5-2020

Second Messenger Cyclic-di-GMP Regulation in Acinetobacter baumannii

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by
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August 2019 – April 2020

An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
University Honors Program
Honors College
East Tennessee State University

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ABSTRACT

Over time, “superbugs,” or bacteria that have become resistant to antibiotics, have become a great concern in modern medicine. Viable alternates are currently being looked into as effective and safe ways to prevent or treat infections caused by these superbugs. One such method is through the utilization of the second messenger molecule cyclic-di-GMP (c-di-GMP) that has been shown to regulate phenotypes within other bacteria that may control surface colonization in *Acinetobacter baumannii*. Through a series of experiments, the active enzymes that create c-di-GMP - diguanylate cyclases - and break down c-di- GMP - phosphodiesterases - have been inactivated in mutants to test phenotypes including biofilm formation, motility, antibiotic resistance, and desiccation survival. The research’s objective is to show that manipulation of c-di-GMP within the multi-drug resistant strain of *Acinetobacter baumannii* may serve as a means to control this bacteria.

ACKNOWLEDGEMENTS

I would like to extend my greatest gratitude to Dr. Erik Petersen in the Health Sciences Department at East Tennessee State University. He was willing to take on an honors student's thesis as a recent hire at the beginning of the year, and he allowed the use of his research lab to conduct experiments throughout the school year. The patience and time invested into me and my work is greatly appreciated. I would also like to dedicate a few words to Dr. Charlotte Powers for her willingness to take part as well under extenuating circumstances. I would like to acknowledge Moriah Arnold, a previous undergraduate researcher who helped compiled some of the preliminary data that my thesis extends upon. Lastly, I would like to thank ETSU Honors College and the ETSU Office of Research and Sponsored Programs for awarding the Student-Faculty Collaborative Grant as it helped fund the materials needed for the experiment to take place.

TABLE OF CONTENTS

INTRODUCTION

Discovery of cyclic-di-GMP:

Cellulose, or poly-β-D-glucose is one of the most abundant biopolymers found across the world [8]. Most people already know that cellulose is found in plants; however, it has been found that some bacteria may also be capable of producing this biopolymer. The enzyme cellulose synthase, the enzyme responsible for cellulose synthesis, may have originated from cyanobacteria that has been postulated to form the symbiotic relationships with plants and is now the organelle chloroplast. Studies conducted by Manfred Aschner and Shlomo Hestrin at the Hebrew University of Jerusalem looked into the production of pure microcrystalline cellulose fibers from *Komagataeibacter xylinus,* an alpha-proteobacteria. Isolation of the cellulose synthase enzyme was attempted, but was determined that the enzyme required membrane-bound and soluble components to become active. Benziman and his colleagues were in search of the conditions that would activate cellulose synthase. It was his group that found that a Guanosine Triphosphate analog, guanosine 5'-[gamma-thio]triphosphate], was an effective stimulator to this enzyme. This was their key that lead them to be able to solubilize active cellulose synthase complexes that compared catalytically to a whole cell form. Once this was found, they set out to determine the structure of the activating factor. They had prior knowledge that it was a cyclic structure with a 3' to 5' bond. They soon found this molecule to be c-di-GMP as the activator factor. Benziman soon went on to find the GGDEF and EAL domains, which are diguanylate cyclases and phosphodiesterases involved in c-di-GMP turnover. These domains, along with curli fimbriae, played a key role in biofilm formation in other bacterial species such as *Salmonella* Typhimurium*, Escherichia coli, Citrobacter, Enterobacter,* and *Klebsiella* genera.

Mechanisms of cyclic di-GMP regulation:

Since Benziman's work, it has become more apparent that c-di-GMP plays a crucial role in the bacterial life cycle, as well as the virulence properties they possess that allow them to persist in today's world [8]. In addition, this signaling molecule is rudimentary to other important pathways such as quorum sensing and phosphorylation pathways. C-di-GMP consists of two GMP moieties bound by a 5'-3' cyclic ring and is one of the most well understood and researched cyclic dinucleotides. C-di-GMP levels within bacteria are controlled by both internal and environmental cues. This control is provided by the activity of two enzymes within the bacterium. These enzymes, diguanylate cyclases (DGCs) and c-di-GMP-specific phosphodiesterases (PDEs), can be found in all of the major bacterial phyla showing its prevalence as signaling proteins in the bacterial kingdom. The GGDEF domains of the DGCs arrange with one GTP, and dimerization allows them to perform condensation to create the c-di-GMP molecule. The EAL domain, which relates to the PDEs, is responsible for the hydrolysis of c-di-GMP to yield a linear pGpG molecule that is eventually broken down to two GMPs. Dimerization of these proteins are orchestrated by the N-terminal sensory domains. This would explain why so many different proteins are present within the genome.

C-di-GMP generates phenotypic changes through interaction with c-di-GMP-binding effectors that include transcriptional regulators, mRNA riboswitches, proteins that contain either GGDEF or EAL non-enzymatic domains, and proteins that contain a PilZ domain. Phenotypes regulated by c-di-GMP vary among bacterial species, but the motile-sessile transition and biofilm formation is a broadly conserved feature of c-di-GMP. Typically, it is shown that low levels of c-di-GMP are associated with motility, while the reverse can be said for biofilm formation and production. However, this system does not simply work as a "on/off" switch

within the bacteria. Motility and biofilm formation are regulated through various mechanisms depending on the bacteria of study. For instance, in *E. coli* and *Salmonella* Typhimurium increased levels of c-di-GMP interferes with both rotor-stator interface and the c-di-GMP-binding flagellar brake YcgR. Motile conditions activate PDEs to increase their affinity to c-di-GMP, permitting degradation and allowing effectors to increase motility. In terms of biofilm formation, bacteria can change their behavior when in contact with a surface. They begin exposing adhesins and producing an extracellular matrix to protect themselves. This behavior can be controlled by c-di-GMP at various levels within the cell. In addition, it is important to note that the gammaproteobacterial relative of *Acinetobacter baumannii, Pseudomonas aeruginosa,* exhibits isolates from human patients with cystic fibrosis showing mutations that lead to the upregulations of DGCs [8,10]. The use of second messenger molecules is a pretty straightforward system when compared to the protein-protein interactions that take places in a cell [8]. It is also easy, from an evolutionary standpoint, to incorporate changes into that system. While research on how c-di-GMP is incorporated into other pathways such as quorum sensing and phosphorylation continues, overlap of these systems suggests a broad role for c-di-GMP in bacterial signaling. Several bacterial virulence factors have been shown to be tied to c-di-GMP levels, indicating that modulation of c-di-GMP levels may show promise in possible future treatments of patients.

***Acinetobacter baumannii*:**

Acinetobacter baumannii is a Gram-negative bacillus [1]. It is an anaerobic, non-flagellated organism that is typically found within water and the soil. Due to the introduction of the notorious antibiotic penicillin by Alexander Fleming, people began treating everything with penicillin [6]. However, due to the over use of antibiotics throughout time since its discovery, it

has expedited the process in which bacteria handle medications. *Acinetobacter baumannii* has developed tactics such as porins, β-lactamase, modifications of aminoglycosides, upregulation of efflux pumps, and so much more in order to protect itself from antibiotics [10]. Today, *A. baumannii* is also a common nosocomial infection that produces resilient biofilms on many surfaces within hospitals [1]. Biofilm formation can take place on ventilators and catheters. Immunocompromised people and those with open wounds from surgery can be at serious risk if infected with this opportunistic, multi-drug resistant pathogen. Due to its virulence properties, it can persist and survive in a hospital environment despite the sterilization methods that take place to combat this pathogen. In addition, *Acinetobacter* has been nicknamed the "Iraqibacter" due to its infection of United States Military members in Iraq and Afghanistan. Due to strains of this organism developing multi-drug resistance, having the ability to effectively produce biofilms within hospital environments, and be an opportunistic pathogen, controlling the second messenger molecule cyclic di-GMP in order to control the virulence factors in *Acinetobacter baumannii* can be a new way to prevent nosocomial infections within the human population.

BACKGROUND

The mutants and their domains:

Previous work has been done with c-di-GMP and *Acinetobacter baumannii.* Moriah Arnold, Dr. Erik Petersen, and their colleagues have done a series of experiments with a multi-drug resistant clinical isolate (UW5075). They worked with transposon mutants deleted for each of the DGC- or PDE-encoding genes. Below, Figure 1 shows the mutants that correspond with diguanylate cyclase mutants.

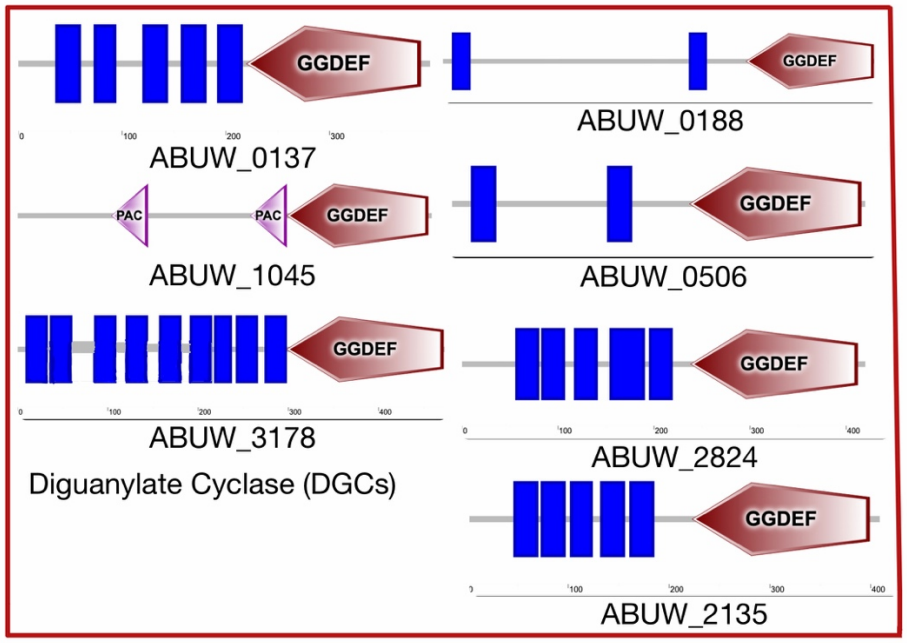

Figure 1: Diguanylate cyclase only proteins within *Acinetobacter baumannii*. Blue bars indicate transmembrane domains. PAC domains are sensory domain components.

In theory, knocking these GGDEF protein domains out will limit the production of c-di-GMP. However, some proteins contain both a GGDEF and an EAL domain. These both possess DGC and PDE activity regulated by their sensory domains.

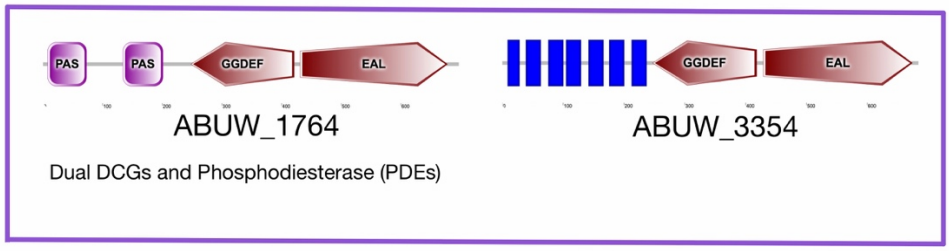

Figure 2: Dual DCG and PDE domains within *A. baumannii* proteins. PAS domains are sensory domains.

In continuation, we come to two strains with degenerate domains. In these cases, the active site residues within the protein have been mutated, but these domains may retain the ability to bind c-di-GMP or act in some other regulatory role. While both appear to contain both

GGDEF and EAL domains, in 1221 the EAL domain is predicted to be active but the GGDEF domain may be inactive due to mutations within the active site. Further, 1138 is inactive at its EAL domain and it is questionable if a GGDEF domain is even present.

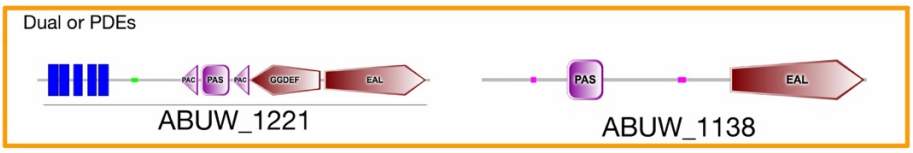

Figure 3: *A. baumannii* proteins containing degenerate enzymatic domains.

Lastly, the 2255 mutant, as shown in figure 4, does not have a DGC or PDE domain but instead has a PilZ domain. As mentioned before, PilZ is a c-di-GMP-binding domain and is responsible for type IV pili motility in other organisms.

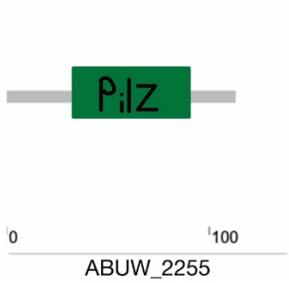

Figure 4: PilZ domain.

Figures 1-4 are used for a better understanding of which phenotypes will be expected of each mutant.

Biofilm assay:

For the biofilm assay, cell cultures were diluted to an 0.05 optical density in lysogeny broth [3,11]. 200 microliters were then placed in a 96 well plate and grown overnight without shaking. They form a barrier along the interface of the water and air to adhere to the plastic.

Excess culture was removed and washed. Crystal violet was used to stain. After additional washing to remove the crystal violet stain, 100% ethanol was added to dissolve the crystal violet and mixed well. Then the intensity of the crystal violet was measured at 590 nanometers and converted in terms of the wild type. As shown in the figure below, our mutants who showed statistical significance were 0137, 1221, and 3354 and had lower levels of biofilm formation. In this case, it is most likely that their protein domains are behaving has DGCs. If this is the case, cyclic-di-GMP levels will decrease and lead to the inhibition of biofilm production and stimulate motility.

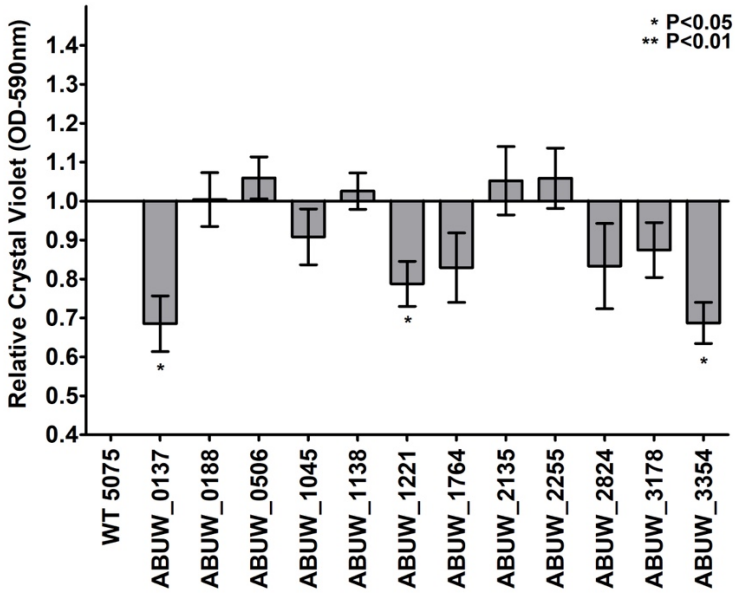

Figure 5: Relative biofilm formation of the *A. baumannii* mutants.

Twitching Motility:

An agar plate was made with a higher water to agar ratio to allow a more liquified plate [3,11]. Then one microliter of 1.0 OD_{600} cell culture was stabbed in the center of the plate and

incubated at 37ºC. *A. baumannii* is able to twitch along the lower surface of the agar at the plastic interface, generating a circular zone. Distance was then measured in centimeters and put in terms to the wild type.

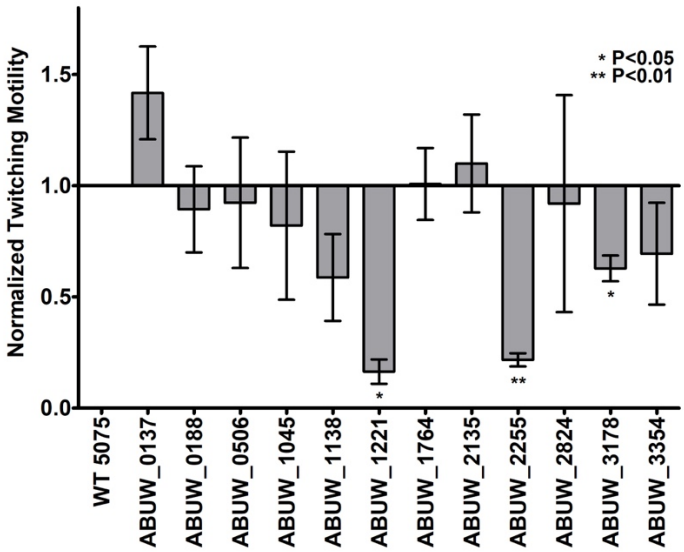

Figure 6: Twitching Motility Plates

The above figure shows that mutants 1221 and 2255 showed severe decrease in motility. In the case of 1221, the protein domain must be acting as a PDE since it is categorized as a dual or only EAL domain. 2255 is the PilZ domain which would conclude why motility decreases.

Rifampicin Assay:

The next assay is determination of the minimum inhibitory concentration (MIC) [3,11]. Very few antibiotics are effective due to this being the hospital isolate strain. However, some mutants showed increased resistance to rifampicin, including 0137. Overnight cultures were diluted to 0.05 OD_{600} and were grown overnight in a 96 well plates. Eleven different two-fold dilutions of rifampicin took place while a twelfth one had no antibiotic. Measurements of relative

optical density compared to the non-treated well was how results was recorded. It was shown that the mutant 0137 grew better with rifampicin when compared to the wild type.

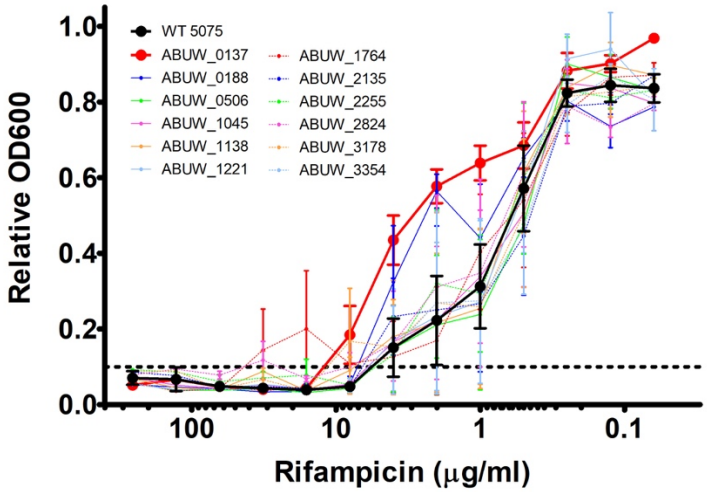

Figure 7: MIC of Rifampicin

MATERIALS AND METHODS

Collection of domain images:

The visual images collected above (Figure 1-4) for the different domains of the mutant knockouts was compiled through use of the Prokaryotic Genome Analysis Tool (PGAT) and the protein database of Simple Modular Architecture Research Tool (SMART) websites [12,13]. Mutants' identification credentials were used on PGAT in order to find the protein sequence. Once the protein sequence was retrieved, it was put into the SMART protein database in order to produce the visual aids.

Desiccation Assay:

The desiccation assay was performed in a similar outline laid out by Biswas and Rather [3]. Frozen cultures of the *Acinetobacter baumannii* mutants were collected and streaked for

isolation onto lysogeny broth plates. Overnight growth cell culture was collected and washed in one milliliter of water three times by centrifugation. Cultures were then place into cuvettes, optical density measured at 600 nm, and samples were diluted to 1.0 optical density. From there, 10 microliters of each sample was placed into their respective wells on a 96 well plate in triplicate. The 96 well plate was then incubated at 37° Celsius until the water had evaporated, then plates were transferred to 25°C. After two days, samples were resuspended in 200 microliters of lysogeny broth through a well plate shaker. From there, fivefold dilutions took place in neighboring wells that contained phosphate-buffered saline. Once dilutions were completed, seven microliter droplets were collected from the dilutions and transferred onto dried petri dishes. The plates were allowed to sit at room temperature and colonies were allowed to grow at 37°C until countable. Colonies were then counted and recorded as shown in the results section. This was done on both inoculum samples as well as day 2 samples.

RESULTS

Once the data was collected, it came to light that some of the mutants were not properly diluted on the inoculum day as planned in the materials and methods section. Therefore, final values were not adjusted to this inoculum count. The raw colony-forming unit per milliliter values were calculated for each particular mutant. They were then converted into a Log_{10} and put through the ANOVA test to determine statistical significance. Everything was then converted to the relevance of the wild type whose survival rate was set to 100% in order to compare the mutants' survival to what was expected of the wild type data. Our negative control *Escherichia coli* wild type is also present within the graph (Figure 8).

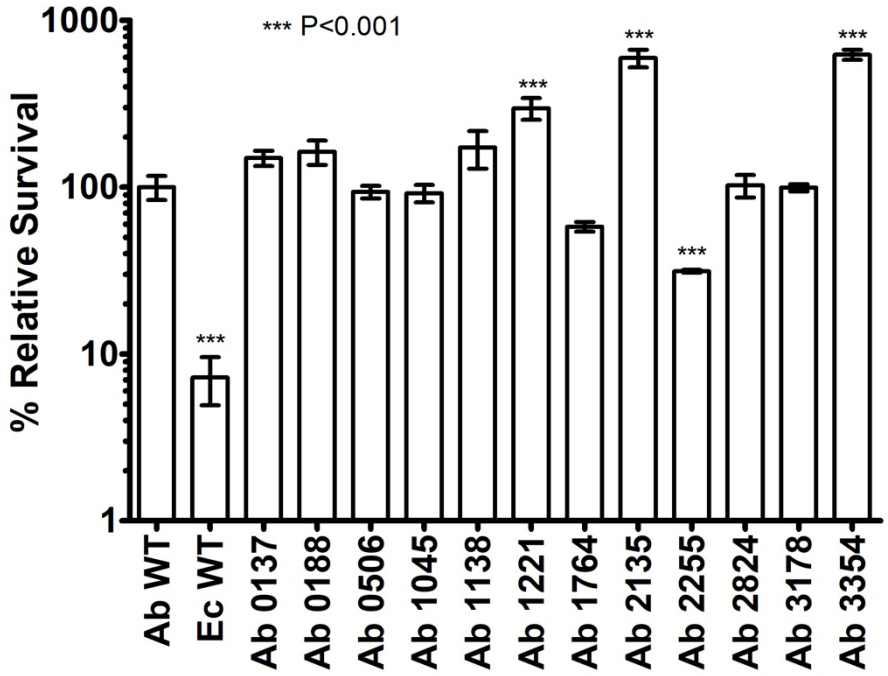

Figure 8: Percent Relative Survival where *E. coli* wild type is used as a negative control. *A baumannii* mutants 1221, 2135, 2255, and 3354 show statistical significance.

DISCUSSION

Desiccation assay:

Mutant 2255 has shown significance in both the desiccation assay as well as the motility assay. This can be speculated that by hindering motility survivability decreases under desiccation. This could be a failure to sense a surface, failure to adhere to the plastic, or send signals to stimulate the pilus interaction needed. This can even be extended to 1221 since it also showed a phenotypic change within the twitching motility assay. However, 1221 shows increased survivability. In the case of 2135, it can be hypothesized that deleting this gene, which is a diguanylate cyclase domain, can decrease concentration of cyclic di-GMP. By decreasing the concentration of this second messenger molecule, survivability under desiccation increases. As

seen before, knocking out the genes within 3354 not only increases survivability, but it also decreased the amount of biofilm *A. baumannii* produced. 3354 and 1221 are interesting mutants to show significance because they show to have both a diguanylate cyclase and the phosphodiesterase protein domains. 1221 one and 3354 shows increased survivability. This can be argued that in this instance they act as DGCs. This can be a complicated matter and leads us to the future steps of the experiment.

For the future:

It is clear from this series of results that more research needs to be done to completely understand the role of cyclic di-GMP in *Acinetobacter baumannii.* An additional test that can be done with the multi-drug resistant strain 5075 is to perform an assay that accounts for concentrations of cyclic di-GMP within the cell. Mutants of 3354 and 1221 would need mutations in one of the active site domains at a time to determine which domain is responsible for motility, biofilm formation, and increased survivability in the desiccation assay. In continuation, complementation tests of the biofilm, twitching motility, antibiotic resistance, and desiccation assays will need to be conducted to ensure that deletion of these genes is responsible for the phenotypes. Further, direct measurement of concentrations of cyclic di-GMP need to performed, and these assays can be replicated in a second strain of *A. baumannii* to determine whether these phenotypes are conserved within other *Acinetobacter* strains. By designing these mutants and performing the series of experiments laid out, a more cohesive answer will emerge on how these different genes play a role in virulence.

CONCLUSION

Acinetobacter baumannii is a multi-drug resist bacterium that is responsible for nosocomial infections in patients by residing on plastics and hard surfaces through biofilms. The rising risk of superbugs that have grown resistance to an array of antibiotics is a growing concern in modern day medicine. It has been shown that the second messenger molecule, cyclic di-GMP, can control virulence factors, such as biofilm formation. The experimentation that has been done so far has shown that cyclic di-GMP does show means of controlling virulence factors within *Acinetobacter baumannii.* This shows promise in combating this multi-drug resistant bacterium and needs further research to be more conclusive on the exact mechanisms that are taking place.

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