Calcium Regulates Virulence of *Pseudomonas aeruginosa* in *Galleria mellonella*

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Honors Thesis

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that is known to infect the lungs of Cystic Fibrosis patients, which have abnormally high levels of calcium (Ca^{2+}). Our lab has shown that elevated Ca^{2+} increases *P. aeruginosa* plant infectivity and its ability to produce several virulence factors, including pyocyanin, pyoverdine, extracellular proteases, and rhamnolipid. Based on these observations, we hypothesized that elevated Ca^{2+} enhances *P. aeruginosa* virulence in an animal host. To test this hypothesis, we have adapted an animal virulence model using larvae of *Galleria mellonella,* also known as wax worm. First, we aimed to determine if Ca^{2+} increases the pathogenicity of *P. aeruginosa*. For this, we determined the half lethal dose (LD_{50}) of *P. aeruginosa*, which is the number of bacterial cells that cause 50% death rate. This information enables comparison between different testing groups. We also generated killing curves that allow comparing rates of the infection. The worms were injected with PAO1, a wild type of *P. aeruginosa*, grown in 0 mM, 5 mM, or 10 mM Ca^{2+}. We found the LD_{50} for 0 mM Ca^{2+} PAO1 is 2 CFUs, which is two-fold higher than that of PAO1 grown in 5 mM Ca^{2+}. The increase of Ca^{2+} concentrations to 10 mM Ca^{2+} further decreased LD_{50} to 0.3 CFUs. This proves that growth at elevated Ca^{2+} makes the pathogen more virulent. We also observed that the worms died faster at higher Ca^{2+} concentrations. We next tested the role of two proteins, CarP and CalC, which previously have been shown to mediate Ca^{2+} regulation of virulence factor production. The *carP*::Tn5 mutant exhibited density-dependent and reduced virulence at 10 mM Ca^{2+}. The *calC*::Tn5 mutant had a slight increase in virulence at 0 mM Ca^{2+}, but a slight reduction at 5 mM Ca^{2+}. These data support the role of CarP and CalC in *P. aeruginosa* Ca^{2+} induced virulence. This knowledge enables characterization of Ca^{2+} regulatory network controlling *P. aeruginosa* virulence, which is a step towards developing novel strategies to fight *P. aeruginosa* infections.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic and nosocomial pathogen, causing a wide range of infections in patients with a compromised immune system. These infections include acute and chronic, often deadly, infections, such as pneumonia, bacteremia, burn and surgery wound infections. In 2013, the CDC estimated around 50,000 *P. aeruginosa* caused nosocomial infections occur each year, with around 13% of these infections being resistant to multiple antibiotics and 400 infections causing death per year (CDC, 2013). *P. aeruginosa* also causes severe infections in the lungs of patients with Cystic Fibrosis. These patients have a mutation in the gene encoding for a cystic fibrosis transmembrane conductance regulator (CFTR). CFTR acts as a chloride channel, and when mutated, causes a problem regulating transport of electrolytes and chloride across epithelial membranes (Lyczak, Cannon, & Pier, 2002). These changes in ion homeostasis lead to generating a layer of thick mucus inside the airways that attracts bacteria. The airway fluids present in the lungs of Cystic Fibrosis patients has been found to have elevated levels of calcium (Ca^{2+}) in comparison to healthy individuals (Flume, 2012).

The greatest problem with *P. aeruginosa* infections is the pathogen's increasing antibiotic resistance and a large number of virulence factors enabling high pathogenicity. A few of these virulence factors relevant to the study are biofilm formation, pyoverdine, pyocyanin, extracellular proteases, and swarming motility. Biofilms are formed when bacterial cells attach to a surface and form a community. This community allows the bacteria to be resistant to antimicrobial reagents as well as to evade host immune responses (Leid, 2009). Pyoverdine is a fluorescent siderophore that sequesters iron thus depleting host cells of iron. Pyocyanin generates reactive oxygen species which are toxic to the host cells. Extracellular proteases are enzymes that break down proteins, and play a key role in tissue damage during *P. aeruginosa* infections. Swarming motility is a type

of motility activated on semi-solid surfaces, allowing *P. aeruginosa* to propagate and form biofilms. Our group has shown that these factors are at least in part controlled by Ca^{2+} (S. Sarkisova, Patrauchan, Berglund, Nivens, & Franklin, 2005).

Ca^{2+} is an ion that has been studied extensively as an intracellular signaling cation acting as a second messenger in eukaryotes. When intracellular concentrations of Ca^{2+} are elevated or the cell is prompted, the cells utilize pumps to eject the Ca^{2+} into the cytosol from the intracellular Ca^{2+} stores. This emission of Ca^{2+} transmits a signal which can regulate different mechanisms in these cells such as cell cycle, gene expression, metabolism, and transport. The Ca^{2+} signal is recognized by binding to Ca^{2+}-binding proteins, which further transmit the "signal" mostly *via* protein-protein interactions (Permyakov & Kretsinger, 2009). Ca^{2+} also plays a role in prokaryotes by regulating different mechanisms (Naseem, Wann, Holland, & Campbell, 2009). One of these mechanisms suggested by our current data is quorum sensing, which is the ability of a cell to communicate with other cells via chemical signals (Williams & Cámara, 2009). Our lab also has shown that *P. aeruginosa* maintains its intracellular Ca^{2+} homeostasis. We collected the evidence supporting that the changes in the intracellular Ca^{2+} regulate virulence of the pathogen.

Patrauchan lab has observed that a large number of genes of *P. aeruginosa* are positively regulated by elevated concentrations of Ca^{2+}. They include virulence factors associated with *P. aeruginosa* biofilms, pyocyanin, pyoverdine (S. Sarkisova et al., 2005). We have also observed that Ca^{2+} induces swarming motility in *P. aeruginosa* (Guragain, Lenaburg, Moore, Reutlinger, & Patrauchan, 2013). These virulence factors aid the pathogen in establishing a successful infection in a host. In addition to *in vitro* experiments, we tested the effect of Ca^{2+} on P. aeruginosa virulence in a plant model (S. A. Sarkisova et al., 2014). The necrosis in lettuce leaves was increased when infected with *P. aeruginosa* grown in elevated Ca^{2+} compared to no Ca^{2+}. This finding built the

foundation for further studies in an animal model to better understand the role of Ca^{2+} in *P. aeruginosa* virulence. The model selected for this purpose was *Galleria mellonella*.

Galleria mellonella larvae, also known as wax moth, has been used as an animal model for studying virulence of bacterial pathogens such as *Escherichia coli*, *Bacillus cereus, Staphylococcus aureus,* as well as *Pseudomonas aeruginosa* (N. Ramarao, Nielsen-Leroux, C., & Lereclus, D. , 2012). When choosing an animal model, several factors must be considered, such as the life span, maintenance, and complexity of the model's systems. *G. mellonella* can be injected with quantifiable doses due to their size, and can grow from 20-37 °C. The model has a short life span which makes them manageable for screening multiple mutants in a short amount of time, and are economically sensible. They also have a relatively complex innate immune system comparable to that of a mammal which is important to providing a relevant host environment for pathogens (Jander, 2000).

Since previous experiments showed an induction of *P. aeruginosa* virulence factors by elevated Ca^{2+}, we hypothesized that elevated Ca^{2+} would also induce the virulence in *G. mellonella*. The first goal of my research project was to determine if elevated extracellular Ca^{2+} levels would induce the virulence of *P. aeruginosa* in *G. mellonella*. The first step towards my goal was optimizing a protocol to use *G. mellonella* as an animal model, then utilizing the protocol to test the virulence of the wild type *P. aeruginosa* in *G. mellonella* at different Ca^{2+} concentrations. The second goal was to determine the role of two proteins produced by *P. aeruginosa*, CarP and CalC, in Ca^{2+}-induced virulence by the pathogen. CarP is a putative phytase that plays a role in Ca^{2+}-induced production of pyocyanin, pyoverdine, and swarming motility. CalC is a putative Ca^{2+}-leak channel that regulates intracellular Ca^{2+} homeostasis and plays a role in Ca^{2+} regulation of production of pyoverdine and swarming motility. This research allowed for

the adaptation of an animal virulence model for use in future experiments in the Patrauchan lab to test other conditions and mutants. This knowledge enables characterization of Ca^{2+} regulatory network controlling *P. aeruginosa* virulence, which is a step towards developing novel strategies to fight *P. aeruginosa* infections.

MATERIALS AND METHODS

Pseudomonas aeruginosa culture

A wild type strain of *P. aeruginosa,* PAO1, was struck onto a Luria Bertani (LB) agar plates from a frozen stock, and a preculture (PC) was inoculated from the plate into 5 mL of Biofilm Minimal Medium (BMM) (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, 145 mM NaCl, 20 µl trace metal solution, and 1 ml vitamin solution) and grown shaking for 12 hours at 37 °C. For culturing mutant strains, the corresponding frozen stock cultures were struck on an LB + tetracycline$_{60µg}$ plate and incubated at 37 °C for 24 hours. Once grown for 12 h, PCs were normalized to an optical density (OD) $_{600}$ = 0.3 by measuring absorbance in 1 mL of the PC in a Biomate 3 spectrophotometer and adjusting the volume with BMM. 500 µL of normalized PC was inoculated into 125 mL flasks containing 50 mL of BMM at 0 mM, 5 mM, and 10 mM Ca^{2+}. For *calC*::tn5, the bacteria were grown in 0 and 5 mM Ca^{2+}. For *carP*:tn5, the bacteria were grown in 0 and 10 mM Ca^{2+} BMM. The main cultures (MC) were then incubated shaking at 37 °C for 16 hours. The MC was normalized to an OD_{600} = 0.1.

Serial Dilutions and estimating infectious dose

Serial dilutions were performed by transferring 20 µL of the normalized MC into 180µL of PBS at the corresponding Ca^{2+} concentration in the first well of a 96-well plate. The cells were mixed well by pipetting up and down several times, then 20 µL was transferred to the next well. These steps were repeated from 10^{-1} to 10^{-6}. 10 µL of the dilutions from 10^{-4} to 10^{-6} were plated onto LB plates split into 4 quadrants, and spread evenly. The plates were incubated at 37 °C for approximately 20 hours, then the colony forming units (CFUs) were counted.

Galleria mellonella injection

This protocol was modified from a protocol obtained from Dr. Barbier at West Virginia University (N. Ramarao, Nielson-Leroux, C., Lereclus, D., 2012). *G. mellonella* larvae were ordered from Speedy Worm, and kept in the dark at 4 °C without food for no more than 48 hours before injection. Active worms 2-3 cm long were chosen for injection. For each experiment, 25 worms were removed from the fridge in a chilled container, and kept on ice. The worms were washed in a glass petri dish with 70% ethanol then with rifampicin (1mg/mL) by dropping 2 drops on each worm using a Hamilton 25 µL syringe with a Hamilton 12 gauge needle. After washing, the needle was inserted into 5 worms to serve as an injection control. Next, the PBS controls were injected, 5 worms were injected with 5 µL of PBS at the required Ca^{2+} concentration. Finally, 10 experimental worms were injected with 5 µL of cell suspension grown at 0 mM, 5 mM or 10 mM Ca^{2+} and normalized to contain 1 (10^{-6}) or 10 CFU (10^{-5}). To verify the infection dose, the normalized cultures were serially diluted, plated, and the CFUs were counted. In addition, 5 worms were injected per Ca^{2+} concentration to be analyzed for hemolymph bacterial load, Ca^{2+} concentration, and immune response (protocols detailed below). Between each worm, the syringe was cleaned with 70% ethanol by aspirating up and down in a flask, then the syringe was rinsed

with sterile 10 mM $MgSO_4$ by aspirating. The worms were incubated at 37 °C for 24 hours and monitored for death every 2 h, identified by observing movement in response to turning them over with a sterile toothpick. Times of death (TOD) were recorded for each worm. Dead worms and those surviving after 24 h were placed in microcentrifuge tubes and frozen at -20 °C for further analyses.

Calculation of LD_{50}

Lethal dose 50 (LD_{50}) was calculated using the Miller and Tainter method (Miller & Tainter, 1944). This method requires mortality percentages below 50% and above 50%. For each experiment, a table was generated, as exemplified below including the number of live and dead worms in each group. The % or mortality was calculated as:

$$\%\ Mortality = \frac{\#\ dead\ worms\ x\ 100}{\#\ total\ worms}$$

CFU/worm	# total worms	# dead	# alive	% Mortality
1	10	3	7	30%
10	10	7	3	70%
100	10	10	0	100%

Next, the Fractional titer was calculated using the formula:

$$\boldsymbol{Fractional\ titer\ (f.t.)} = \frac{\mathbf{50} - (\%\ \boldsymbol{mortality} < \mathbf{50}\%)}{(\%\ \boldsymbol{mortality} > \mathbf{50}\%) - (\%\boldsymbol{mortality} < \mathbf{50}\%)}$$

The LD_{50} titer was calculated using the following formula:

$$\boldsymbol{LD_{50}\ titer} = \mathbf{log_{10}}(\boldsymbol{dose\ causing} < \mathbf{50}\%\ \boldsymbol{mortality}) + \boldsymbol{f.t} \times \boldsymbol{log_{10}}\left(\frac{\boldsymbol{dose\ causing} > \mathbf{50}\%\ \boldsymbol{mortality}}{\boldsymbol{dose\ causing} < \mathbf{50}\%\ \boldsymbol{mortality}}\right)$$

The LD_{50} was calculated by: $\boldsymbol{LD_{50}} = \mathbf{10}^{\boldsymbol{LD_{50}\ titer}}$

In this example: $f.t. = (50 - 30)/(70 - 30) = 0.4$

$LD_{50}\ titer = log_{10}(1) + 0.4 \times log_{10}(10/1) = 0.4$

$LD_{50} = 10^{0.4} = 2.51$ CFU

Homogenization and Bacterial Load

The worms were thawed, and 500µL of 10mM $MgSO_4$ was transferred into the 1.5 mL centrifuge tube holding the worm. Worms were homogenized by using a BT Labs handheld homogenizer. Serial dilutions were performed using the method described above from 10^{-1} to 10^{-4}. Ten µL of each dilution was pipetted onto one quadrant of an LB plate split into four quadrants, and spread using a sterile loop. Plates were incubated at 37 °C for 20 hours. After 20 hours, they were removed and CFUs were counted to determine the bacterial load in each worm.

Prophenoloxidase (PPO) Assay

Five worms collected at 11 h after injection were removed and frozen for infection dose. These worms were thawed, and 15 µL of the hemolymph was removed from each. 10 µL each sample were centrifuged at 1,500 g for 10 min at 4 °C. The remaining 5 µL was collected to measure free Ca^{2+} (described below). Immediately after centrifugation, the samples were diluted tenfold in Tris-Buffered Saline (50 mM Tris-HCl pH 6.8, 1mM NaCl), and 2 µL of this mixture was added to 18 µL of TBS with 5 mM $CaCl_2$ in a 96-well plate. The samples were then incubated at room temperature for 20 minutes. One hundred and eighty µL of 2 mM dopamine in 50 mM sodium phosphate (pH 6.5) was then added to the samples, and absorbance was measured every 15 minutes for 45 minutes using a Biotek microplate reader.

Measurement of free Ca^{2+}

To measure changes in free Ca^{2+}, 5 µL of the above described hemolymph sample was used for subsequent estimation of free Ca^{2+} by using QuantiChrom™ calcium assay kit following the manufacture's protocol.

RESULTS

Optimization of *G. mellonella* Protocol to Study the Virulence of *P. aeruginosa*

To investigate the role of Ca^{2+} in the virulence of *G. mellonella*, we first needed to optimize the *G. mellonella* infection protocol shown in Figure 1.

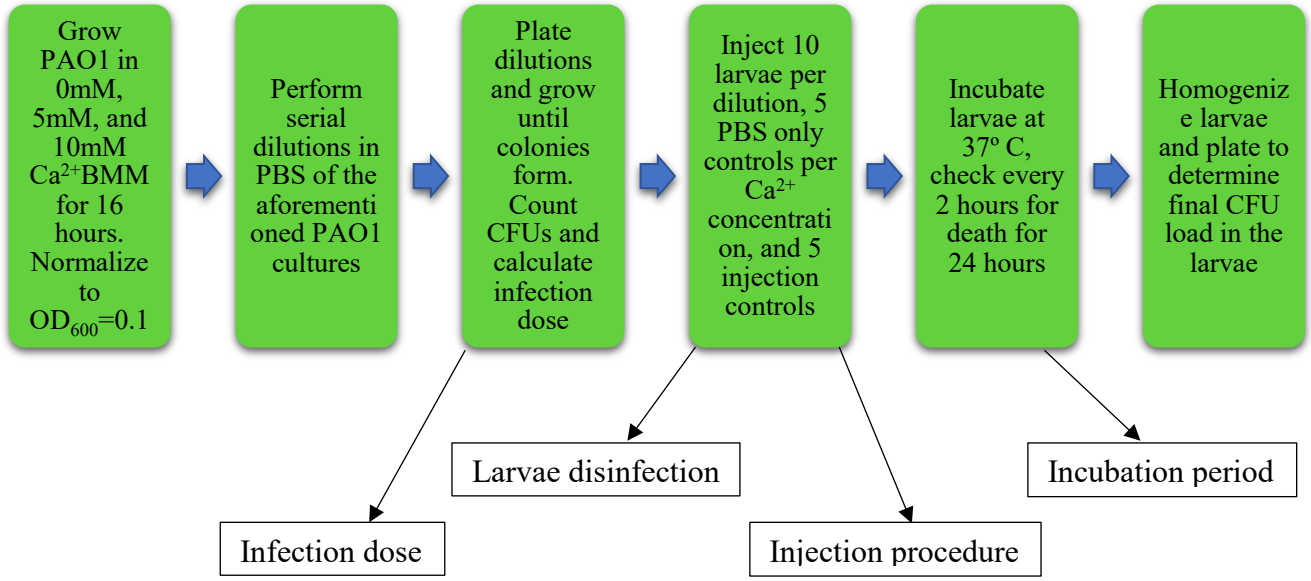

Figure 1: *G. mellonella* infection protocol. Arrows indicate points of optimization.

Infection dose. To optimize the infection dose, we first determined the optical density (OD_{600}) of PAO1 to attain $1x10^6$ CFUs/5 µL. To do so, PAO1 cultures were normalized to an OD_{600} equal to 0.3, 0.2, and 0.1. Each normalized culture was diluted from 10^{-1} to 10^{-6} in a 96-well plate and 10 µL was plated of each dilution from each OD_{600}. We discovered that an OD_{600} equal to 0.1 generated $1x10^6$ CFUs/5 µL. Next we aimed to determine what infection dose would give the best

ratio of live to dead larvae for comparison among testing groups, or the dose that killed over 50% and the dose that killed under 50% of the larvae. To discover this, we performed serial dilutions from 10^{-1} to 10^{-6} and injected *G. mellonella* larvae with 1,000 CFUs/5 µL, 100 CFUs/5 µL, 10 CFUs/5 µL, and 1 CFU/5 µL. The dilution that produced over 50% death was 10^{-5} which corresponded to 10 CFUs/5µL, and the dilution that produced under 50% death was 10^{-6} which corresponded to 1 CFU/5µL.

Larvae injection and disinfection. Larvae for the infection model were obtained from Speedy Worm, and used within 48 hours of delivery. After 72 hours, larvae begin to lose firmness and die. For ideal firmness of larvae for injection, the larvae were warmed up at 25 °C for 1-2 minutes in a glass petri dish. If warmed for a longer period, worms were too active to successfully inject. When injecting the larvae with PAO1, we wanted to avoid contamination from the surface of the larvae by commensal bacteria. To prevent contamination, we cleaned the site of injection using first 70% ethanol then 1 mg/mL Rifampicin in drops from a plastic syringe. To avoid drowning the larvae, only one to two drops of 70% ethanol and 1 mg/mL Rifampicin, an antibiotic that acts in inhibiting bacterial RNA polymerases. Larvae were injected into the last left proleg (Figure 2) slowly to avoid puncturing any vital structures.

Figure 2: *G. mellonella* injection

Larvae incubation. Larvae began cocooning at 72 hours, and death ceased to occur after 24 hours. To avoid cocooning and unnecessary incubation time, we began observation of the larvae 6 hours after injection, then every 2 hours for survival up to 24 hours.

Elevated Ca^{2+} Induces Virulence of PAO1 in *G. mellonella*

After optimizing the *G. mellonella* infection protocol, we aimed to determine the effect of elevated Ca^{2+} on the virulence of PAO1 in the model. For this, larvae were injected with PAO1 grown at three Ca^{2+} concentrations: 0 mM, 5 mM, and 10 mM. The larvae were monitored over the course of 24 hours to generate survival curves and calculate LD_{50}.

PAO1			*carP*::Tn5		*calC*::Tn5	
0 mM Ca^{2+}	5 mM Ca^{2+}	10 mM Ca^{2+}	0 mM Ca^{2+}	10 mM Ca^{2+}	0 mM Ca^{2+}	5 mM Ca^{2+}
2 CFUs	1 CFU	0.3 CFU	5 CFUs	6.1 CFUs	2.51 CFUs	1.28 CFUs

Table 1: Calculation of lethal dose 50% for PAO1, *carP*::Tn5, and *calC*::Tn5

To compare different conditions, we calculated the LD_{50} which is the infection dose that caused 50% death to the larvae. The LD_{50} determined for PAO1 at 0 mM, 5 mM, and 10 mM Ca^{2+} are shown in Table 1. The LD_{50} decreases as Ca^{2+} concentration increases. We also plotted killing curves (Fig. 3). Larvae injected with 10 CFUs of PAO1 grown at 0 mM Ca^{2+} began to die at 20 hours post infection (hpi), and had 10 % survival. At 5 mM Ca^{2+}, the larvae began to die 4 hours earlier with 20 % survival, and at 10 mM Ca^{2+} they began to die even earlier at 13 hpi with 0 % survival. Similarly, at infection dose of 1 CFU, each increase in Ca^{2+} concentration exhibited a faster death rate and lower survival (Fig. 3). Overall more death in the larvae was observed at 10 mM Ca^{2+} compared to 0 mM Ca^{2+}. The experiment was conducted with 0 mM Ca^{2+} three times, with 5 mM Ca^{2+} two times, and one time with 10 mM Ca^{2+}.

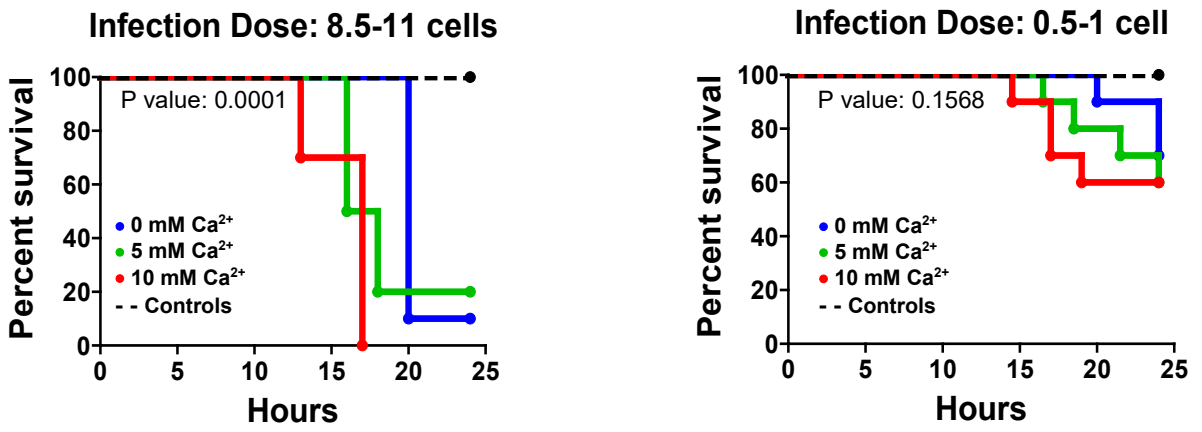

Figure 3: Graphs show percent survival for *G. mellonella.* Ten larvae per Ca^{2+} concentration were injected with 5µL of PAO1 grown at 0 mM Ca^{2+} (•), 5 mM Ca^{2+} (•), and 10 mM Ca^{2+} (•). Five larvae were injected with PBS, and five larvae were punctured (- -).

carP* Plays a Role in Virulence in *G. mellonella

carP is a gene previously found to play a role in mediating Ca^{2+} regulation of production of virulence factors, such as pyocyanin and pyoverdine, as well as swarming motility, in *P. aeruginosa* (Guragain et al., 2016). The gene codes for a protein predicted to function as a phytase. We hypothesized that *carP* also plays a role in the ability of *P. aeruginosa* to kill *G. mellonella.* Therefore, we expected that the mutant with disrupted *carP* would show reduced virulence. Considering our recent data suggesting that *carP* is regulated by quorum sensing, we aimed to test the role of the gene at different cell densities by injecting worms with two infection doses: 1 and 10 CFU.

To study the role of *carP* in *P. aeruginosa* virulence, *G. mellonella* larvae were injected with 10 CFUs and 1 CFU of *carP*::Tn5 grown at 0 mM Ca^{2+} and 10 mM Ca^{2+}. For larvae injected with 10 CFU, the calculated LD_{50} for *carP*::Tn5 (Table 1) was 2.5-fold higher at 0 mM Ca^{2+} and 20-fold higher at 10 mM Ca^{2+} than those for PAO1. The mutant *carP*::Tn5 grown in 0 mM Ca^{2+} exhibited death beginning 4 hours earlier than PAO1, but had 20% higher survival than PAO1 (Fig. 4). *carP*::Tn5 grown in 5 mM Ca^{2+} exhibited a similar relationship, with larvae dying 2 hours earlier than PAO1 and exhibiting 20% higher survival.

At the infection dose of 1 CFU, larvae injected with *carP*::Tn5 grown at 0 mM Ca^{2+} began dying at 11 hpi, 9 hours before larvae injected with PAO1. Larvae injected with *carP*::Tn5 at 1 CFU had 40 % survival, while larvae injected PAO1 had 70 % survival. At 10 mM Ca^{2+} and 1 CFU/5 µL, larvae injected with *carP*::Tn5 showed 40 % more survival than larvae injected with PAO1. The experiment was conducted twice with 0 mM Ca^{2+}, and once with 10 mM Ca^{2+}.

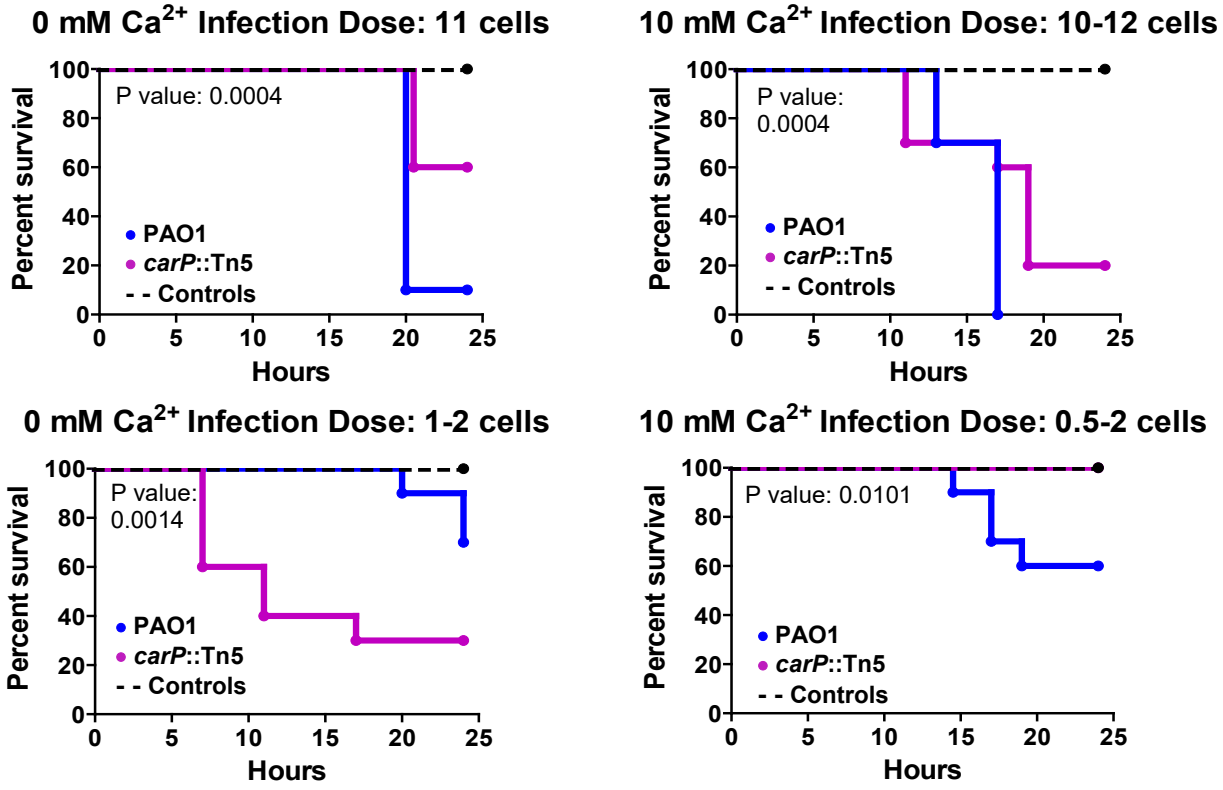

Figure 4: Graphs show percent survival for *G. mellonella*. Ten larvae per condition were injected with 5 µL of PAO1(•) or 5 µL of the *carP*::Tn5 mutant (•) at 0 mM Ca^{2+} and 10 mM Ca^{2+}. Five larvae were injected with PBS, and five larvae were punctured (- -).

calC::Tn5 Mutant Has a Slight Reduction in Virulence in *G. mellonella*

Our group collected data showing that *calC*, a gene coding for a putative Ca^{2+} leak channel, contributes to Ca^{2+} regulation of virulence factor pyoverdine and swarming motility. It is responsible for generating intracellular Ca^{2+} transients. We hypothesized that *calC* plays a role in Ca^{2+} induction of *P. aeruginosa* virulence in *G. mellonella*.

To study virulence of *calC*::Tn5 mutant, we injected *G. mellonella* larvae with *calC*::Tn5 grown at concentrations of 0 mM Ca^{2+} and 5 mM Ca^{2+}. The *calC*::Tn5 mutant exhibited a slightly higher LD_{50} compared to PAO1 at both 0 mM Ca^{2+} and 5 mM Ca^{2+} (Table 1). At 0 mM Ca^{2+} and 10 CFUs/5 µL, the larvae injected with *calC*::Tn5 began to die at 15 hpi and exhibited 10 % survival. At 5 mM Ca^{2+}, the larvae injected with *calC*::Tn5 exhibited 10 % more survival than PAO1, and a slower mortality rate (Fig. 5).

At 1 CFU/5 µL, The larvae began to die at 16 hpi when injected with the *calC*::Tn5 mutant at 0 mM Ca^{2+}, but both *calC*::Tn5 and PAO1 had 70% survival (Fig. 5). At 5 mM Ca^{2+}, larvae injected with the *calC*::Tn5 mutant died slower, but had the same total survival as PAO1 at 60 %. The experiment was repeated three times with both 0 mM Ca^{2+} and 5 mM Ca^{2+}.

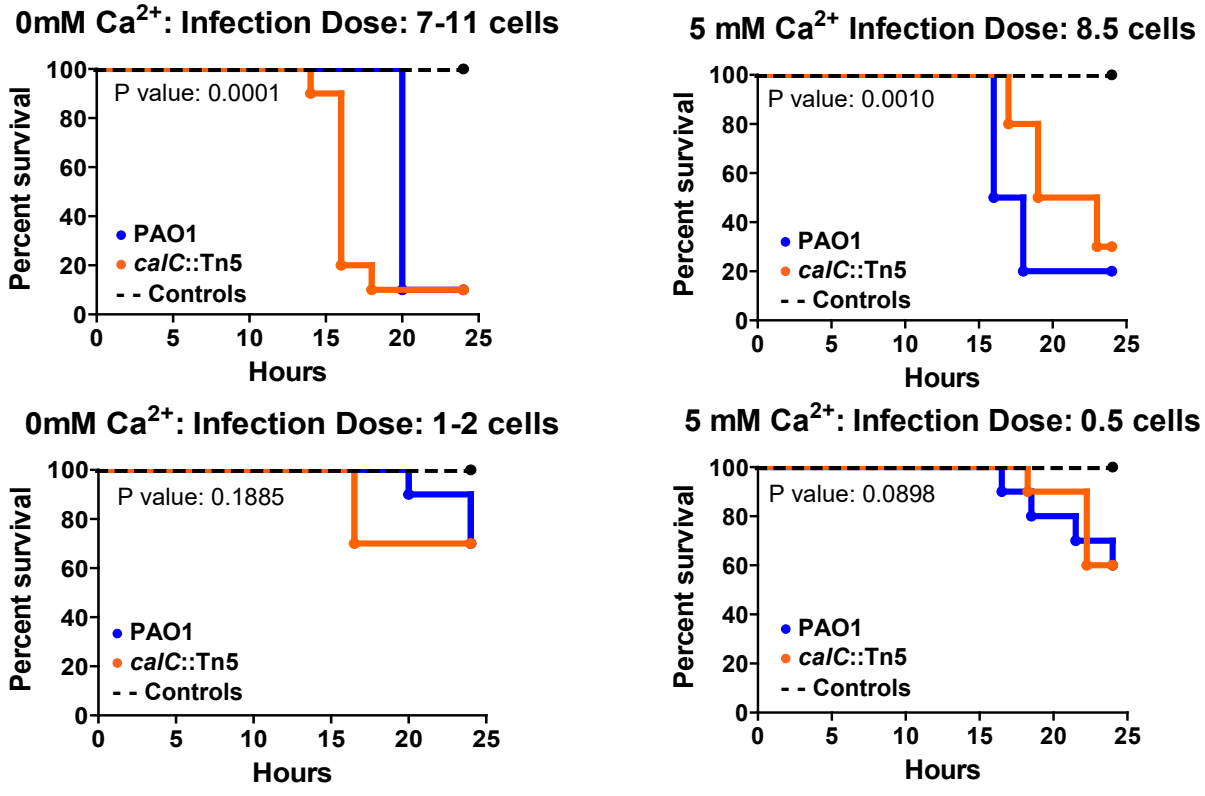

Figure 5: Graphs show percent survival for *G. mellonella*. 10 larvae per condition were injected with 5 µL of PAO1(•) or 5 µL of the *calC*::Tn5 mutant (•) at 0 mM Ca^{2+} and 5 mM Ca^{2+}. Five larvae were injected with PBS, and five larvae were punctured (- -).

DISCUSSION

P. aeruginosa is a pathogen that causes severe chronic infections in the airways of patients with cystic fibrosis. It is also known for causing hospital-acquired. It is highly antibiotic resistant and produces many virulence factors that aid in its pathogenicity, many of which are induced by elevated Ca^{2+}. The virulence factors induced by Ca^{2+} include production of pyocyanin, rhamnolipid, alginate, and extracellular proteases, as well as more complex behaviors such as swarming motility and biofilm growth (S. Sarkisova et al., 2005). Here we confirm that elevated Ca^{2+} enhances virulence of *P. aeruginosa* and its ability to kill larvae of *G. mellonella*. These data support previously described Ca^{2+} induction of PAO1 infectivity in lettuce leaves used as a plant model (S. A. Sarkisova et al., 2014). We explain such induction of virulence by Ca^{2+}-induction of individual virulence factors. For example, the extracellular protease, Elastase B, has been shown to digest proteins and peptides in the hemolymph of *G. mellonella* (Andrejko, 2012). We have previously shown that LasB biosynthesis is increased in the presence of elevated Ca^{2+} (S. Sarkisova et al., 2005). Although LasB abundance was measured *in-vitro*, we expect a similar regulatory event occurring *in-vivo* and shaping pathogenesis of *P. aeruginosa* in *G. mellonella*.

To better understand the behavior of *P. aeruginosa* in *G. mellonella* as a host, we aimed to quantify changes in bacterial load during infection and host response. For this, we plan to perform an immune assay called the prophenoloxidase (PPO) assay. Prophenoloxidase is an enzyme present in the hemolymph of *G. mellonella* that is post-translationally cleaved to form phenoloxidase. The latter activates a pathway that secretes molecules toxic to microbes. One of these molecules is melanin, the production of which can be measured by a colorimetric assay using dopamine as an activation of the cascade (Kopácek, Weise, & Götz, 1995). This assay has been used to characterize the immune response of *G. mellonella* during infection with *P. aeruginosa* (Zdybicka-Barabas & Cytryńska, 2010). The authors showed a correlation between the PPO

activity and immune response to an immune challenge. Collecting these data allows for a comparison between immune responses to PAO1 and the mutants at various Ca^{2+} concentrations. We are also in the process of testing the final bacterial load in the worms at 11 hpi. This will determine the level of replication of PAO1 and the mutants at different Ca^{2+} concentrations.

carP is a gene previously identified as a putative phytase in *P. aeruginosa*. The transcription of *carP* is regulated in a Ca^{2+}-dependent manner by CarSR, a two component system that itself is positively regulated by elevated Ca^{2+} (Guragain et al., 2016). Here we showed that disruption of *carP* gene led to a reduction in virulence at 10 mM Ca^{2+}. No Ca^{2+}-induced virulence was observed in the mutant, indicating the necessity of *carP* gene for Ca^{2+} induction of *P. aeruginosa* virulence. Recent research in my lab suggests that quorum sensing, the system bacteria use to regulate gene expression in response to cell density, is regulated by Ca^{2+}. Quorum sensing regulates expression of *P. aeruginosa* virulence factors, such as extracellular proteases, alginate, formation of biofilms, all of which contribute to pathogenicity in a host (Holm & Vikström, 2014). Furthermore, our most recent data suggests Ca^{2+} induction of *carP* transcription is also dependent upon quorum sensing regulation. Our data showing enhanced virulence in carP::Tn5 at lower density correlates with this data. Overall, these data confirm the role of *carP* in *P. aeruginosa* virulence. Such impact can be explained by the earlier established role of the gene in the production of virulence factors: pyocyanin, and pyoverdine.

Based on sequence similarities to established Ca^{2+} channels and its role in maintaining intracellular Ca^{2+} levels, CalC was predicted to function as a Ca^{2+}-leak channel in *P. aeruginosa*. Earlier, disruption of *calC* has been shown to abolish Ca^{2+} regulation of pyoverdine production, swarming motility, and biofilm production. Our data showed only a slight decrease in virulence of the mutant when compared to the wild type, PAO1. A quicker mortality was present in larvae

injected with the calC::Tn5 mutant compared to PAO1, but the same percent survival was observed. This result suggests that CalC and the intracellular Ca^{2+} transients may contribute to regulation of chronic type of infection, which may require a different animal model. The bacterial load assay and PPO assay will allow for better characterization of the pathogenesis of *P. aeruginosa* in relation to the gene, *calC*.

Overall, this study verified the role of Ca^{2+} in the induction of virulence in the animal model, *G. mellonella*. The experiments with the *carP*::Tn5 and *calC*::Tn5 mutants assisted in further characterization of the role of these genes in regulation of Ca^{2+}-dependent virulence. This study also assisted in optimizing an animal model for further characterization of *P. aeruginosa* virulence by testing a variety of mutants. Further characterization of the mechanisms coordinating Ca^{2+}-dependent virulence will lead to a better understanding of *P. aeruginosa* pathogenesis, and eventually to the development of better methods to kill this deadly pathogen.

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