Abstract

Pseudomonas aeruginosa is an opportunistic bacterial pathogen that infects a variety of eukaryotic organisms. Virulence gene expression in *P. aeruginosa* is regulated by small, CheY-like response regulator proteins PilG and PilH. How these proteins function is unknown. Uncovering the targets with which PilG and PilH interact is essential to understanding the molecular events that control *P. aeruginosa* virulence and lead to subsequent infection. To investigate how PilG and PilH function, we sought to determine their cellular localization patterns using fluorescence microscopy. PilG and PilH were tagged on their carboxyl-termini with an isoform of green fluorescent protein optimized to be monomeric (mGFP). We mated plasmids containing these fusion protein genes into a common strain of *P. aeruginosa* (PAK) that contained a chromosomal reporter gene for measuring virulence factor expression. The PAK strains used were *pilG* or *pilH* deletion mutants such that the inserted plasmid contained the sole copy of *pilG* or *pilH*. Immunoblotting was performed on the experimental PAK strains to determine the conditions necessary to elicit wild type expression levels of the fusion protein PilGmGFP. At wild type expression levels, reporter gene assays that indirectly measure PilG and PilH function show that PilGmGFP does not retain function. Localization data were not collected due to unknown effects of PilGmGFP loss of function. Future investigations will focus on uncovering visual localization techniques that do not negatively impact Pil proteins. Amino-terminal fluorescence tagging is being pursued as a means to study localization without protein disruption.

Introduction

Cystic fibrosis (CF) is a genetic disease characterized by hyperosmolar and abnormally viscous mucus in the lungs (Oliver et al., 2000). The defect is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) (Therien et al., 2001). The lungs traditional bacterial defenses, mucociliary clearance, polymorphonuclear neutrophil phagocytosis, and local cationic peptide release are all considerably impaired in the CF lung (Oliver et al., 2000). It is the impaired immune defense resulting from CFTR mutation that is responsible for CF and the host of bacterial infections that follow, including chronic colonization by *Pseudomonas aeruginosa*.

P. aeruginosa is an opportunistic bacterial pathogen capable of infecting a variety of eukaryotes, including humans (Fulcher et al., 2010; Bertrand et al., 2010). With a pervasive prevalence in water and soil, *P. aeruginosa* stands as a considerable threat to immunocompromised patients (Mandell et al., 2005). The CDC estimates that over 50,000 new cases of *P. aeruginosa* infection occur annually in the United States, with an increasing percentage of these cases being multi-drug resistant infections (CDC, 2014). Of particular importance is the nearly universal infection of cystic fibrosis patients with *P. aeruginosa* (Smith et al., 1996; Goldman et al., 1997).

To further understand *P. aeruginosa* infection, studies have focused on the molecular mechanisms that enable the bacteria to colonize tissue. One such mechanism being studied is Type IV pili (TFP), which are multifunctional filamentous surface appendages produced by *P. aeruginosa.* It has been shown that *P. aeruginosa* TFP act as adhesins that enable bacterial binding to mammalian epithelial cells and respiratory-tract mucins (Strom and Lory, 1993). As a result, TFP-mediated attachment plays a critical role in *P. aeruginosa*'s ability to colonize tissue

(Strom and Lory, 1993). Because virtually all CF patients are infected with *P. aeruginosa*, it is presumed that the CF lung environment is an exceptional niche for *P. aeruginosa* adhesion and subsequent colonization.

Previous studies have shown that TFP proteins are part of a suite of virulence factors that are regulated by second messengers (Beatson et al., 2002; Wolfgang et al. 2003). The second messenger molecule adenosine 3'5'-cyclic monophosphate (cAMP) has been shown to play a critical role in regulating virulence. Synthesized primarily by adenylate cyclase enzyme CyaB[1], cAMP acts as an allosteric regulator of the transcription factor Vfr (Virulence factor regulator) (Fulcher et al., 2010). Together, cAMP and Vfr are considered global regulators of virulent gene expression in *P. aeruginosa* (Suh et al., 2002), controlling multiple systems including TFP regulation (Beatson et al., 2002; Wolfgang et al., 2003). In summation, TFP production and function, key components of *P. aeruginosa* infection, are dependent on intracellular cAMP and Vfr levels. We focus on cAMP production and its relationship to the TFP proteins PilG and PilH.

PilG and PilH are two CheY-like response regulators that are part of the Chp chemosensory system (Fultcher et al., 2010). The Chp chemosensory system has been shown to both positively and negatively regulate CyaB-dependent cAMP levels (Fulcher et al., 2010). *PilG* deletion mutants show a severe drop in cAMP (~80% drop from WT levels) while *pilH* deletion mutants show a roughly three-fold increase in cAMP (Fulcher et al., 2010).

The objective of this study is to develop fluorescent fusion proteins to investigate the localization patterns and molecular targets of PilG and PilH in *P. aeruginosa.* It is because of PilG and PilH's tremendous effect on intracellular cAMP and, thus, on TFP production, function,

[1] A second adenlyate cyclase, enzyme CyaA, synthesizes a marginal amount of cAMP in *P. aeruginosa*. However, studies have shown that CyaB is the primary regulator of cAMP for virulence pathways (Wolfgang et al., 2003). To more precisely study cAMP as it relates to virulence, our working strains are Δ*cyaA*.

and overall virulence that we have chosen to study this system. The mechanisms by which PilG and PilH function are unclear; however, CheY-like proteins such as PilG and PilH are typically regulated through phosphorylation (Bertrand et al., 2009). It has been proposed that PilG modulates pilus extension and PilH allosterically modulates pilus retraction (Bertrand et al., 2009). Because of function and preliminary testing, it is presumed that PilG localizes to the leading pole of the cell and that PilH is a diffuse intracellular protein. Our plan was to test protein localization using GFP fluorescent tagging. To uncover potential PilG and PilH targets, we sought to screen mutant strains and look for changes in localization.

Materials and Methods

All plasmids for this study were isolated using Promega Wizard Plus SV Miniprep DNA Purification System. PCR amplicons were purified using Qiagen QIAquick PCR Purification Kit. Cloning was performed using Invitrogen Gateway LR and BP clonase II. Refer to the appendix for a summary of plasmids, primers, and strains used in this study.

Construction of a stable, monomeric GFP. Plasmid pRGFP was isolated from *Escherichia coli* strain DH5α as a source of *GFPmut3*.[2] The plasmid was used as a template for splice by overlap extension mutagenesis. Primers 1 and 2 were used to amplify a portion of *GFP*; primers 3 and 4 were used to amplify the remaining portion of *GFP* by PCR.[3] Primers 2 and 3 introduced a point mutation (L221K) in the amplified PCR products. The introduced point mutation was shown to effectively eliminate any potential for GFP dimerization and

[2] GFPmut3 is an isoform of GFP optimized for expression in bacteria. All GFP used in this study are mut3 and will henceforth be referred to as GFP.

[3] See appendix for full primer sequences.

unpredictable localization at high concentrations (Zacharias et al., 2002). The mutation is referred to as *monomericGFP*, or *mGFP*.

The two *GFP* PCR products with the introduced point mutation (L221K) were spliced together and amplified using primers 5 and 6. This amplification product was cloned into pDONR201 plasmid by way of BP Gateway Cloning. Following cloning, *E. coli* strain DH5α was transformed with pDONR201-*mGFP*. Plasmid pDONR201-*mGFP* was isolated and sequence confirmed using primers 11 and 12.

***pilGmGFP* and *pilHmGFP* construction via splice by overlap extension**. In *pilGmGFP* construction, *mGFP* was amplified from pDONR201-*mGFP* by PCR with primers 4 and 9. Plasmid pMMBV1-*pilG* was used to amplify *pilG* via PCR using primers 7 and 8. Primers 8 and 9 were designed with an overlapping region encoding SGGGG as a strong, flexible linker between PilG and mGFP. Primers were designed to attach mGFP to the carboxyl-terminus of PilG.

Constructing *pilHmGFP* was performed using the same methods as *pilGmGFP*. PCR amplification of *mGFP* was done on pDONR201-*mGFP* using primers 4 and 13. Plasmid pMMBV1-*pilH* was used to amplify *pilH* via PCR using primers 14 and 15. As in *pilGmGFP* construction, primers 13 and 14 were designed with an overlapping region encoding SGGGG as a flexible linker between PilH and mGFP. Primers were designed to attach mGFP to the carboxyl-terminus of PilH.

Amplified *pilG* was spliced to *mGFP,* and amplified *pilH* was spliced to *mGFP* in individual PCR reactions using primers 5 and 6. These fusion proteins were cloned into pDONR201 plasmid using BP Gateway Cloning. Following cloning, *E. coli* strain DH5α was

transformed with pDONR201-*pilGmGFP* and pDONR201-*pilHmGFP*, independently. Both plasmids were isolated from DH5α and sequence confirmed using primers 11 and 12.

Cloning into inducible plasmids and mating with *P. aeruginosa*. *pilHmGFP*, *pilGmGFP*, and *mGFP* were cloned from their pDONR201 plasmids into two plasmids, pMMBV1GW and pMMBGW, which have different degrees of inducible expression in *P. aeruginosa*. The destination pMMB- plasmids were linearized with restriction enzyme BspEI (New England Biolabs Inc.). *pilGmGFP*, *pilHmGFP*, and *mGFP* were inserted into pMMB- plasmids using LR Gateway Cloning. Following cloning, *E. coli* strain DH5α was transformed.

pMMB- plasmids containing *pilGmGFP* and *pilHmGFP* constructs were individually mated into *P. aeruginosa* PAK strains with chromosomal *pilG* or *pilH* deletions such that the introduced plasmids contained the sole copy of *pilG* or *pilH*. The PAK strains used also contain a chromosomal reporter gene for measuring virulence factor expression. A helper *E. coli* strain pRK2013 was used to assist in a tri-parental mating. At this time plasmid pMMB67EH was mated into PAK strains as an empty vector control for future experiments.

PCR. PCR was conducting using phusion hotstart II polymerase and following reaction setups recommended by the manufacturer (Thermo Fischer). Initial denaturation was performed at 98° C for 1 minute. The following was performed for 35 cycles: denaturation at 98° C for 15 s, annealing at 58° C for 20 s, and extension at 72° C for 45s. A final extension was performed at 72° C for 3 minutes to finish replication.

B-galactosidase assays. The PAK strains used in this study contained a background chromosomal insertion, *lacP1⊿lacI-lacZ,* that enables indirect measurement of cAMP levels by measuring B-galactosidase activity. In *E. coli*, CRP binds to the P1 promoter region of the *lac* operon and facilitates transcription of *lacZ* in the presence of cAMP. Similarly, in *P. aeruginosa*, Vfr functions as a CRP homolog that interacts with the P1 promoter and, in the presence of cAMP, facilitates the transcription of *lacZ* (Fulcher et al., 2010). In summation, PilG and PilH regulate cAMP levels positively and negatively, respectively. cAMP acts as a cofactor for the transcription of *lacZ*, which codes for B-galactosidase. Therefore, B-galactosidase activity reflects cAMP levels, and can be used to surmise the functionality of fusion proteins PilGmGFP and PilHmGFP. The assumption being made is that if PilGmGFP or PilHmGFP are able to regulate cAMP in a wild type fashion, then the fluorescent tags are not inhibiting their function.

P. aeruginosa strains with pMMB- *pilGmGFP* inserts were streaked for isolation and cultured overnight on LB plates with carbomycin (150 ug/ml). Single colonies were then picked and inoculated in LB broth with carbomycin (150ug/ml) overnight at 37° C and 300 rpm. Overnight inoculations were diluted 1:100 in LB broth with low dose antibiotic (carbomycin 50 ug/ml) and a gradient of IPTG[4] concentrations. An IPTG gradient was used to determine the induction concentration that produced wild type expression levels of *pilGmGFP*. Samples were grown to $OD_{600} \approx 0.5$ and placed on ice to arrest growth. At this point, 1 ml samples were taken for immunoblotting.

B-galactosidase assays were conducted with three technical replicates per sample. To 0.05 ml of sample, 0.95 ml of Z buffer + B-mercaptoethanol ($1000_{Z\text{-}Buffer}$:$3.58_{B\text{-}ME}$) were added and vortexed briefly. To each sample, 15 µl of 0.1% SDS and 15 µl of chloroform were added to

[4] IPTG is a synthetic sugar that induces expression of our pMMB- plasmids. IPTG is not readily metabolized, so its cellular concentration remains relatively constant throughout experiments.

lyse cells, with brief vortexing between each addition. Samples were incubated at 37° C for 5 minutes. To develop samples, 0.2 ml of developing solution (4 mg ONPG/ml Z-buffer + B-ME) were added. Samples were vortexed briefly and returned to a 37° C water bath for timed developing. Developing was stopped by the addition of 0.5 ml 1M Na_2CO_3 and brief vortexing after the sample had turned a straw-yellow color, or if an hour had passed. Samples OD_{420} was then measured and Miller Units[5] calculated.

Immunoblotting. Aliquots of 1 ml were taken from B-galactosidase assay samples of *P. aeruginosa* with pMMB- *pilGmGFP* inserts grown to $OD_{600} \approx 0.5$. Aliquots were pelleted, supernatant removed, and the pellet re-suspended in 50 µl of loading buffer ($950_{\text{SDS-Page 2x Loading Buffer}}$:$50_{\text{B-ME}}$). Samples were then boiled before being loaded onto 12% acrylamide SDS-PAGE gels. Gels were run using10 µl of sample, separated at 150V for roughly 1 hour. Proteins were subsequently transferred to nitrocellulose at 100V for 1 hour. PilG rabbit antibodies (1:10,000) and 800 nm anti-rabbit secondary antibodies (1:10,000) were used to develop blots.

Results

The objective of this study was to construct PilG and PilH fluorescent fusion proteins to study their localization patterns in *P. aeruginosa*. Sequencing showed success in point mutating *GFP* (L221K), creating a monomeric copy of GFP that does not dimerize or localize at high concentrations (Zacharias et al., 2002). *PilGmGFP* and *pilHmGFP* fusions were sequence confirmed to ensure no mutations had occurred during the splicing process. By observing fluorescence in *E. coli* expressing PilHmGFP, we surmised that the fluorescent tag was

[5] A Miller Unit is a unit-less dimension that represents B-galactosidase activity normalized for differences in OD_{600} and developing time.

functional (figure 1). Conducting B-galactosidase assays and immunoblotting tested PilGmGFP function and expression in *P. aeruginosa*. The mechanisms underlying the B-galactosidase assay can be found in the methods. In general, we compared trends in B-galactosidase activity between mutants and wild type controls.

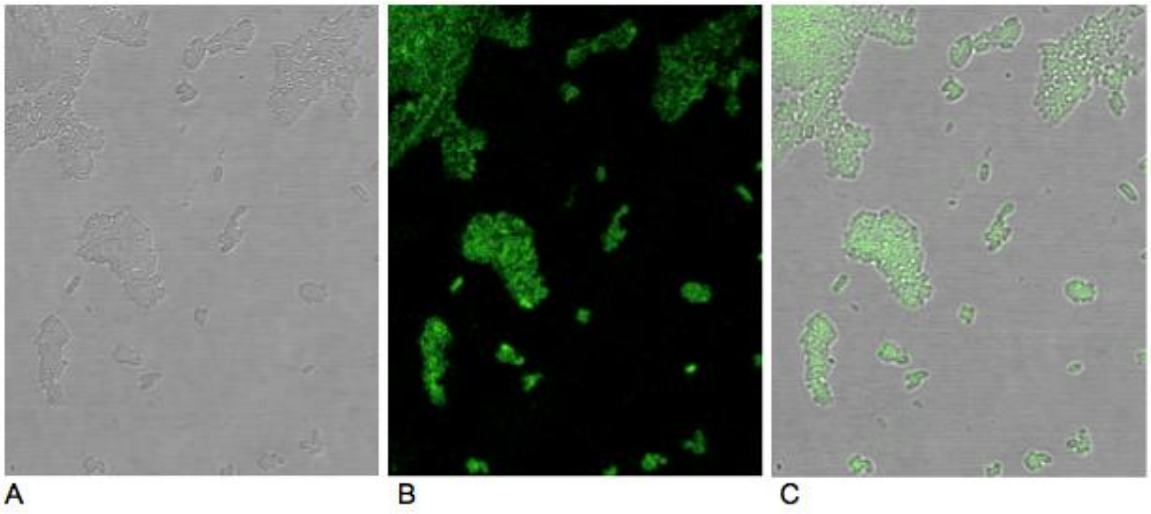

Figure 1. *E. coli* strain DH5α pDONR201-*pilHmGFP* inoculated with 50 μmol IPTG. Image captured using Leica TES SP8 microscope. Image **C** (100x magnification) is an overlay of images **A** (captured without fluorescence) and **B** (captured fluorescence at 480 nm).

B-galactosidase assay and immunoblotting for pMMBV1GW-*pilGmGFP* complementation

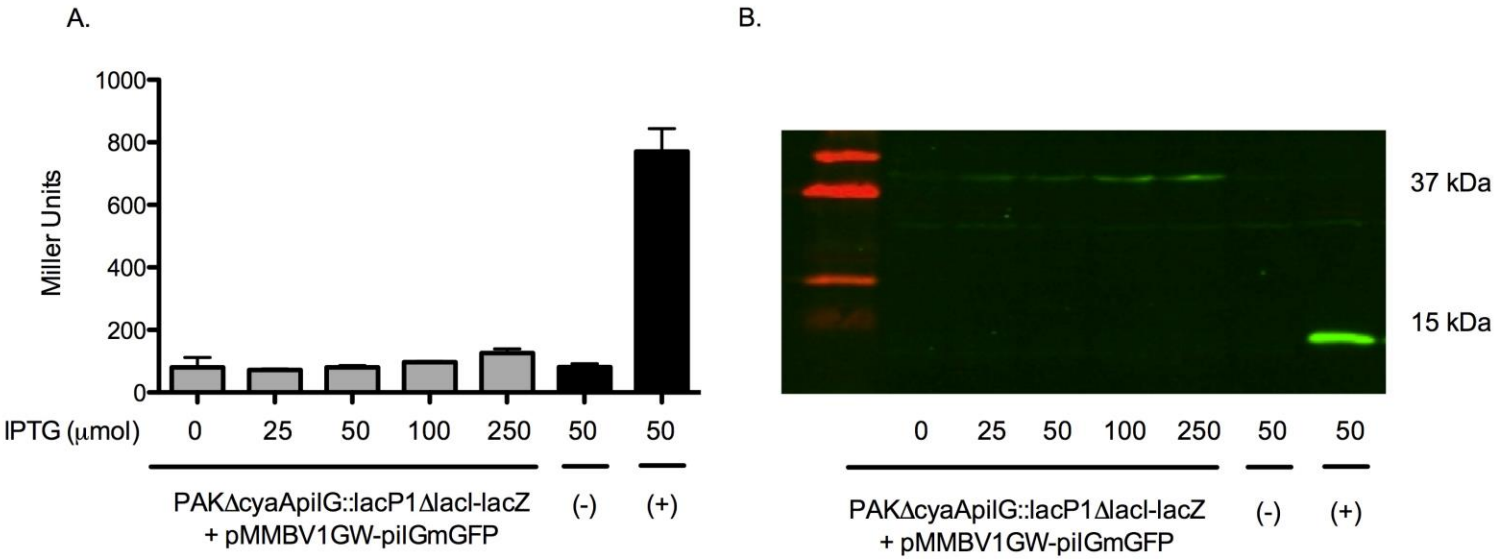

Figure 2A. B-galactosidase activity across an IPTG induction gradient to test PilGmGFP functionality. The (+) label refers to a pseudo-WT positive control and (-) refers to a *ΔpilG* negative control (appendix). Error bars represent SD between three technical replicates. **Figure 2B** represents an immunoblot of the same samples depicted in **2A.** Bands at ~38 kDa represent PilGmGFP and bands at ~14 kDa represent PilG.

The data in figure 2A show that mutants complemented with pMMBV1GW-*pilGmGFP* were not restored to wild type B-galactosidase activity. Instead, complemented mutants reported similar B-galactosidase activity as the negative control Δ*pilG* mutants. The expression of *pilGmGFP* for these samples was tested through immunoblotting (figure 2B). Qualitative analysis of figure 2B reveals increasing *pilGmGFP* expression matching the increasing IPTG gradient. Comparisons between experimental and positive control bands indicate that *pilGmGFP* expression in the complemented strain is not restored to wild type levels. For this reason, it could not be concluded whether deficient B-galactosidase activity in pMMBV1GW-*pilGmGFP* complemented mutants was a result of low *pilGmGFP* expression levels or the result of malfunctioning PilGmGFP. These data prompted the utilization of a more inducible plasmid,

pMMBGW. Fluorescent constructs were cloned into pMMBGW; B-galactosidase assays and immunoblotting were repeated.

B-galactosidase assay and immunoblotting for pMMBGW-*pilGmGFP* complementation

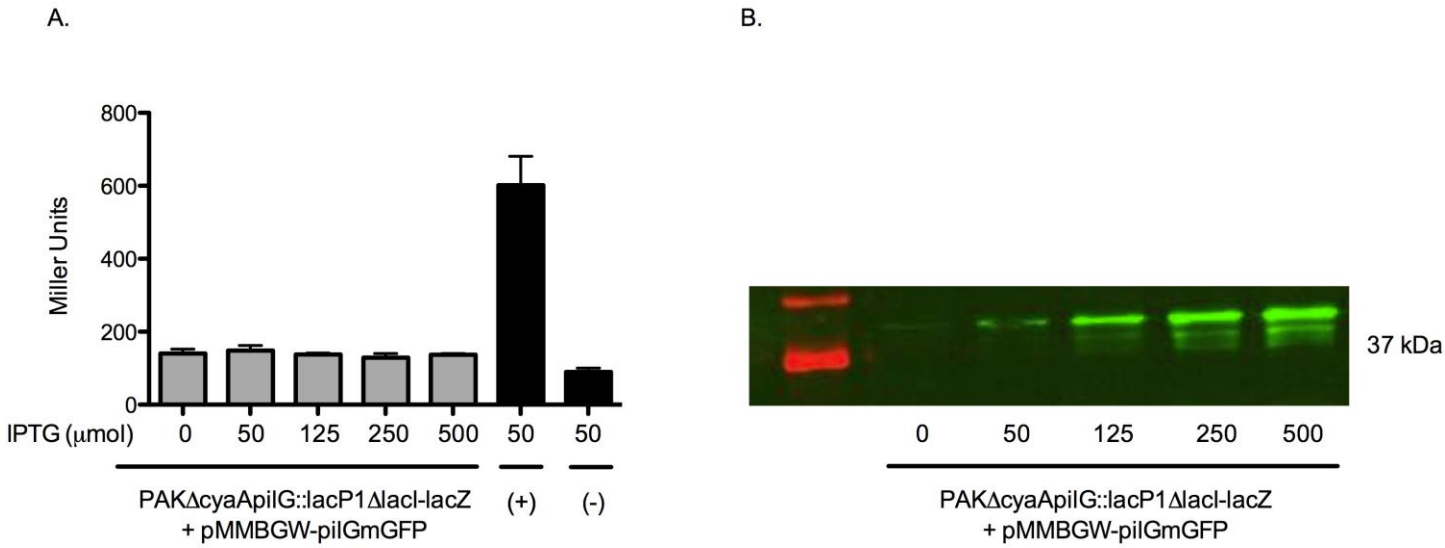

Figure 3A. B-galactosidase activity across an IPTG induction gradient to test PilGmGFP functionality. The (+) label refers to a pseudo-WT positive control and (-) refers to a *ΔpilG* negative control (appendix). Error bars represent SD between three technical replicates. **Figure 3B** represents an immunoblot of the same samples depicted in **3A.** The band at ~38 kDa represents PilGmGFP.

Data from figure 3A indicate that mutants complemented with pMMBGW-*pilGmGFP* were not rescued to wild type B-galactosidase activity. The data follow the same trend as the pMMBV1GW based assays in which B-galactosidase activity of complemented strains was near that of the Δ*pilG* negative control. The immunoblot for these samples illustrates *pilGmGFP* expression levels across a broad range; however, no trend was observed between *pilGmGFP* expression levels and B-galactosidase activity (figure 3A, B). B-galactosidase activity is entirely independent of *pilGmGFP* expression in this system.

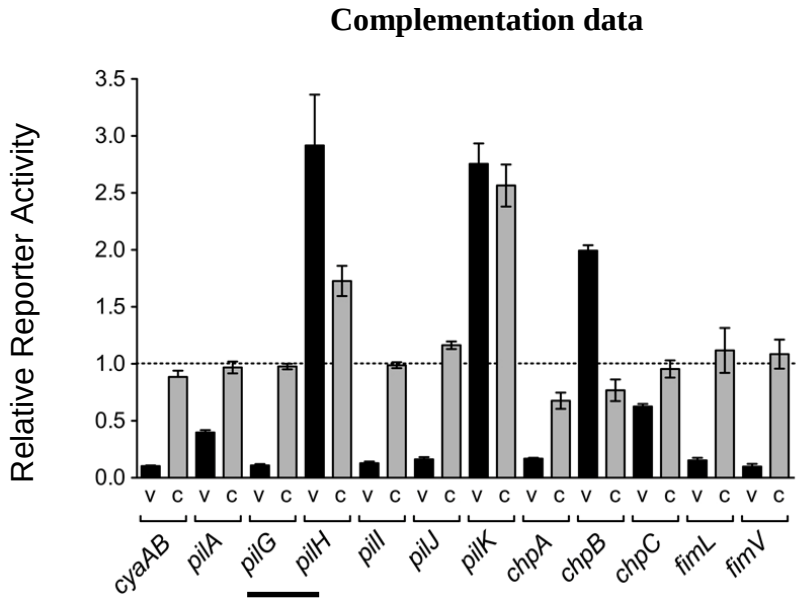

Figure 4. This figure is borrowed from previous studies conducted in our laboratory (Fulcher et al., 2010). Relative reporter activity is reported as the B-galactosidase activity of an individual mutant strain divided by that of the Δ*cyaA* mutant parent stain harboring pMMB67EH empty vector (Fulcher et al., 2010). V indicates deletion mutants and C indicates deletion mutants rescued with pMMB based plasmids.

Figure 4 from a previous study was included here to demonstrate that Δ*pilG* mutants could be rescued with pMMB based complementary plasmids (Fulcher et al., 2010). These data indicate that, in this study, failure to rescue mutants by complementation is a result of fluorescent tagging. Because PilG protein function is severely compromised localization data were not collected.

Discussion

The results of this study provide insight into an artificial means of tracking protein localization patterns in *P.* aeruginoas. Fundamental to experimental design is the caution taken to preserve the fidelity of the naturally functioning system. Thus, careful procedures were enacted

in this work to determine if our means of testing protein localization were inherently disruptive to native protein function.

Sequencing results indicated successful creation of a monomeric version of GFP that does not dimerize or localize at high concentrations (Zacharias et al., 2002). Sequencing also indicated successful splicing of *mGFP* to *pilG* and *pilH*. Confocal microscopy was utilized to determine that mGFP was functional when fused to PilH. These preliminary findings prompted the use of B-galactosidase assays and immunoblotting to measure intracellular cAMP levels and *pilGmGFP* expression, respectively. In wild type systems, PilG positively regulates cAMP levels. In contrast, our results show that PilGmGFP has no capacity to regulate cAMP levels at any expression threshold. It was decided that fusing mGFP to the carboxyl-terminus of PilG was too disruptive to native protein function for a faithful interpretation of localization patterns. For these reasons, PilGmGFP localization data were not collected.

PilHmGFP function and expression were not sampled due to a high degree of homogeneity between *pilG* and *pilH*. Because of biochemical and primary sequence similarities between the PilG and PilH, it is presumed that pilHmGFP would be impaired similarly to PilGmGFP. Future studies can further investigate this presumption by employing B-galactosidase assays and immunoblotting for PilHmGFP.

The mechanism behind PilGmGFP loss of function is not known. Importantly, mGFP retained its fluorescent characteristics when fused to PilH. For this reason, it is not believed that mGFP is misfolding or aggregating in these fusion systems. We suggest steric hindrance is the cause of PilGmGFP loss of function. In protein tagging, there is a possibility that fluorescent proteins will obscure key active sites on native proteins. To resolve this issue, a current study is being conducted that tests PilGmGFP and PilHmGFP function and expression when mGFP is

fused to the amino-termini. Amino tagging may reduce or eliminate PilGmGFP loss of function observed here, serving as a more faithful representation of the natural system. Additionally, it was observed that the rabbit PilG antibody has affinity for PilGmGFP and does not target PilH despite the high degree of homogeneity between the two proteins. These observations demonstrate the possibility of conducting PilG and PilH localization studies on fixed cells using antibodies should amino-terminal fluorescent tagging prove unsuccessful.

In conclusion, this study fell short of its ultimate goal of tracking PilG and PilH localization patterns in *P. aeruginosa*. We were successful in constructing PilGmGFP and PilHmGFP fusion proteins; however, unexpected PilGmGFP loss of function attributed to the fluorescent tag has led to redesigning our approach to study localization. Our current investigation into amino-terminal tagging may prove to remedy the protein loss of function observed here.

Future studies into the nature of the many TFP proteins in *P. aeruginosa* and other bacteria can benefit from these findings. It is suggested that future work on small Pil proteins employ amino-terminus tagging. In lesser-studied systems, it is suggested that investigators create both carboxyl- and amino-termini tags simultaneously. This redundancy will save time should one fluorescence orientation prove disruptive. Lastly, these findings underscore the importance of developing smaller and more efficient protein tracking tools.

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Appendix

Primer	Name	Sequence 5'-3'
1	5' mGFPmut3	TCGAGGAGGATATTCATGAGTAAAGGAGAAGAACTTTTCAC
2	3' mGFPmut3 SOE	GTTGGGATCTTTCGAAAGCTTAGATTGTGTGGACAGGTAATG
3	5' mGFPmut3 SOE	CATTACCTGTCCACACAATCTAAGCTTTCGAAAGATCCCAAC
4	3' mGFPmut3	CAAGAAAGCTGGGTTTCATTTGTATAGTTCATCCATGCC
5	AttB1-02	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGAGGAGGATATTC
6	AttB2-02	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCA
7	5' pilG	TCGAGGAGGATATTCATGGAACAGCAATCCGACGGTTTGAAAG
8	3' pilGmGFPmut3 SOE	CTCCTTTACTGCCACCGCCACCGCTGGAAACGGCGTCCACCGGGG
9	5' pilGmGFPmut 3 SOE	CGCCGTTTCCAGCGGTGGCGGTGGCAGTAAAGGAGAAGAACTTTT
10	3' pilG	CAAGAAAGCTGGGTTTCAGGAAACGGCGTCCACCGGGGTGAAG
11	ForwardSeq201	GTTAACGCTAGCATGGATCTC
12	ReverseSeq201	GTAACATCAGAGATTTTGAGACAC
13	5' pilHmGFPmut3 SOE	GCTGGCGGGCAGCGGTGGCGGTGGCAGTAAAGGAGAAGAACTTT
14	3' pilHmGFPmut3 SOE	CTCCTTTACTGCCACCGCCACCGCTGCCCGCCAGCACCGCATTGATG
15	5' pilH	TCGAGGAGGATATTCATGGCTCGTATTTTGATTGTTGATG
16	3' pilH	CAAGAAAGCTGGGTTTCAGCCCGCCAGCACCGCATTGATGG

Table 1. Exhaustive list of primers used in this study.

Strain/plasmid	Description
DH5α pMMBV1-pilG	Initial source of *pilG*
DH5α pMMBV1-pilH	Initial source of *pilH*
DH5α pRGFP	Initial source of *GFPmut3*
DH5α pMMBV1GW	Initial source of plasmids used for cloning
DH5α pMMBGW	
DH5α HB101 pMMB67EH	Empty vector control plasmid

Table 2. A list of initial plasmids used to construct inserts for this study.

Constructs Created or Utilized	
***E. coli* DH5α**	***P. aeruginosa***
pDONR201-mGFPmut3	**In PAKΔcyaA::lacP1ΔlacI-lacZ Background:**
pDONR201-pilGmGFPmut3	pMMBV1GW-mGFPmut3
pDONR201-pilHmGFPmut3	ΔpilG + pMMBV1GW-pilGmGFPmut3
pMMBV1GW-mGFPmut3	ΔpilG + pMMBGW-pilGmGFPmut3
pMMBV1GW-pilGmGFPmut3	ΔpilH + pMMBV1GW-pilHmGFPmut3
pMMBV1GW-pilHmGFPmut3	ΔpilH + pMMBGW-pilHmGFPmut3
pMMBGW-mGFPmut3	pMMB67EH **(positive control)**
pMMBGW-pilGmGFPmut3	ΔpilG + pMMB67EH **(negative control)**
pMMBGW-pilHmGFPmut3	

Table 3. A list of plasmid constructs either assembled here or used from previous studies.

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