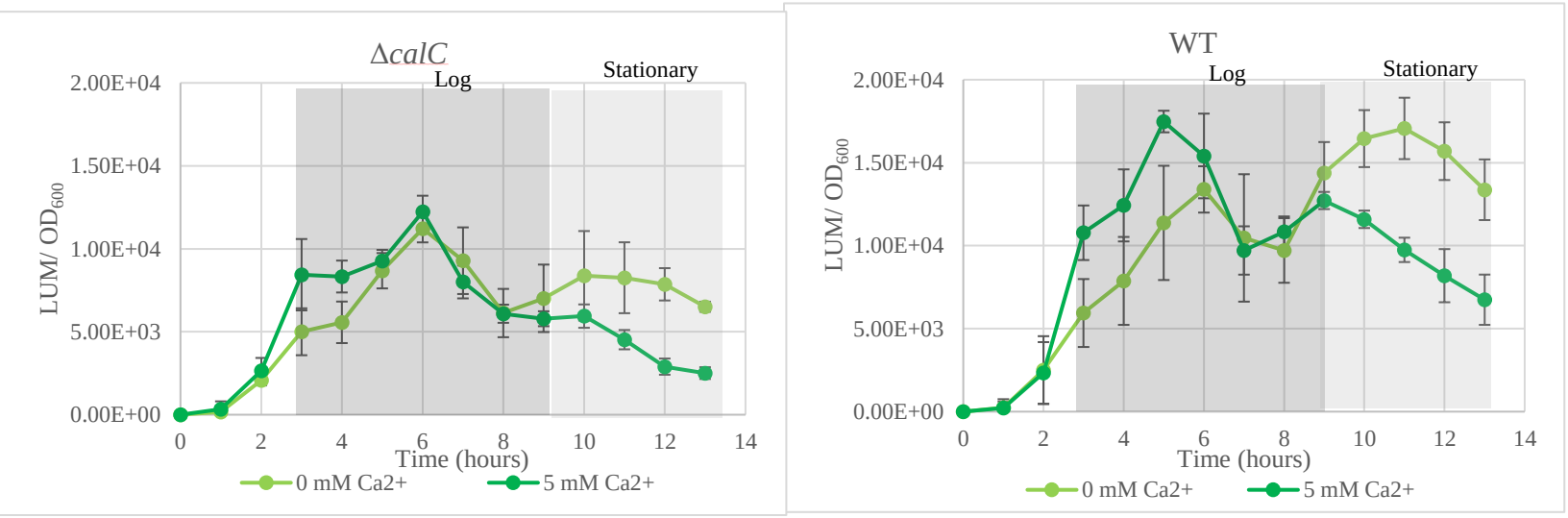


Figure 10: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*calC* in the presence and absence of Ca^{2+}

In contrast, there was no significant difference in *efhP* promoter activity in the *ΔcalC* mutant at no added Fe^{2+} (Figure 11). However, in the presence of 4 µM Fe^{2+}, the mutant showed slightly increased promoter activity, again suggesting a potential regulatory link between Ca^{2+} and Fe^{2+}. Additionally, we found that iron has an inhibitory effect on both strains at log phase.

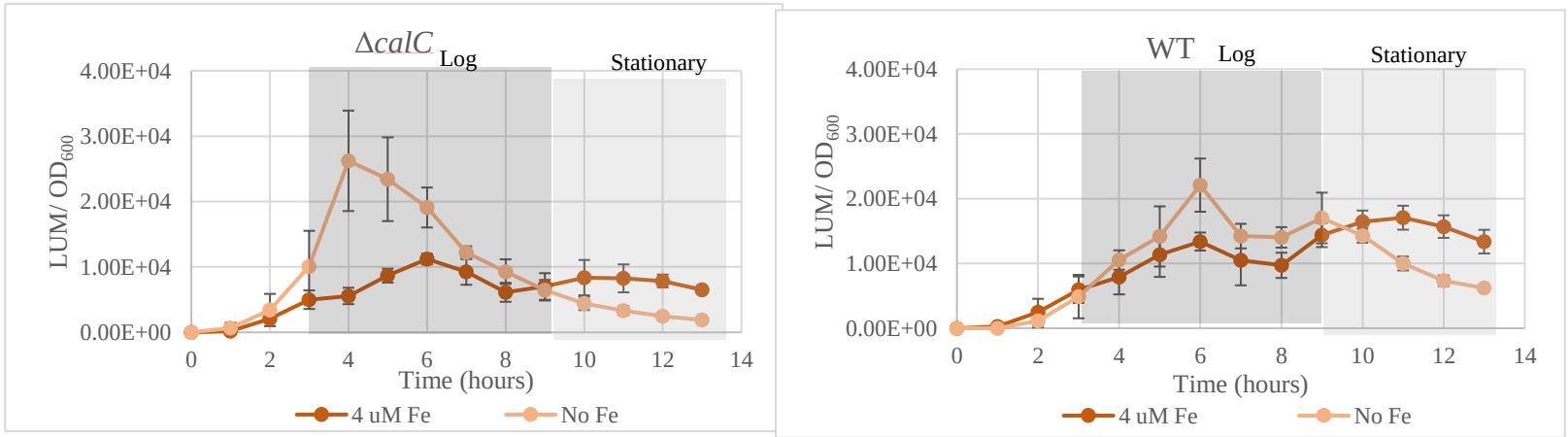


Figure 11: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*calC* in the presence and absence of Fe^{2+}

The next selected protein was CarR, which a transcriptional regulator and a component of the CarSR two component system (TCS) in *P. aeruginosa* [8]. Earlier we showed that the TCS is positively regulated by elevated Ca^{2+}. CarR plays a role in Ca^{2+} homeostasis by transcriptionally regulating *carP* [8]. With this, we predicted that *carR* may be involved in regulating transcription of *efhP* as well. In agreement, a slight increase in promoter activity was observed in the mutant, suggesting a negative impact on *efhP* transcription (Figure 12).

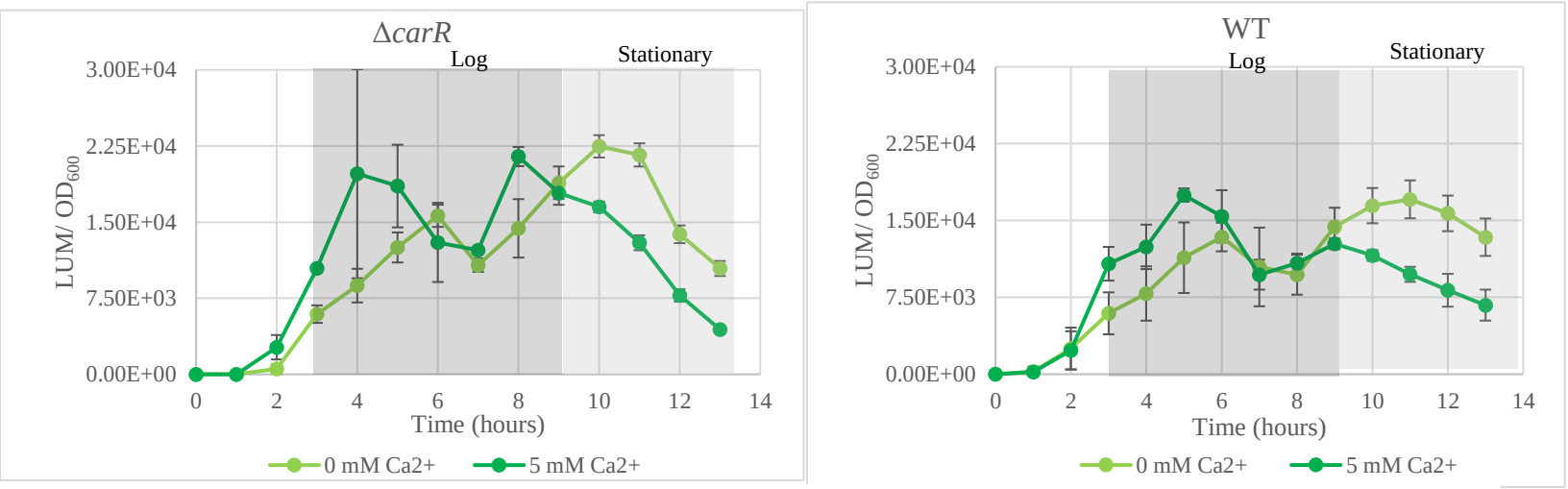


Figure 12: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*carR* in the presence and absence of Ca^{2+}

Interestingly, we observed a significant increase in *efhP* promoter activity in the *carR* mutant at no added Fe^{2+}, but not in the presence of 4 µM Fe^{2+} (Figure 13). This suggests that addition of Fe^{2+} may inhibit the negative impact of CarR on *efhP* expression.

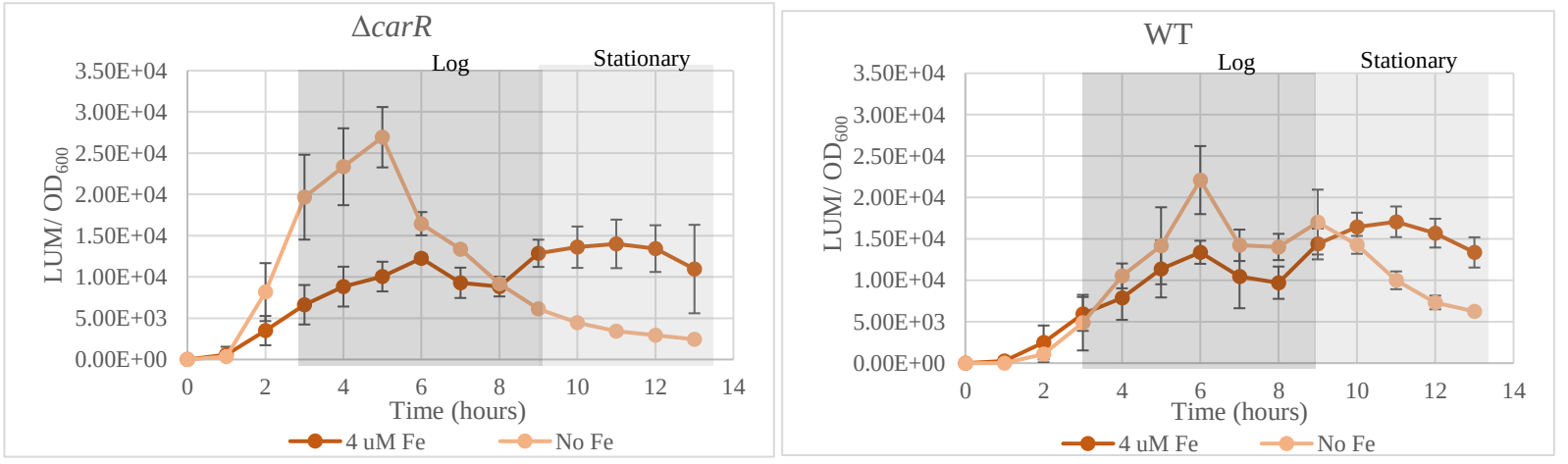
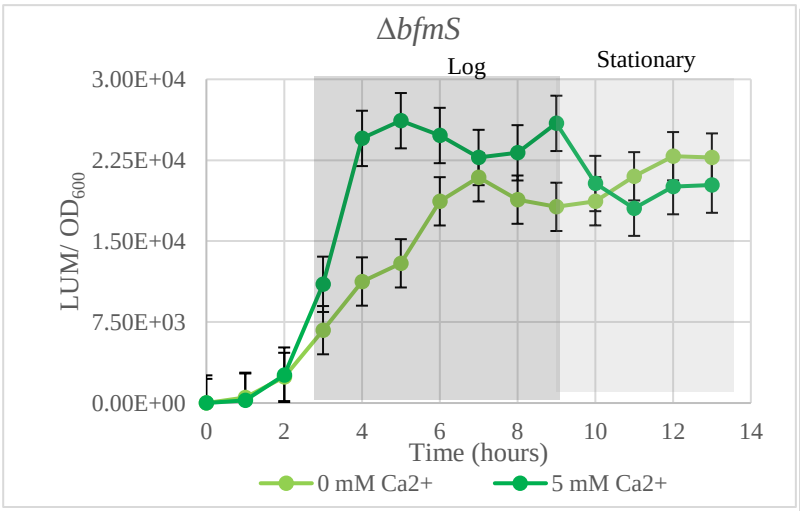


Figure 13: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*carR* in the presence and absence Fe^{2+}

The last protein that we tested using the promoter activity assay is BfmS. BfmS is a sensor kinase and a component of the TCS called BfmRS in *P. aeruginosa* [12]. This TCS plays a role in *P. aeruginosa* biofilm formation and regulates biofilm progression and maturation [12]. Additionally, scientists have learned that BfmRS affects quorum sensing and is an activator and mediator in the developments of acute or chronic *P. aeruginosa* infections [12].

We hypothesized that BfmRS and BfmS play a role not only in the virulence of this pathogen but the transcriptional regulation of *efhP*. We found that the mutant lacking *bfmS*, showed a significant increase in promoter activity at both Ca^{2+} conditions (Figure 14) and a slight increase in the absence or presence of Fe^{2+} (Figure 15). The data illustrates the regulatory role of BfmS in *efhP* transcription and once again suggests a regulatory connection between the two ions. Further studies are imperative to study such connection.



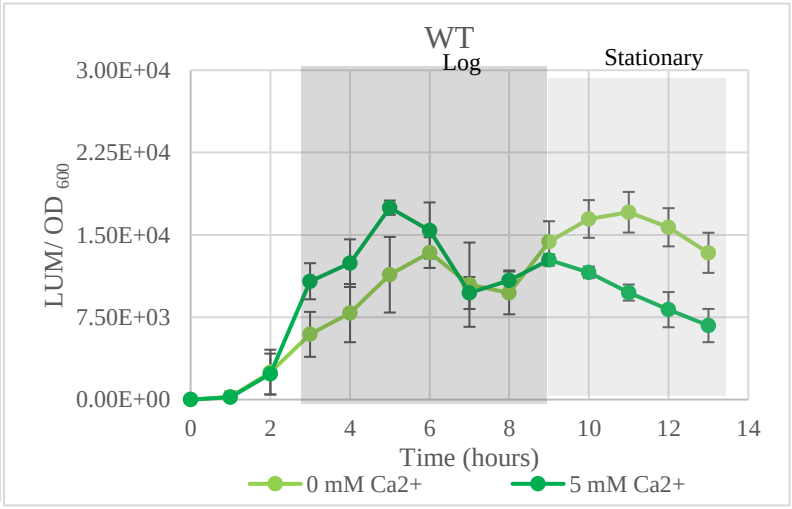
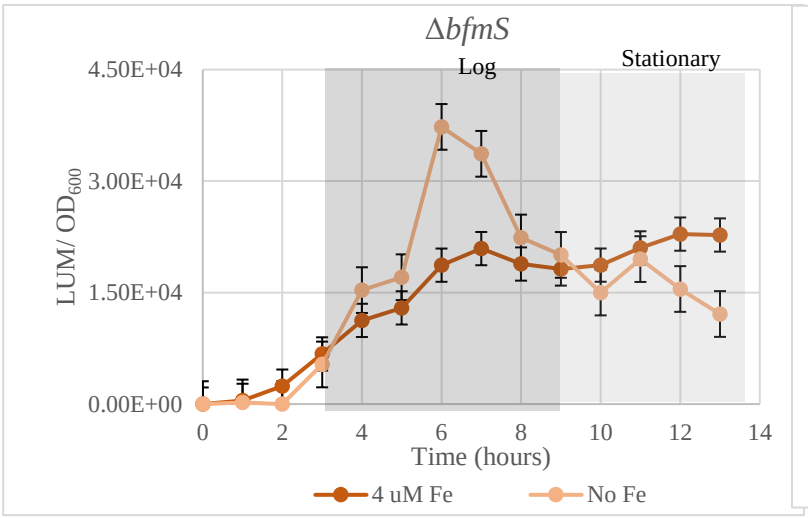


Figure 14: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*bfmS* in the presence and absence of Ca^{2+}



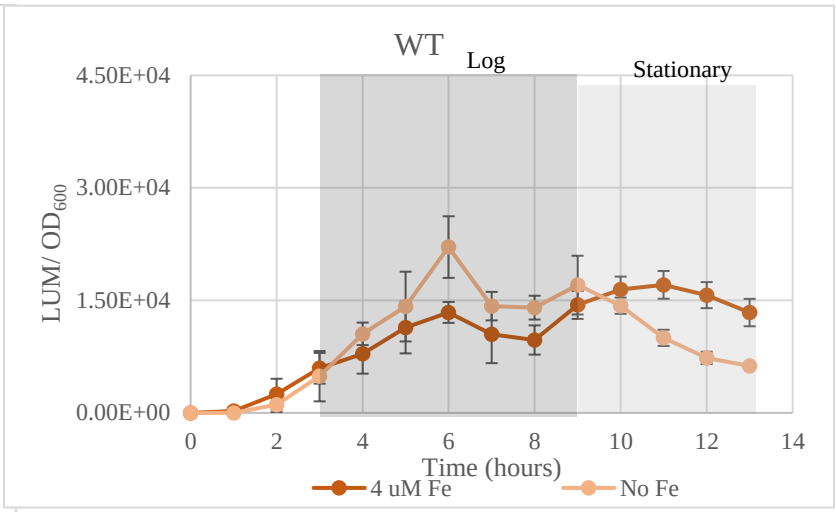


Figure 15: Normalized Luminescence for *efhP* promoter construct in the wild type PAO1 and Δ*bfmS* in the presence and absence Fe^{2+}

DISCUSSION AND CONCLUSIONS

EfhP was predicted to bind Ca^{2+} and shown to play a role in Ca^{2+}-induced virulence in an important human pathogen *P. aeruginosa*. This study has been necessary in characterizing EfhP, and in obtaining a better understanding of Ca^{2+}-signaling in bacteria. We have shown mediatory role of EfhP in Ca^{2+} regulation of virulence in *P. aeruginosa*. We determined that there is potential regulatory connection between Ca^{2+} and Fe^{2+} and confirmed that both ions are involved in the regulation of *efhP* transcription. Additionally, we identified several proteins that have a role in regulating *efhP* transcription, specifically CarP, CarR and BfmS. These findings pertaining to transcriptional regulation of *efhP* will allow for a better understanding on how to inhibit EfhP functions.

Obtaining information about the signaling pathways involving EfhP will give insight into Ca^{2+} signaling mechanisms in prokaryotes, which are not well defined. With this knowledge, we will gain a better understanding into the mechanisms of the pathogenicity of bacteria. Considering the importance of Ca^{2+} signaling in a host, this understanding provides insight into the regulatory systems engineering virulence and pathogenic interactions with a host. This is essential for future development of scientific and medicinal approaches for preventing or controlling *P. aeruginosa* infections, reducing the number of fatalities caused by the pathogen and minimizing the threat posed by antibiotic resistance.

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