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Cyclic-di-GMP-binding proteins regulate Acinetobacter baumannii motility

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Cyclic-di-GMP-binding proteins regulate *Acinetobacter baumannii* motility

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Abstract

Acinetobacter baumannii is a prevalent nosocomial pathogen where, like many other infectious bacteria, *A. baumannii* is increasingly considered a multi-drug resistant pathogen. This research study was designed to find a way to affect the persistence of *A. baumannii* such that it can be applied to a hospital setting to prevent further nosocomial infections. One regulatory mechanism potentially used by *A. baumannii* to persist on hospital surfaces is through the use of the bacterial second messenger cyclic-di-GMP (c-di-GMP). This nucleotide signal is regulated in response to environmental conditions, and then activates c-di-GMP-binding proteins that induce phenotypic changes. One c-di-GMP-regulated phenotype is bacterial motility, and reducing motility may alter *A. baumannii*'s ability to colonize and persist on hospital surfaces. I hypothesized that *A. baumannii* uses c-di-GMP-binding proteins to regulate motility. *A. baumannii* encodes two potential c-di-GMP-binding proteins of interest, one that contains a sole c-di-GMP-binding PilZ domain and another that pairs a PilZ domain with a hydrolase enzymatic domain. I am also testing two *A. baumannii* strains: AB5075, a recent multi-drug resistant military hospital isolate and 17978, an established lab strain. A notable difference between these two strains is that AB5075 demonstrates twitching motility where it utilizes type IV pili, while 17978 demonstrates swarming motility that has unknown mechanisms. Both c-di-GMP-binding proteins were tested for their role in motility for the particular *A. baumannii* strain. While I am still generating the deletion strain for the c-di-GMP-binding hydrolase enzyme in AB5075, the sole PilZ domain protein is required for twitching motility, while both c-di-GMP-binding proteins are required for 17978 swarming motility. Future plans include determining the role of the c-di-GMP-binding hydrolase enzyme in twitching motility and identifying the role that these proteins play through binding of c-di-GMP.

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INTRODUCTION

Acinetobacter baumannii

Acinetobacter baumannii is an aerobic, Gram-negative bacterium that is increasing in prevalence as a pathogen and is cause for concern because of its extraordinary resilience. Strains of *A. baumannii* are repeatedly surfacing that are either multidrug-resistant (MDR) or pandrug-resistant (PDR). *A. baumannii* is primarily a nosocomial, opportunistic pathogen that is not cause for concern for those who are not immunocompromised or immunosuppressed. However, those that are in the hospital setting are typically immunocompromised or immunosuppressed due to a preexisting condition. This is where *A. baumannii* commonly causes infections of the lung, soft tissue, blood stream, and urinary tract (1). Specifically, *A. baumannii* becomes cause for concern because of this virulence that is a result of its increasing drug resistance and persistence.

As a result, *A. baumannii* has become increasingly hard to treat with most available antibiotics, which necessitates a need for an alternate form of treatment. Although *A. baumannii* accounts for only 2% of healthcare-associated infections in the United States and Europe, 45% of isolates globally are considered MDR, climbing to nearly 70% of isolates in Latin America and the Middle East (2). Due to its significantly increased prevalence in infections of soldiers and veterans who have served in Iraq and Afghanistan, *A. baumannii* has earned the name "Iraqibacter" (3). Finally, *A. baumannii* infections primarily occur in the intensive care unit (ICU) and are now accounting for nearly 20% of ICU infections worldwide (3).

A. baumannii belongs to the class of gammaproteobacteria, placing it in close proximity to other common pathogenic bacteria like *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella*. An arsenal of factors play into *A. baumannii*'s ability to survive and infect. Of

particular interest are *A. baumannii's* mechanisms for persistence on a surface such as biofilm formation, desiccation survival, and motility. A biofilm is akin to a community of bacteria that are joined together through what is referred to as an extracellular matrix (2). These biofilms are formed using capsular exopolysaccharides that are excreted from the bacterial cells out where they form a sticky layer or net that allows for cell adhesion to the matrix itself and to other surfaces (3). To assist with attachment to surfaces, adhesion molecules called adhesins are also employed. These biofilms offer a selection of advantages when it comes to resistance and persistence in unfavorable conditions. In particular, biofilms allow for survival on the abiotic surfaces found throughout healthcare settings notably endotracheal tubes, polycarbonate, and stainless steel where conditions are dry and there are little to no nutrients (2). Persistence, unlike resistance, is not necessarily a bacterial cell's ability to resist treatment with antibiotics or cleaning agents through a specific mechanism but is the result of the population surviving through the sacrifice of those cells that are outermost or proximal to the treatment so that the inner cells may live and carry the population. With all of this in mind, *A. baumannii's* ability to form biofilms extremely well is now being considered one of its primary virulence factors (4).

A more complex virulence factor of *A. baumannii* is desiccation survival. Simply put, desiccation survival is the ability of the microbe to remain viable in dry conditions, although it is multifactorial and ill defined (2). Despite the lack of knowledge of the exact mechanisms, it is known that *A. baumannii* has a remarkable ability to survive extended periods of desiccation, and there is a hypothesis that the capsular polysaccharides used in a biofilm have a role in water retention thereby enhancing desiccation survival (2). There are other hypothesized mechanisms beyond the water retention that are thought to contribute to the desiccation survival capabilities

of *A. baumannii*. Further understanding of mechanisms that factor into desiccation survival are yet another avenue to be explored in an effort to combat this resilient MDR pathogen.

Lastly, many bacteria use motility mechanisms to help colonize and persist on surfaces. *Acinetobacter* as a genus is considered to be nonmotile, and its name translates from Greek to mean nonmotile rod. While not possessing flagellar motility, *A. baumannii* is a species that demonstrates not only motility but different forms of motility. The two strains of *A. baumannii* being used in this study both demonstrate one of these two forms of motility. AB5075 is a multidrug-resistant recent clinical isolate that demonstrates twitching motility, while 17879 is a common laboratory strain that demonstrates swarming motility. Twitching motility in *A. baumannii* is surface associated and type IV pilus-mediated because these pili are used to extend out onto the surface, tether or attach, and retract; this can be thought of as dragging itself along the surface (5). However, the swarming motility phenotype demonstrated in 17978 does not have a known mechanism, although flagellar genes are not present within either genome. It can be seen from this that motility can serve as another virulence factor that allows *A. baumannii* to migrate along surfaces in settings such as those that are healthcare associated.

Cyclic-di-GMP

Cyclic-di-GMP (c-di-GMP) is a bacterial second messenger that is regulated by environmental stimulus in order to produce phenotypic changes – including biofilm formation and motility regulation – that give the bacteria a better chance at surviving environmental conditions (6). C-di-GMP does this by binding to specific c-di-GMP-binding effector proteins that are then able to regulate and produce certain phenotypes as a result of increased or decreased c-di-GMP levels. These c-di-GMP-binding proteins are of interest in this study because of their

ability to produce not just a phenotype but a clinically relevant phenotype that has the potential to affect *A. baumannii's* ability to remain on less than ideal surfaces in hospital settings. Breaking down the entire signaling pathway for c-di-GMP starts with GTP molecules that are converted to c-di-GMP by diguanylate cyclases (DGCs) (6). These DGCs contain a GGDEF domain and dimerize to condense two GTP molecules to form a single c-di-GMP molecule (6). These c-di-GMP molecules are then able to bind the specific c-di-GMP binding proteins or effector molecules. Additionally, c-di-GMP levels also have to have a way to be down regulated to reverse these phenotypes produced by increased c-di-GMP levels. This is done by phosphodiesterases (PDEs) which are composed of an EAL or HD-GYP domain (6). EAL domains function to hydrolyze c-di-GMP to produce pGpG while HD-GYP domains hydrolyze c-di-GMP into GMP (6). Both DGCs and PDEs are often regulated through the presence of N-terminal sensory domains, linking c-di-GMP enzymatic activity to environmental sensing (7). The levels of c-di-GMP present and thus the amount binding to these effector molecules is then able to produce a phenotype. Through these mechanisms c-di-GMP is downregulated, and thus a phenotype is regulated. Biofilm formation as well as motile-to-sessile transition is generally believed to be associated with increased levels of c-di-GMP (6). It follows that knocking out or disabling one of the c-di-GMP-binding proteins that are associated with the production of a certain phenotype would serve to reveal the association itself as well as revealing some information about the mechanism(s) at play when the effector domain(s) are bound by c-di-GMP.

Cyclic-di-GMP-Binding Effectors

Once c-di-GMP has been made, there must be further mechanisms that use or involve c-di-GMP such that it regulates bacterial processes. The most common mechanism and the one being looked at in this study is the binding of c-di-GMP to effector domains or proteins. C-di-GMP binding to these effector proteins causes them to produce a phenotypic change within the cell that is advantageous to its survival in the current environment. Effectively, then, c-di-GMP is able to regulate certain phenotypes based on environmental stimulus. This is where the clinical importance of *A. baumannii's* ability to survive through certain phenotypes such as motility meets the mechanisms of c-di-GMP. If there is a known protein that functions to regulate a phenotype like motility through some mechanism and binds c-di-GMP, then being able to affect that protein or mechanism would have potential to be a treatment to prevent *A. baumannii* from spreading due to surface colonization.

This is where the PilZ c-di-GMP-binding domain that has been studied in other bacteria comes into play (7). In this study, there are two genes of interest that are annotated and appear to be conserved throughout the *A. baumannii* species. The first of these genes is annotated A1S_0490 in AB17978 and ABUW_3388 in AB5075. This gene contains a PilZ c-di-GMP-binding domain in addition to a hydrolase domain. The annotated hydrolase domain resembles those of the alpha-beta fold that is able to cleave a number of different potential unknown substrates (8). Because of this wide variety of functions and the lack of knowledge, it is unknown what role the hydrolase domain plays individually or in relation to the PilZ domain. These genes will be referred to as PilZ/Hyd. The other gene of interest is annotated as A1S_1559 in AB17978 and ABUW_2255 in AB5075, and this gene contains just a lone PilZ domain. This gene will be called PilZ. The PilZ domains are named after a protein that functions to regulate the type IV

pili-mediated motility in other organisms in the "dragging" action previously described (7). Originally discovered in *Pseudomonas aeruginosa*, PilZ was noted to remove twitching motility and susceptibility to a fimbria specific phage and reversed these phenotypes when complemented back (9). Since this, PilZ c-di-GMP-binding domains have been discovered in various proteins that carry out a variety of functions. My hypothesis is then that these genes may regulate the twitching motility, swarming motility, or both motility phenotypes of *A. baumannii*.

METHODS AND MATERIALS

Bacterial Growth Conditions

A. baumannii cultures were grown in lysogeny broth (LB) medium at 37°C shaking at 250 rpm unless noted otherwise. The following antibiotics were used for selective media: tetracycline (10 μg/mL, Tet_{10}), kanamycin (50 μg/mL, Kan_{50}), rifampicin (50 μg/mL, Rif_{50}), ampicillin (100 μg/mL, Amp_{100}), chloramphenicol (34 μg/mL, Cam_{34}) and gentamicin (30 μg/mL, $Gent_{30}$). *Escherichia coli* strains Mach1 and DH5α were used for cloning purposes. These strains were grown in LB typically or super optimal broth with catabolite repression (SOC) for transformations and shaken at 250 rpm and 37°C unless noted otherwise. Motility media used in motility plates for 17978 mutants were made using 0.5x LB (12.5 g/L LB Nutrient Powder) and 0.4% agar (4g/L of bacteriological agar). 5075 motility assays used normal LB plates. All plates were incubated at 37°C, and motility plates were placed in the incubator for approximately 24 hours.

Name	Genotype	Source
Mach1	*E. coli* str. W Δ*recA1398 endA1 fhuA Φ80*Δ*(lac)M15* Δ*(lac)X74 hsdR(rK–mK+)*	Invitrogen

DH5α	*E. coli fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Thermo Fisher
pRK2013	Helper plasmid for triparental mating	ATCC
17978	*A. baumannii* 17978 wild type	ATCC
5075	*A. baumannii* 5075 wild type	(10)
GRAB054	*A. baumannii* 17978, pMMBRif	This study
EPAB008	*A. baumannii* 5075, pMMBRif	This study
GRAB007	*A1S_0490::KanR* (ΔPilZ/Hyd)	This study
GRAB076	*A1S_0490::KanR*, pMMBRif-*A1S_0490* (pPilZ/Hyd)	This study
GRAB077	*A1S_0490::KanR*, pMMBRif (pV)	This study
GRAB012	*A1S_1559::KanR* (ΔPilZ)	This study
GRAB046	*A1S_1559::KanR*, pMMBRif-*ABUW_2255* (pPilZ)	This study
GRAB045	*A1S_1559::KanR*, pMMBRif (pV)	This study
AB05915	*ABUW_2255::Tn26Tet* (ΔPilZ)	(11)
GRAB035	*ABUW_2255::Tn26Tet* / pMMBRif-*ABUW_2255* (pPilZ)	This study
GRAB036	*ABUW_2255::Tn26Tet* / pMMBRif (pV)	This study

Table 1: Bacterial Strains Used.

Cloning

Deletion of A1S_0490 (PilZ/Hyd) and A1S_1559 (PilZ) was generating using double homologous recombination. The plasmid vector was modified to perform a double homologous recombination such that it would be homologous to the target region in the bacterial genome where the gene is located except for the gene itself. Deletion vectors were constructed through Gibson cloning techniques (12), and primers are included in **Table 3**. Genomic DNA was prepared from the *A. baumannii* 17978 strain using the MasterPure Genomic DNA Isolation Kit (Lucigen), and PCR fragments of adjacent regions of homology to the genes of interest were generated using Phusion DNA polymerase and primers corresponding to each fragment (**Table 3**). The suicide vector used to generate 17978 PilZ/Hyd & PilZ knockouts was pEX18Gm, a suicide vector which contains a SacB counterselection marker and gentamicin resistance cassette on the backbone (13). In short, homologous genomic PCR fragments were generated from *A. baumannii* 17978 genomic DNA and the kanamycin marker from pKD4 and were then ligated together with PCR fragments corresponding to the pEX18Gm vector using Gibson cloning (12).

Deletion vectors were then transformed into chemically competent or electrically competent *E. coli* cells. Successful transformations were selected using Kan_{50} plates as a result of the cloned Kanamycin resistance cassette expressed from the integrated vector. Successful mutants were then conjugated into *A. baumannii* using the pRK2013 conjugation helper strain (14). Due to the natural resistance of *A. baumannii* to chloramphenicol, this antibiotic was used to remove the plasmid donor *E. coli* strain after conjugation. After initial selection on LB+Kan_{50}+Cam_{34} agar plates, colonies were grown up in LB media to permit recombination of the deletion vector out of the genome. This culture was struck to LB+Kan_{50} plates containing 6%

sucrose to select for loss of the SacB counter selection marker. Successful deletion of the gene of interest was first determined through selection of kanamycin-resistant, gentamicin-sensitive colonies, and then confirmed through colony PCR.

Complementation of deleted genes was performed using the pMMBRif vector to allow varying levels of expression of the gene using isopropyl β-D-thiogalactopyranoside (IPTG) induction. Similar to the gene knock outs, primers were used to create Gibson PCR fragments that have regions of homology using Phusion DNA polymerase, and genomic *A. baumannii* 5075 DNA was prepared using the MasterPure Genomic DNA Isolation Kit (Lucigen). These fragments were then Gibson cloned together and transformed into chemically or electrically competent *E. coli* cells. Successful transformations were selected using Rif_{50} agar plates due to the rifampicin resistance cassette expressed by the pMMBRif vector. Helper strain pRK2013 was then used to conjugate the successful mutants into *A. baumannii,* and successful mutants were selected using LB+Rif_{50}+Cam_{34} agar plates. Successful conjugation was confirmed through the selection of rifampicin resistant colonies and PCR with confirmation primers.

Name	Plasmid	Ab Resistance	Description
pEP377	pEXTet-1221	Tet	Suicide vector to generate clean deletion of ABUW_1221
pEX18Gm	pEX18Gm	Gent	Parent vector to be modified (13)
pMMBRif	pMMBRif	Rif	Empty vector for *Acinetobacter* expression (15)
pKD4	pKD4	Kan/Amp	Source of Kan resistance gene (16)

pGR001	pEX18Gm-490KO-Kan	Kan/Gent	Deletion vector for A1S_0490 from 17978
pGR024	pMMBRif-A1S_0490	Rif	Complementation vector for the hydrolase PilZ in 17978
pGR006	pEXTet18Gm-1559KO-Kan	Kan/Gent	Deletion vector for A1S_1559 from 17978
pGR022	pMMBRif-ABUW_2255	Rif	Complementation vector for A. baumannii ABUW_2255 and A1S_1559 in pMMBRif

Table 2: Plasmids Used.

Primer Name	Primer Sequence	Primer Purpose
A1S_0490 KO 5' GFwd	ACGGCAGGTAAGCTAATTCCGCCGCCAAGATAGCTGC	5' Gibson fragment primers for deletion of 0490 in the 17978 Ab genome
A1S_0490 KO 5' GRvs	GAAGCAGCTCCAGCCTACACCATGTGGTACGAGCTGGC	
A1S_0490 KO 3' GFwd	CTAAGGAGGATATTCATATGCCAGAAACAAGCAGCTTAACG	3' Gibson fragment primers for deletion of 0490 in the 17978 Ab genome
A1S_0490 KO 3' GRvs	ACGACAGGAGCACGATCATGGTTTGACTCTAACCGCCCAC	
A1S_0490-KOConf-Fwd	GCTCAAGACGATCAAGTAAGGC	Primers outside the original deletion region to

A1S_0490-KOConf-Rvs	CAGTCGATTTATTTACCGAAGCC	confirm deletion of A1S_0490
A1S_1559 KO 5' GFwd	ACGGCAGGTAAGCTAATTCCCATTTAGCGGAAACAGCATCAG	5' Gibson fragment primers for deletion of 1559 in the 17978 Ab genome
A1S_1559 KO 5' GRvs	GAAGCAGCTCCAGCCTACACGACCTGAATAATTCCACCCATTTG	
A1S_1559 KO 3' GFwd	CTAAGGAGGATATTCATATGGGTAGTATGTCTTTAGATCGCCC	3' Gibson fragment primers for deletion of 1559 in the 17978 Ab genome
A1S_1559 KO 3' GRvs	ACGACAGGAGCACGATCATGCCACCGTATCATGTTTTGCTG	
A1S_1559-KOConf-Fwd	GAGCCAGGTGAACGC	Primers outside the original deletion plasmid to confirm deletion of A1S_1559
A1S_1559-KOConf-Rvs	AATACCATGTGTAGATTGTTCAGCTCG	
ABUW_2255Comp-Fwd	ATATTAGAATTCATGCAACCACAAATGGGTG	Primers to generate Gibson fragment of complementation vector for ABUW_2255
ABUW_2255Comp-Rvs	ATATTAAAGCTTCTTTCCTATCTGCTTACATGGT	

Table 3: Primers Used.

Motility Assays

Motility assays for strain 17978 were completed using an overnight culture of the wild type strain or PilZ/Hyd and PilZ mutants. Overnight cultures were vortexed, and the OD_{600}

reading was taken. Overnight cultures were diluted in LB in a microcentrifuge tube to create 1.0 OD_{600}. 1 μL of 1.0 OD_{600} mix was then pipetted onto the surface of the LB 0.4% agar plates. Distance traveled from motility was measured 24 hours later, and both length and width were measured and averaged to determine the spread diameter. Motility assays for 5075 were similar except for the use of specific motility plates, and the sample was stabbed through the agar to the plastic plate. This is done due to the difference in swarming and twitching motility respectively.

RESULTS

Both the PilZ and PilZ/Hyd proteins regulate strain 17978 swarming motility

To determine whether the predicted c-di-GMP-binding proteins PilZ and PilZ/Hyd regulate swarming motility in *A. baumannii* strain 17978, deletion mutants for each respective protein were compared to wild type (WT) on 0.4% LB agar swarming motility plates. PilZ and PilZ/Hyd mutants both showed significantly reduced motility in reference to 17978 WT. The measured diameters were relativized to WT with WT being 100% distance traveled (**Figure 1**). This indicates that both PilZ and PilZ/Hyd play a role in the motility for 17978.

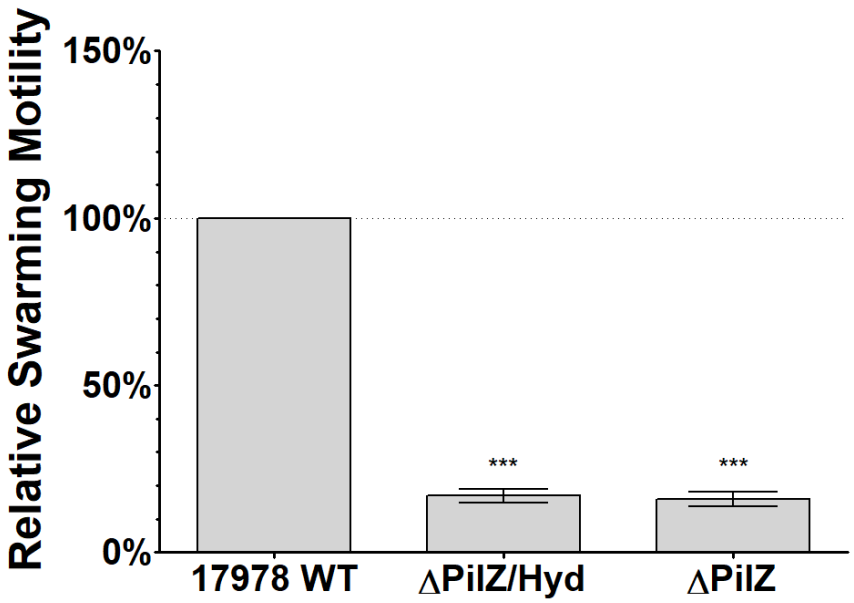

Figure 1: Normalized swarming motility for PilZ/Hyd & PilZ KO mutants in 17978. Mutants are normalized relative to WT being 100%. Samples were compared using one-way ANOVA with Dunnett's post hoc test. n=7 *** = P<0.001

The PilZ protein regulates twitching motility in strain 5075

While deletion of the PilZ/Hyd protein is still on-going in strain 5075, I sought to investigate whether the PilZ protein regulated twitching motility in this strain. The PilZ mutant in 5075 showed a statistically significant decrease in twitching motility in relation to WT. Twitching distances were relativized to the 5075 WT with it being 100%. The PilZ mutant knockout almost completely removed all motility (**Figure 2**). Additionally, the PilZ gene was complemented back into the 5075 PilZ deletion strain and almost completely restored the twitching motility in 5075. This indicates that the PilZ gene plays a pivotal role in 5075 twitching motility.

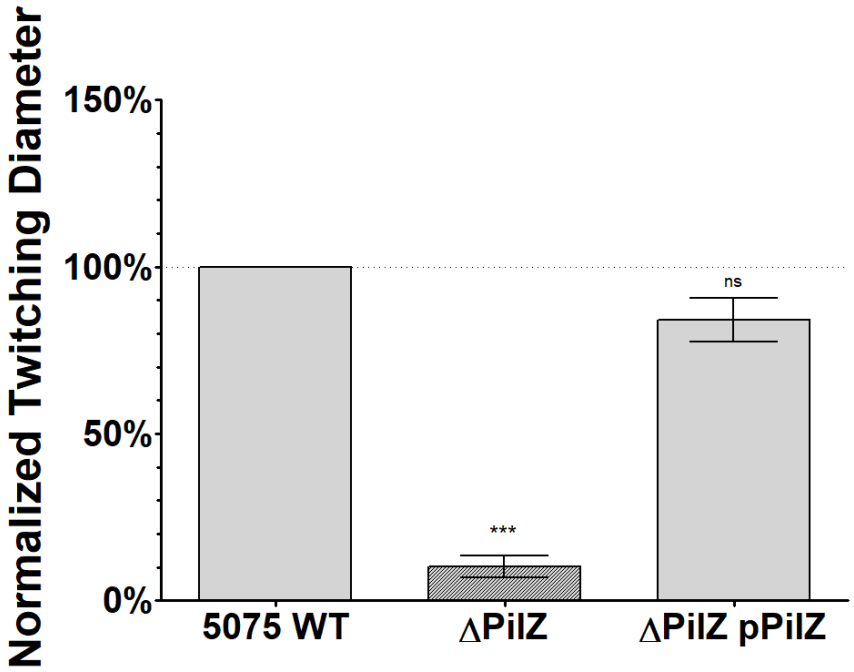

Figure 2: Normalized twitching motility for PilZ and the PilZ complemented mutants in 5075. Mutants are normalized relative to WT being 100%. Samples were compared using one-way ANOVA with Dunnett's post hoc test. n=3 *** = P<0.001

DISCUSSION

Because of *A. baumannii's* increasing prevalence as a nosocomial pathogen, membership of the ESKAPE pathogens, and incredible ability to take up DNA and develop drug resistance, there is an increasing need for research in order to find novel techniques and treatments that can be used to treat the strains of *A. baumannii* that are developing multidrug-resistance. This research investigates annotated c-di-GMP-binding proteins in *A. baumannii* to see if and what role they play in the motility of *A. baumannii* to then be implemented in an effective treatment for *A. baumannii*.

The annotated PilZ and PilZ/Hyd domains in *A. baumannii* were promising genes because of other research done in other genera and species where they were shown to be c-di-GMP binding and to have various phenotypic effects such as regulation of motility (5, 7, 9).

Specifically, the PilZ gene was named for its essential function with the fimbrial biogenesis of type IV fimbriae in *Pseudomonas aeruginosa* (9). Further research on the PilZ protein in conjunction with FimX in *Xanthomonas citri* revealed that PilZ with FimX interact with PilB in the process of type IV pili biogenesis, however the exact mechanism was unknown (17). In *Xanthomonas* and *Pseudomonas*, PilZ, which is generally known to be a c-di-GMP binding protein, does not bind c-di-GMP (17). Instead, in these genera FimX instead binds c-di-GMP despite having degenerate forms of REC, PASS, GGDEF, and EAL domains with the EAL domain retaining its ability to bind c-di-GMP (17). This study also showed that in *X. citri* and *Xanthomonas campestris*, PilZ and FimX complex directly but in *P. aeruginosa* did not show the same direct interactions and instead showed direct interactions of FimX and PilB (17). All of this research tends to indicate that PilZ plays a significant role in pili biogenesis in addition to other phenotypes. This combination of a significant role in motility and other phenotypic regulation makes PilZ a high priority in terms of research on disruption and understanding interactions and roles for future treatment development.

The PilZ/Hyd gene contains a hydrolase domain that resembles those of the alpha-beta fold which is known to have a vast selection of substrates that it is able to cleave (8). The data shown in **Figure 1** demonstrates that the PilZ/Hyd gene is essential for swarming motility in 17978, but this data also demonstrates that the PilZ gene is also essential for swarming motility in 17978. It would appear that both the PilZ and PilZ/Hyd genes are needed to maintain the swarming motility phenotype as knocking either out individually significantly decreases swarming motility. Evidence, then, is not conclusive that hydrolase activity itself is essential for the swarming phenotype. Further research is needed such that the hydrolase part of this gene is removed or disrupted in order to test whether or not it plays a significant role in the motility

phenotype of 17978. At present, no research has been done on this dual PilZ/Hydrolase protein as no studied homologs exist in other organisms.

The PilZ domain in The PilZ domain in 5075 follows suit to the PilZ domain in 17978 by almost entirely abolishing the twitching motility in 5075 when the gene was knocked out as seen in **Figure 2**. This helps to construct a narrative of PilZ playing a significant role in motility in *A. baumannii* through an unknown means. Further research is needed to determine the mechanism that PilZ plays for motility whether it be through the binding of a secondary protein to form a complex or some other more direct interaction that may also play a role in extension and/or retraction. Complementation of the PilZ gene shows almost complete restoration of twitching motility phenotype in 5075, further strengthening the idea that PilZ is playing a pivotal role in 5075 twitching motility.

Of great interest is whether or not the PilZ/Hyd protein in 5075 regulates the twitching motility phenotype just as it did swarming motility in 17978. If it does, there may be a conserved mechanism that PilZ/Hyd serves in *A. baumannii*. If knocking out the PilZ/Hyd gene in 5075 did not function to down regulate the twitching phenotype in 5075, this may indicate that the hydrolase domain plays a distinct role in swarming motility that makes it essential for one but not the other. These investigations may also begin to uncover the mechanism of swarming motility in those *A. baumannii* strains that exhibit this alternative motility phenotype.

Data not shown reveals that the deletion of PilZ in 5075 decreases the 48 hour desiccation survival. Although this does not reveal any specifics about the mechanism that PilZ plays in motility and/or desiccation survival, it does indicate that it appears to play some role in the multifactor desiccation phenotype. Knowing that PilZ likely plays some role in the biogenesis or function of pili, it follows that disrupting part of that system could also translate

into an altered phenotype for desiccation as this desiccation likely relies on biofilm formation. As previously stated, biofilm formation involves the secretion of exopolysaccharides, motile to sessile transition, and the adherence to a surface. This adherence could be inhibited by the loss or malfunction of pili that typically function for that surface attachment. PilZ may play a role in signaling the transitions between motile and sessile states, and the removal of PilZ may eliminate a signal that continuous upregulates motility unless a sessile state is more advantageous.

Future Work

More work is needed to better understand what role and the exact mechanisms PilZ, PilZ/Hyd, and the individual hydrolase domain are playing in the various motility phenotypes. Generating the PilZ/Hyd deletion mutant in *A. baumannii* strain 5075 is needed in order to test whether it plays a role in the twitching motility phenotype. To move toward a better understanding of their mechanism, c-di-GMP binding assays and mutagenesis of their c-di-GMP binding sites are also needed to see whether these proteins are binding c-di-GMP and what loss of c-di-GMP binding causes in terms of phenotypes. Making complementation mutants of the 17978 deletion mutants with an expression inducible vector such as pMMB is needed in order to confirm that the deletion of the various genes is the cause of the altered phenotype(s). Finally, in the quest to find genes that can be targeted to treat *A. baumannii*, more genes of interest need to be explored that have the potential to be vital for *A. baumannii* survival and are relatively easy to treat.

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