MINOR PILINS REGULATE VIRULENCE VIA FimS-AlgR

PSEUDOMONAS AERUGINOSA MINOR PILINS REGULATE VIRULENCE VIA MODULATION OF FimS-AlgR ACTIVITY

By

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TITLE: *Pseudomonas aeruginosa* minor pilins regulate virulence via modulation of FimS-AlgR activity

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LAY ABSTRACT

Pseudomonas aeruginosa is a bacterium that causes dangerous infections, including lung infections in cystic fibrosis patients. The bacteria use many strategies to infect their hosts, one of which involves a grappling hook-like fibre called the type IV pilus. There are many components involved in assembly and function of the pilus, including five proteins called “minor pilins” and a larger protein called PilY1 that may help the pilus detect surface attachment. We used a roundworm infection model to show that loss of PilY1 and specific minor pilins leads to delayed killing, while loss of other pilus proteins has no effect on worm survival. This effect was due to increased activation of a regulatory system called FimS-AlgR that inhibits expression of other factors used by this bacterium to infect its hosts. By studying how *P. aeruginosa* causes infection, we can design better strategies to disarm it and reduce the severity of infections.

ABSTRACT

The type IV pilus is a motility organelle found in a range of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa*. These flexible fibres mediate twitching motility, biofilm maturation, surface adhesion, and virulence. The principle structural protein of the pilus is the major pilin, PilA, while a set of low abundance "minor pilins" are proposed to constitute the pilus tip. The minor pilins, FimU and PilVWXE, along with the non-pilin protein PilY1, prime assembly of surface-exposed pili. The *fimU-pilVWXY1E* operon is positively regulated by the FimS-AlgR two-component system. Independent of pilus assembly, PilY1 is an adhesin and mechanosensor that, along with PilW and PilX, triggers virulence upon surface attachment. Here, we aimed to uncover the mechanism for PilWXY1-mediated virulence. We hypothesized that loss of PilWXY1 would relieve feedback inhibition on FimS-AlgR, resulting in increased transcription of the minor pilin operon and dysregulation of virulence factors in the AlgR regulon. *Caenorhabditis elegans* slow killing assays revealed that *pilW, pilX*, and *pilY1* mutants had reduced virulence relative to a *pilA* mutant, implying a role in virulence independent of pilus assembly. FimS-AlgR were required for the increased promoter activity of the minor pilin operon upon loss of *pilV*, *pilW*, *pilX*, or *pilY1*. Overexpression or hyperactivation of AlgR by point mutation led to reduced virulence, and the virulence defects of *pilW*, *pilX*, and *pilY1* mutants were dependent on FimS-AlgR expression. We propose that PilWXY1 inhibit

their own expression at the level of FimS-AlgR, such that loss of *pilW, pilX,* or *pilY1* leads to FimS-mediated activation of AlgR, and reduced expression of acute-phase virulence factors. Accumulation of mutations in the minor pilin operon may represent an evolutionary strategy for *P. aeruginosa* populations in chronic lung infections, as loss of PilWXY1 would upregulate the expression of AlgR-dependent virulence factors – such as alginate – characteristic of such infections.

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LIST OF ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
c-di-GMP	Cyclic diguanosine monophosphate
CF	Cystic fibrosis
IM	Inner membrane
MP	Minor pilin
MPP	Minor pseudopilin
OM	Outer membrane
PCR	Polymerase chain reaction
SK	Slow killing
SM	Swarming motility
Spp	Species
T4P	Type IV pilus/type IV pili
T2SS	Type II secretion system
T3SS	Type III secretion system
TCS	Two-component system
TM	Twitching motility
WT	Wild type

DECLARATION OF ACADEMIC ACHIEVEMENT

Ylan Nguyen and Quinn Demik designed the deletion constructs pEX18Gm-*fimU*, pEX18Gm-*pilV*, pEX18Gm-*pilW*, pEX18Gm-*pilX*, pEX18Gm-*pilY1*, pEX18Gm-*pilE*, and created the mutants PA14 *fimU*, PA14 *pilX*, PA14 *pilY1*, and PA14 *pilE*. Ylan also cloned pBADGr-*pilX*, pUT18C-*pilA*, pUT18C-*pilV*, pUT18C-*pilW*, and pUT18C-*pilX*.

Sara Kilmury performed the cloning and mating of pEX18Gm-*algR* to create PA14 *algR*. Sara also cloned pKT25-*fimS* and performed the bacterial two-hybrid assays.

Hanjeong Harvey performed the cloning and mating of pEX18Gm-*pilA* to create PA14 *pilA*.

CHAPTER 1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen, recently listed as one of the highest priority antimicrobial-resistant threats by the World Health Organization, due to its high intrinsic antibiotic resistance and recalcitrance to therapy (1). *P. aeruginosa* is the causative agent of sepsis in severe burn victims, acute ulcerative keratitis in contact lens users, and chronic lung infections in cystic fibrosis (CF) patients (2-5). It frequently infects immunocompromised patients such as those with HIV/AIDS or cancer (6, 7). In CF patients, *P. aeruginosa* infections are the leading cause of morbidity and mortality (5). *P. aeruginosa* can produce an arsenal of virulence factors that contribute to its broad host range and pathogenesis, enabling this pathogen to cause both acute and chronic infections (5, 8). Among these virulence factors is a filamentous surface appendage called the type IV pilus (T4P) (9).

1.1. Type IV pilus assembly and function

The T4P system is a sophisticated biological nanomachine that is broadly distributed among bacteria and archaea (10). In *P. aeruginosa*, T4P facilitate dynamic cellular behaviours including surface and host cell adhesion, biofilm and microcolony formation, virulence, and twitching motility (TM), a form of surface-associated motility facilitated by cycles of extension, adhesion, and retraction of the T4P fibre (10, 11) (Fig. 1A). T4P can generate forces of over 100 pN

(sufficient to move a mass 100,000X that of the cell), making this system one of the strongest molecular motors known (12, 13). T4P are composed of hundreds to thousands of copies of small proteins called major pilins, named PilA in *P. aeruginosa* (14). In addition to PilA, *P. aeruginosa* expresses a subset of low abundance pilins, termed the "minor pilins" (MPs), which includes FimU, PilV, PilW, PilX, and PilE (15-18). The MPs are encoded in a polycistronic operon with the *pilY1* gene that codes for a large ~125 kDa non-pilin protein. Pilins feature a highly conserved N-terminal α-helix, which faces the interior of the helical T4P fibre, and a variable globular C-terminal head domain ending in a disulfide-bonded loop, facing the exterior (19). Pilins are inserted into the inner membrane (IM) by the Sec system, after which they are cleaved and methylated by the prepilin peptidase, PilD, while PilY1 is thought to be processed by signal peptidase 1 (20-23) (Fig. 1B). Mature pilins are assembled into a T4P fibre through a dedicated, envelope-spanning assembly system, where individual PilA subunits are added or removed at the platform protein, PilC, via action of the ATPases PilB and PilT, respectively (9, 24).

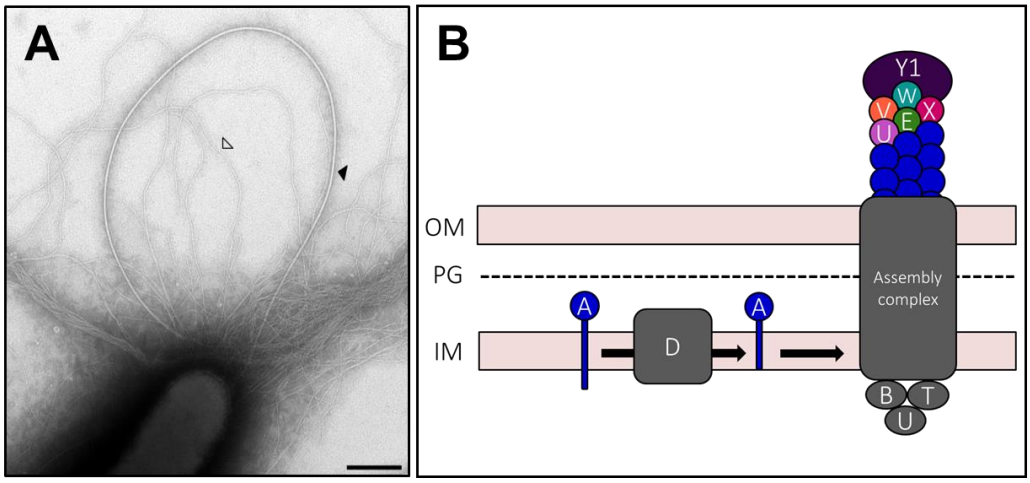

Figure 1. The T4P and assembly machinery. (**A**) Hyper-piliated *P. aeruginosa* cells imaged by transmission electron microscopy. The narrow flexible filaments are individual T4P (open arrow), while the thicker filament is the single polar flagellum (closed arrow). Scale bar = 0.5 μm. Image courtesy of Poney Chiang. (**B**) Simplified model of the T4P system. Pilins are processed by PilD in the IM, followed by assembly into T4P by the assembly complex. PilB powers extension, PilT powers retraction, and PilU controls directionality. Abbreviations: PilA, A (blue); FimU, U (purple); PilV, V (orange); PilW, W (cyan); PilX, X (pink); PilY1, Y1 (dark purple); PilE, E (green); ATPases PilBTU, B, T, U (grey); Prepilin peptidase PilD, D (grey); OM, outer membrane; IM, inner membrane; PG, peptidoglycan.

The T4P system is important in the initial stages of *P. aeruginosa* adherence and colonization (25-29). There are several lines of evidence that implicate T4P and TM in host cell adherence and virulence. For example, Zoutman et al. (30) showed that wild type (WT) *P. aeruginosa* displayed greater adherence to damaged canine tracheal cells compared to non-piliated mutants, but did not adhere to healthy tracheal cells. Using non-piliated and retraction-deficient mutants, both T4P and TM were shown to be important for cytotoxicity against

epithelial cells and for virulence in a mouse model of acute pneumonia (31). A role for functional T4P in virulence was also demonstrated in corneal epithelial cells, where retraction-deficient, hyper-piliated mutants had attenuated virulence in a murine corneal infection model (32). T4P also contribute to biofilm development, although their exact role is less clear. T4P mutants were identified in a screen for biofilm-defective *P. aeruginosa* PA14 strains over the course of 24 h (33). However, Chiang et al. (34) showed that a retraction-deficient *pilT* mutant was capable of seeding biofilms, demonstrating that twitching is not required. Heydorn et al. (35) showed that T4P mutants were capable of forming biofilms in a 6 day flow chamber, but had a heterogenous structure relative to the smooth biofilms formed by WT *P. aeruginosa* strain PAO1. Thus, T4P may be one of multiple adhesion factors that can seed biofilms, and are important for normal biofilm maturation.

Despite the established role for T4P in virulence, *P. aeruginosa* CF isolates are more frequently non-twitching compared to environmental isolates (36). However, *P. aeruginosa* CF lung populations are hyper-mutable and highly heterogeneous; thus, caution should be exercised when interpreting these studies (37). During early stages of lung infection, *P. aeruginosa* populations are generally motile, cytotoxic, and antibiotic-sensitive, and produce virulence factors including pyoverdine, rhamnolipids, proteases, type III secretion system (T3SS) effectors, and quorum sensing molecules (38). Initial *P. aeruginosa* isolates from

CF lung infections are often piliated and exhibit TM; however, these traits tend to be lost as chronic infections are established, despite the continued presence of the *pilA* gene and intracellular PilA in many isolates (25, 36, 39). These data imply that a defect in T4P assembly, rather than PilA expression, can be responsible for loss of piliation and TM in CF isolates. The loss of T4P may be an adaptation to the CF lung, as the energetically expensive act of TM may no longer be required once a biofilm infection is established (39, 40). Further, loss of T4P increases resistance to macrophages and bacteriophages, which may contribute to the persistence of *P. aeruginosa* CF lung infections (41, 42). A defining feature of *P. aeruginosa* isolates from CF lungs is development of a mucoid phenotype, associated with poor prognosis due to increased resistance to antibiotics and host immune defences (38, 43, 44). Overproduction of the polysaccharide alginate via the *algD* operon is responsible for mucoidy (45). The *algD* operon is under positive regulation by the alternative sigma factor AlgU, and the transcriptional regulator AlgR (46, 47). AlgR and the putative sensor kinase FimS (also called AlgZ), positively regulate the MP operon, such that T4P function is lost when FimS or AlgR is absent (48-50). Why these T4P genes are under the control of FimS-AlgR, a two-component system (TCS) associated with chronic infections in the context of CF, is not yet known but may hint at the function of the MPs and PilY1.

1.2. PilY1 and the minor pilins

The MPs and PilY1 are required for T4P function in various bacterial species, including *P. aeruginosa*, *Escherichia coli*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Myxococcus xanthus* (15-18, 51-54). However, whether they are required for initial pilus formation, or if they stabilize the pilus to promote anti-retraction, is a matter of ongoing debate. A crystal structure of the PilVWX homologues in the type II secretion system (T2SS), minor pseudopilins (MPPs) GspIJK, inspired the hypothesis that the MPs or MPPs form an assembly-priming complex to initiate pilus polymerization (55). This idea was supported by a study in *Klebsiella oxytoca* showing that GspIJK self-assemble in the IM, leading to partial extraction of GspJ (56). Our lab recently showed that in *P. aeruginosa*, PilY1 and the MPs are detectable in sheared surface fractions, and that the loss of PilV, PilW, PilX, or PilY1 excludes the other three components from the pilus (23). Thus, PilVWXY1 are thought to form the core assembly-initiation complex, while FimU and PilE connect this complex to PilA (Fig. 2). Studies of retraction-deficient *pilT* mutants challenged the notion that PilY1 and the MPs are required for T4P assembly, as loss of PilT leads to some surface piliation when the individual MPs, or PilY1 (or its homologues in *Neisseria* spp., PilC1/2), are absent (21, 52, 53, 57, 58). However, Nguyen et al. (23) showed that deletion of the MPPs in a background lacking the MPs completely abolished assembly of T4P, suggesting the MPs and PilY1 act as an assembly-priming complex, but that

the MPPs can perform this role in the absence of retraction. After priming assembly, FimU-PilVWXY1E likely remain at the pilus tip, with PilY1, the largest component, at the distal position. The positioning of PilY1 at the pilus tip supports the hypothesis that PilY1 is a T4P-associated adhesin, as demonstrated by Heiniger et al. (57).

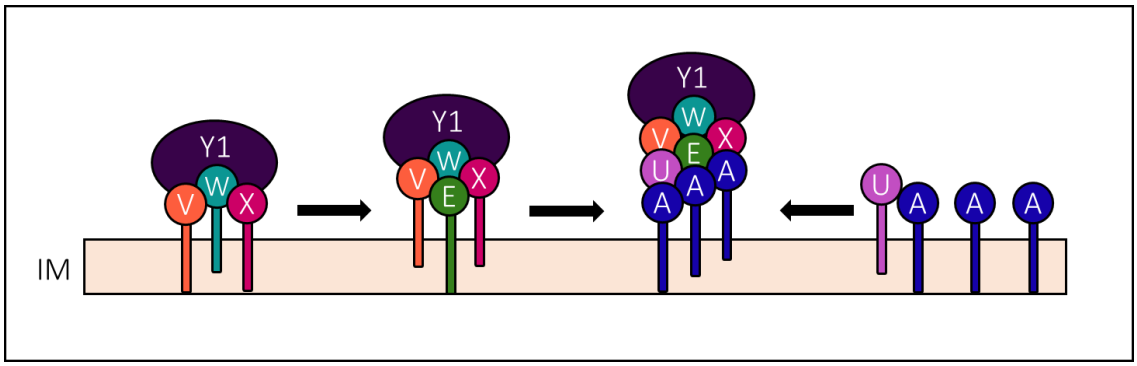

Figure 2. Model for PilY1 and the MPs priming T4P assembly. PilVWXY1 form the core assembly-priming complex, to which PilE can then bind. FimU associates with the pilus independent of the other MPs, and is therefore a proposed connector of the core priming complex to PilA subunits. Adapted from Nguyen et al., 2015 (23). Abbreviations: PilA, A (blue); FimU, U (purple); PilV, V (orange); PilW, W (cyan); PilX, X (pink); PilY1, Y1 (dark purple); PilE, E (green); IM, inner membrane.

The MPs prime T4P assembly and have pilin-like architectures, suggesting these proteins are pilus-associated (23). However, there are conflicting reports regarding the localization of PilY1. In *Neisseria* spp., the PilY1 homologues are thought to localize to the outer membrane (OM) (59, 60). Some studies in *P. aeruginosa* have demonstrated that PilY1 is secreted into the extracellular environment (61, 62). However, there are also data showing that PilY1 can be

detected in sheared surface pilus fractions, indicating localization to the cell surface, and probable association with T4P (15, 23, 63). These data are more parsimonious with PilY1's proposed role as an adhesin. Wolfgang et al. showed that loss of the PilY1 homologue of *Neisseria* spp. abolished adherence to epithelial cells, regardless of whether *pilT* was present (58). These findings were later mirrored in a study by Heiniger et al., who showed that unlike a *P. aeruginosa pilT* mutant, a *pilY1 pilT* mutant could not adhere to epithelial cells, even though both strains were piliated (57). These data suggest that T4P lacking PilY1 are adherence-deficient, and that PilY1 specifically mediates adherence to epithelial cells. There is no consensus yet for PilY1 localization, but we argue that positioning this protein at the tip of the pilus is consistent with its role as a T4P-associated adhesin (64). This idea is supported by data suggesting PilVWXY1, or their homologues, interact (23, 55, 56). Alternatively, PilY1 may localize to multiple sites, where free PilY1 is in the extracellular environment or OM, while PilVWXY1 is tip-associated. Using electron cryotomography, Chang et al. recently demonstrated that the MPs are detectable within T4P assembly complexes lacking surface-exposed pili, where they may assemble prior to the addition of PilA subunits (54). When T4P retract, it is possible that PilY1 and the MPs remain exposed at the exterior face of the secretin, ready for another round of extension. Therefore, PilY1 that appears OM-associated may actually represent tip-associated PilY1 of retracted pili, further supporting the hypothesis that PilY1 and the MPs are required for T4P biogenesis and function.

1.3. PilY1 and the minor pilins regulate virulence

PilY1 and the MPs (and by extension, FimS-AlgR) are important for T4P biogenesis and function, and therefore for T4P-mediated virulence (15-18, 45, 48). However, recent studies hinted at a more enigmatic mechanism of virulence regulation by PilWXY1. Bohn et al. (61) showed that in the non-piliated *P. aeruginosa* strain TBCF10839, loss of *pilY1* led to reduced virulence in a *Caenorhabditis elegans* fast killing assay and in a murine airway infection model. Thus, PilY1 has a specific role in virulence that does not require functional pili. Subsequent studies using *C. elegans* infection models suggested that MP and *pilY1* mutants had attenuated virulence relative to WT, and in one case, to a non-piliated mutant (65-67). Recently, Siryaporn et al. (68) showed that PilWXY1 were required for virulence towards amoebae, while other non-piliated mutants had WT virulence. Of particular note was the WT virulence of a *pilD* mutant, which demonstrated that PilD-processing was not required for PilWXY1-mediated virulence. As PilD-mediated removal of the positively-charged N-terminus is essential for extraction of pilins from the IM and incorporation into the T4P fibre, this result further supported the argument that the role of PilWXY1 in regulation of virulence is independent of their role in T4P assembly (23, 69, 70).

Studies by Siryaporn et al. (68) demonstrated that surface-attached *P. aeruginosa* cells, but not planktonic cells, were virulent towards amoebae. To

probe the mechanism behind surface-triggered virulence, they compared transcriptional profiles of surface-grown versus planktonic cells. The genes of the MP operon were among the most highly transcribed in surface-grown versus planktonic cells, and loss of *pilY1* led to loss of virulence. Other studies revealed that the N-terminal region of PilY1 had limited sequence similarity to the eukaryotic von Willebrand factor A (VWFa) domain, which can be deformed by shear forces (62). VWFa-containing proteins are involved in diverse functions, including platelet adhesion, cell adhesion, DNA repair, protein degradation, and transcription (62, 71, 72). Siryaporn et al. (68) showed that planktonic cells with a *pilY1* variant lacking only the VWFa domain became virulent toward amoebae, implicating this domain in sensing surfaces. The authors proposed that PilY1 is a "mechanosensor" and that deformation of its VWFa domain upon surface interaction induces expression of virulence factors. In an effort to determine which downstream virulence factors were responsive to PilY1-mediated surface detection, a series of mutants lacking known virulence factors were tested for virulence towards amoebae. However, all mutants tested had WT virulence, including an *algR* mutant. Although loss of AlgR leads to a loss of TM due to reduced expression of the MP operon, it is conceivable that PilY1 levels in an *algR* mutant, though lower than WT, are sufficiently high to trigger virulence upon surface contact (49, 73). However, this idea has not been verified, and alternate hypotheses for the mechanism of PilY1-mediated virulence have not been tested.

1.4. PilWXY1 in mechanosensation and c-di-GMP regulation

PilY1 has been implicated in the mechanosensation of surfaces and expression of virulence factors, a process that involves the second messengers cyclic adenosine monophosphate (cAMP) and cyclic diguanosine monophosphate (c-di-GMP) (68, 74). cAMP synthesis is induced after ~1-2 h of surface association, which stimulates expression of early-phase virulence factors, such as those involved in motility, attachment, and secretion (74-76). c-di-GMP synthesis is induced downstream of cAMP, after which expression of those virulence factors is repressed. In turn, c-di-GMP promotes expression of genes associated with a biofilm lifestyle. When c-di-GMP levels are low, cells can exhibit a form of motility known as swarming motility (SM) (62, 77). Diguanylate cyclases such as SadC are involved in the synthesis of c-di-GMP, while phosphodiesterases including BifA degrade c-di-GMP (78, 79). In a *bifA* mutant (high c-di-GMP), loss of *pilW*, *pilX*, or *pilY1* leads to reduced biofilm formation and hyper-swarming in a SadC-dependent manner, indicative of lower c-di-GMP levels compared to the *bifA* single mutant (62, 77). Importantly, loss of *pilA* in the *bifA* background only partially suppressed these high c-di-GMP phenotypes; thus, it appears to be specifically the loss of PilWXY1 – not the loss of piliation – that leads to reduced c-di-GMP levels. As with virulence towards amoebae, PilD processing was not required for swarming repression, showing that PilWXY1 do not need to be incorporated into the T4P fibre to mediate these phenotypes (68,

77). To further explore the PilWXY1-SadC pathway, Luo et al. (74) performed a genetic screen to identify mutants with altered *pilY1* promoter activity. In their model, PilY1 is secreted through the T4P assembly machinery into the extracellular environment, where it initiates a surface-triggered signalling cascade (74). Upon surface association, PilY1 signals (by an as-yet unknown mechanism) through the T4P assembly proteins PilMNOP to promote c-di-GMP production via IM-localized SadC. This model is consistent with work by Rodesney et al. (80) who showed that c-di-GMP levels increased in response to shear forces, and that functional T4P were required for this phenomenon. However, unlike *pilW*, *pilX*, and *pilY1* mutants, a *sadC* mutant has WT virulence towards amoebae, suggesting the PilWXY1-SadC pathway may be important for surface-sensing, but not necessarily surface-activated virulence (68). It is also possible that PilWXY1 regulate multiple factors in a hierarchical signalling cascade, rather than a linear pathway, such that the loss of one downstream factor (SadC) does not appreciably affect the system.

Though PilY1 is a proposed surface-sensor, transcription of *pilY1* and the MP genes is also thought to be partially dependent on an unknown surface-associated signal (68, 74, 80). Luo et al. (74) showed that this signal may be detected by the Pil-Chp chemosensory system, which promotes cAMP production. It was later proposed this signal may be a physical modification of the T4P resulting from tension generated during retraction of surface-attached filaments

(75). This signal leads to the activation of Vfr (virulence factor regulator), a major transcription factor that positively regulates acute-phase virulence factors (74, 76). Finally, Vfr promotes the expression of FimS-AlgR, and Vfr and AlgR together then activate transcription of *fimU-pilVWXY1E*. This signalling cascade is non-linear; although Vfr promotes FimS-AlgR expression, AlgR represses *vfr* transcription, perhaps as a fine-tuning mechanism (76, 81, 82). Siryaporn et al. (68) showed that *fimS* and *algR* transcript levels were over 5-fold higher in a *pilY1* mutant relative to WT PA14, and that the MP genes were upregulated over 200-fold. Several studies showed that loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to elevated expression of PilY1 and the MPs (21, 61, 74). Loss of *algR* abolished *pilY1* promoter activity in all strains tested, consistent with a role downstream of Pil-Chp and cAMP-Vfr, and as a positive regulator of *fimU-pilVWXY1E* transcription. Taken together, these data suggest that PilVWXY1 can feedback-inhibit their own expression, and that this process involves FimS-AlgR (68, 74). However, the detailed mechanism of this putative feedback inhibition remains largely uncharacterized.

1.5. The FimS-AlgR two-component system

Several groups showed that the FimS-AlgR TCS promotes the expression of PilY1 and the MPs; however, though essential for T4P function, FimS-AlgR are best known for their role in alginate production in the context of CF *P.*

aeruginosa infections (47, 49). Loss-of-function mutations in *mucA*, an anti-sigma factor, lead to increased activity of the alternative sigma factor, AlgU (83, 84). In turn, its activity leads to increased *algR* transcription, and eventually, alginate biosynthesis (47). In TCSs, sensor kinases are generally membrane-bound, homodimeric proteins with a periplasmic sensor domain, and cytoplasmic dimerization and ATP-binding auto-phosphorylation domains (85). Some sensor kinases also have phosphatase activity, mediating de-phosphorylation of a response regulator. Response regulators have a receiver domain that catalyzes the transfer of the phosphate from the conserved histidine of the sensor kinase to the conserved aspartate on the response regulator, leading to activation of its DNA-binding effector domain (85). As a response regulator, AlgR has a CheY-like receiver domain, and a LytR family DNA binding domain (45). AlgR can be phosphorylated at residue D54, leading to increased binding affinity at some – but not all – of its target sequences (48, 50, 86, 87). For example, a phospho-inactivating D54N mutation leads to a loss of TM due to reduced AlgR binding at the MP operon, but does not affect AlgR binding to the *algD* promoter (48, 86). The membrane-bound sensor kinase FimS belongs to an unorthodox family of histidine kinases, as it lacks the canonical motif involved in ATP-coordination, which mediates auto-phosphorylation (45, 88). However, it is possible that FimS undergoes phosphorylation by other means, potentially via an uncharacterized mechanism of auto-phosphorylation (45). Alternatively, another sensor kinase may phosphorylate the conserved FimS H175 site; this mechanism of trans-

phosphorylation has been demonstrated in *P. aeruginosa* for the sensor kinase GacS, which can be phosphorylated by another sensor kinase, LadS (89). It is unknown whether FimS can de-phosphorylate AlgR; it contains one E/DxxN/T phosphatase motif (EMPT), but this falls within the first 150 residues which are predicted to form transmembrane helices, rather than adjacent to phospho-accepting H175 (45, 90, 91) unlike other sensor kinases with known phosphatase activity, where the E/DxxN/T motif is adjacent to the phospho-accepting histidine (91). In FimS, H175 is followed by FLFN, which could potentially be a non-canonical phosphatase motif. Alternatively, reduced FimS activity may occur via inhibition of phosphorylation via another sensor kinase, as is the case for RetS-GacS (92). Currently, no direct interaction between FimS and AlgR has been demonstrated, nor has phospho-transfer between them been observed. Rather, indirect evidence for FimS as the sensor kinase for AlgR comes from genetic studies demonstrating that *fimS* mutants behave similarly to a point mutant lacking the AlgR phosphorylation site (48, 49, 93).

The transcriptional regulation of *fimS-algR* is complex; there are two transcriptional start sites within the *fimS* gene, regulated by AlgU and RpoS, and two sites upstream of *fimS*, the first of which is regulated by Vfr (Fig. 3) (81). The transcriptional regulator for the second start site upstream of *fimS* remains elusive, but is proposed to be constitutively active. These multiple layers of regulation indicate that FimS-AlgR levels are tightly controlled. *fimS* transcription is not

affected by AlgU; therefore, in mucoid strains of *P. aeruginosa*, one would expect higher AlgR levels relative to FimS, and potentially, lower relative levels of phosphorylated versus un-phosphorylated AlgR (81). Interestingly, loss of *fimS* leads to increased AlgR-dependent alginate production, suggesting that un-phosphorylated AlgR may activate *algD* transcription more robustly than its phosphorylated form (94).

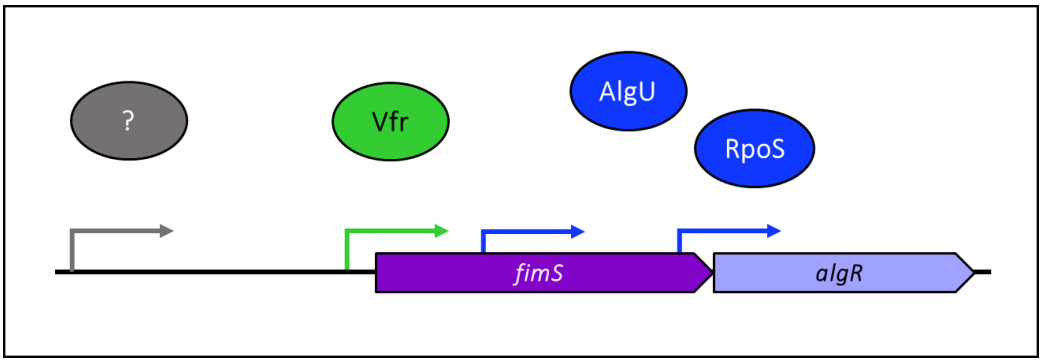

Figure 3. Transcriptional regulation of *fimS-algR*. There are two promoters within the *fimS* coding region responsive to AlgU and RpoS (in blue). The promoter directly upstream *fimS* is bound by Vfr, independent of AlgU or RpoS (in green). There is another *fimS-algR* promoter ~1 kb upstream *fimS* that is not activated by AlgU, RpoS, or Vfr; it is proposed to be constitutively active, or activated by an unidentified transcriptional regulator. Adapted from Pritchett et al. (81).

FimS and AlgR are proposed to promote expression of genes important for production of alginate, biofilms, and c-di-GMP (95, 96). Conversely, they inhibit the expression of virulence factors such as the T3SS, pyocyanin, and quorum sensing (82, 95, 97). FimS-AlgR activity in biofilms is low under laboratory conditions, as evidenced by the lack of detectable alginate or expression of

alginate biosynthetic genes; rather, alginate production is thought to be activated specifically during CF lung infections (98). AlgR was shown to directly bind 156 distinct loci, directly or indirectly regulating the expression of over 900 genes (73, 95, 96). Regulation by AlgR is not straightforward; for example, loss of *algR* leads to decreased hydrogen cyanide production in mucoid *P. aeruginosa*, but increased hydrogen cyanide in non-mucoid backgrounds (73, 93, 99). AlgR positively regulates RsmA, a post-transcriptional regulator that inhibits genes associated with chronic infection, but the small RNAs RsmY and RsmZ, which competitively bind RsmA, are also under positive regulation by AlgR (82, 97, 100). Stacey et al. (100) showed that a phospho-inactive $AlgR_{D54N}$ mutant is capable of binding *rsmA*; thus, differences in AlgR phosphorylation status may account for some of these contradictory results.

In the context of pathogenesis, AlgR levels are important for virulence in a murine model of acute septicemia, as either the deletion or overexpression of *algR* resulted in attenuated virulence (101). This suggests that dynamic regulation of AlgR activity may be important for the proper progression of infection in complex infection models. However, an *algR* mutant had WT virulence towards amoebae (68). In that case, virulence traits associated with chronic infection – under positive regulation by AlgR – may have minimal impact on amoebae killing, an acute *P. aeruginosa* infection. Due to the WT virulence of the PA14 *algR* mutant, Siryaporn et al. ruled out AlgR's involvement in PilY1-mediated virulence (68).

However, they also showed that the loss of *pilY1* promotes *fimS* and *algR* transcription, hinting at a possible mechanism for the attenuated virulence of the *pilW*, *pilX*, and *pilY1* mutants towards amoebae. It is possible that hyperactivation or increased expression of AlgR, rather than loss of AlgR, is responsible for the loss of virulence upon loss of PilWXY1.

1.6. Hypothesis

Although several studies showed that PilY1 and the MPs are important for virulence, none have clearly defined the underlying mechanism (61, 65-68). Here, we tested if PilWXY1 were important for virulence in *C. elegans*, as they were in amoebae (68). We hypothesized that loss of *pilW*, *pilX*, or *pilY1* would relieve feedback inhibition on FimS-AlgR, such that activated AlgR would repress virulence factors important for *C. elegans* pathogenesis. We performed *C. elegans* slow killing (SK) assays, which involve an infection-like process that occurs over multiple days that correlates with the accumulation of live bacteria in the intestine (102-104). We found that the loss of *pilW*, *pilX*, or *pilY1* led to attenuated virulence towards *C. elegans* compared to WT or a *pilA* mutant. A *sadC* mutant had WT virulence, suggesting PilWXY1-mediated virulence is unrelated to c-di-GMP regulation via SadC. Using luminescent reporter assays, we showed that loss of *pilV*, *pilW*, *pilX*, or *pilY1* leads to increased expression of the MP operon, and that FimS-AlgR were both required for this process. Hyperactivation (via

point mutation) or overexpression of AlgR led to reduced virulence, and the reduced virulence of *pilW*, *pilX*, and *pilY1* mutants was dependent on FimS-AlgR. We conclude that the loss of PilWXY1 relieves the feedback inhibition on expression of the AlgR regulon, resulting in dysregulation of virulence factors that are important for *C. elegans* pathogenesis. This regulatory circuit may reflect a potential adaptation for *P. aeruginosa* in the CF lung, where loss of PilWXY1 may inactivate the T4P system and upregulate AlgR-dependent virulence factors.

CHAPTER 2. MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

Strains and plasmids used in this work are listed in Table 1. Bacteria were grown at 37°C for 16 h in 5 ml Luria Bertani Lennox (LB) broth, or on 1.5% agar LB plates, unless otherwise specified. Plasmids were transformed into chemically-competent *E. coli* by heat-shock, and into *P. aeruginosa* by electroporation (105). Where appropriate, gentamicin (Gm) was added at 15 μg/ml for *E. coli*, and 30 μg/ml for *P. aeruginosa*. Kanamycin (Kan) was added at 50 μg/ml for *E. coli*, and 150 μg/ml for *P. aeruginosa*. Ampicillin (Amp) was added at 100 μg/ml for *E. coli*. L-Arabinose was added at 0.05% where indicated to induce expression from the pBADGr promoter (106).

Table 1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristics	Source
Plasmids		
pEX18Gm	Suicide vector for gene replacement	(107)
pEX18Gm-*pilA*	Deletion construct for PA14 *pilA*	This work
pEX18Gm-*fimU*	Deletion construct for PA14 *fimU*	This work
pEX18Gm-*pilV*	Deletion construct for PA14 *pilV*	This work
pEX18Gm-*pilW*	Deletion construct for PA14 *pilW*	This work
pEX18Gm-*pilX*	Deletion construct for PA14 *pilX*	This work
pEX18Gm-*pilY1*	Deletion construct for PA14 *pilY1*	This work
pEX18Gm-*pilE*	Deletion construct for PA14 *pilE*	This work
pEX18Gm-*sadC*	Deletion construct for *sadC*	This work
pEX18Gm-*fimS*	Deletion construct for *fimS*	This work
pEX18Gm-*algR*	Deletion construct for *algR*	This work
pEX18Gm-*algR*$_{D54A}$	Mating construct for *algR* D54A substitution	This work

pEX18Gm-*algR*$_{D54E}$	Mating construct for *algR* D54E substitution	This work
pBADGr	Arabinose-inducible complementation vector	(106)
pBADGr-*pilW*	Complementation construct for *pilW*	(108)
pBADGr-*pilX*	Complementation construct for *pilX*	This work
pBADGr-*pilY1*	Complementation construct for *pilY1*	This work
pBADGr-*sadC*	Complementation construct for *sadC*	This work
pBADGr-*algR*	Complementation construct for *algR*	This work
pBADGr-*algR*$_{D54A}$	Complementation construct for *algR*$_{D54A}$	This work
pMS402	Transcriptional reporter vector carrying the promoterless *luxCDABE* genes	(109)
pMS402-P*fimU*	Transcriptional reporter for *fimU* promoter	This work
pKT25	Vector encoding T25 fragment of *B. pertussis* CyaA	(110)
pKT25-*fimS*	Vector encoding *fimS* fused to T25	This work
pKT25-*pilA*	Vector encoding *pilA* fused to T25	(23)
pKT25-*fimU*	Vector encoding *fimU* fused to T25	(110)
pKT25-*pilV*	Vector encoding *pilV* fused to T25	(23)
pKT25-*pilW*	Vector encoding *pilW* fused to T25	(23)
pKT25-*pilX*	Vector encoding *pilX* fused to T25	(23)
pKT25-*pilE*	Vector encoding *pilE* fused to T25	(23)
pUT18C	Vector encoding T18 fragment of *B. pertussis* CyaA	(111)
pUT18C-*fimS*	Vector encoding *fimS* fused to T18	(90)
pUT18C-*pilA*	Vector encoding *pilA* fused to T18	This work
pUT18C-*fimU*	Vector encoding *fimU* fused to T18	(23)
pUT18C-*pilV*	Vector encoding *pilV* fused to T18	This work
pUT18C-*pilW*	Vector encoding *pilW* fused to T18	This work
pUT18C-*pilX*	Vector encoding *pilX* fused to T18	This work
pUT18C-*pilE*	Vector encoding *pilE* fused to T18	(112)
***E. coli* strains**		
DH5α	*F$^-$φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk$^-$, mk$^+$) phoA supE44 thi-1 gyrA96 relA1 λ−*	Invitrogen
SM10	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu (Km^R)*	Invitrogen
OP50	Uracil auxotroph, *C. elegans* food source	(113)
BTH 101	Bacterial two-hybrid reporter strain	Euromedex
***P. aeruginosa* strains**		
PAO1	WT	(114)
PAO1 *pilA*	ISphoA/hah transposon insertion at position 163	(114)

PAO1 *fimU*	ISlacZ/hah transposon insertion at position 237	(114)
PAO1 *pilV*	ISphoA/hah transposon insertion at position 122	(114)
PAO1 *pilW*	ISlacZ/hah transposon insertion at position 381	(114)
PAO1 *pilX*	ISphoA/hah transposon insertion at position 182	(114)
PAO1 *pilY1*	ISlacZ/hah transposon insertion at position 1407	(114)
PAO1 *pilE*	ISphoA/hah transposon insertion at position 183	(114)
PAO1 *fimS*	Deletion of *fimS*	This work
PAO1 *algR*	Deletion of *algR*	This work
PAO1 $algR_{D54A}$	Phospho-inactive form of *algR*	This work
PAO1 $algR_{D54E}$	Phospho-mimetic form of *algR*	This work
PA14	WT	(115)
PA14 + pBADGr	WT with pBADGr	This work
PA14 + pMS402-P*fimU*	WT with pMS402 containing *fimU* promoter	This work
PA14 *pilA*	Deletion of *pilA*	This work
PA14 *pilA* + pMS402-P*fimU*	Deletion of *pilA* with pMS402 containing *fimU* promoter	This work
PA14 *fimU*	Deletion of *fimU*	This work
PA14 *fimU* + pMS402-P*fimU*	Deletion of *fimU* with pMS402 containing *fimU* promoter	This work
PA14 *pilV*	Deletion of *pilV*	This work
PA14 *pilV* + pMS402-P*fimU*	Deletion of *pilV* with pMS402 containing *fimU* promoter	This work
PA14 *pilW*	Deletion of *pilW*	This work
PA14 *pilW* + pBADGr	Deletion of *pilW* containing pBADGr	This work
PA14 *pilW* + pBADGr-*pilW*	Deletion of *pilW* complemented with *pilW*	This work
PA14 *pilW* + pMS402-P*fimU*	Deletion of *pilW* with pMS402 containing *fimU* promoter	This work
PA14 *pilX*	Deletion of *pilX*	This work
PA14 *pilX* + pBADGr	Deletion of *pilX* containing pBADGr	This work
PA14 *pilX* + pBADGr-*pilX*	Deletion of *pilX* complemented with *pilX*	This work
PA14 *pilX* + pMS402-P*fimU*	Deletion of *pilX* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1*	Deletion of *pilY1*	This work
PA14 *pilY1* + pBADGr	Deletion of *pilY1* containing pBADGr	This work

PA14 *pilY1* + pBADGr-*pilY1*	Deletion of *pilY1* complemented with *pilY1*	This work
PA14 *pilY1* + pMS402-P*fimU*	Deletion of *pilY1* with pMS402 containing *fimU* promoter	This work
PA14 *pilE*	Deletion of *pilE*	This work
PA14 *pilE* + pMS402-P*fimU*	Deletion of *pilE* with pMS402 containing *fimU* promoter	This work
PA14 *sadC roeA*	Deletion of *sadC* and *roeA*	(78)
PA14 *sadC*	Deletion of *sadC*	This work
PA14 *sadC* + pBADGr	Deletion of *sadC* with pBADGr	This work
PA14 *sadC* + pBADGr-*sadC*	Deletion of *sadC* complemented with *sadC*	This work
PA14 *fimS*	Deletion of *fimS*	This work
PA14 *fimS* + pMS402-P*fimU*	Deletion of *fimS* with pMS402 containing *fimU* promoter	This work
PA14 *algR*	Deletion of *algR*	
PA14 *algR* + pBADGr	Deletion of *algR* with pBADGr	This work
PA14 *algR* + pBADGr-*algR*	Deletion of *algR* complemented with WT *algR*	This work
PA14 *algR* + pBADGr-$algR_{D54A}$	Deletion of *algR* complemented with phospho-inactive *algR*	This work
PA14 *algR* + pMS402-P*fimU*	Deletion of *algR* with pMS402 containing *fimU* promoter	This work
PA14 $algR_{D54A}$	Phosopho-inactive form of *algR*	This work
PA14 $algR_{D54E}$	Phospho-mimetic form of *algR*	This work
PA14 *pilW fimS*	Deletion of *fimS* in *pilW* background	This work
PA14 *pilW algR*	Deletion of *algR* in *pilW* background	This work
PA14 *pilW* $algR_{D54A}$	Deletion of *pilW* in phospho-inactive *algR* background	This work
PA14 *pilX fimS*	Deletion of *fimS* in *pilX* background	This work
PA14 *pilX algR*	Deletion of *algR* in *pilX* background	This work
PA14 *pilX* $algR_{D54A}$	Deletion of *pilX* deletion in phospho-inactive *algR* background	This work
PA14 *pilY1 fimS*	Deletion of *fimS* in *pilY1* background	This work
PA14 *pilY1 fimS* + pMS402-P*fimU*	Deletion of *pilY1*/*fimS* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1 algR*	Deletion of *algR* in *pilY1* background	This work
PA14 *pilY1 algR* + pMS402-P*fimU*	Deletion of *pilY1*/*algR* with pMS402 containing *fimU* promoter	This work
PA14 *pilY1* $algR_{D54A}$	Deletion of *pilY1* in phospho-inactive *algR* background	This work

2.2. Cloning procedures

To create pEX18Gm-*pilA*, primers pilA(-1100)F/pilA(52)R and pilA(62)F/pilA(+1056)R were used to amplify ~1000 bp upstream and downstream PA14 *pilA*, respectively (Table 2). The upstream fragment was digested with BamHI/PstI, and the downstream fragment was digested with PstI/HindIII. In a two-step ligation, fragments were sequentially ligated into pEX18Gm digested with BamHI/PstI or PstI/HindIII. Deletion constructs for PA14 *fimU, pilV, pilW, pilX, pilY1,* and *pilE* were synthesized by Genscript in the pUC57Kan vector, containing ~700 bp upstream and downstream of each gene to be deleted. Where appropriate, small portions of the genes to be deleted were retained to prevent loss of ribosome binding sites or stop codons of adjacent genes. Vectors were digested with EcoRI/HindIII and ligated into pEX18Gm. To create *sadC* mutants, primers sadC(-500)F/sadC(+558)R were used to amplify ~500 bp upstream and downstream of PA14 *sadC*. These were used to amplify the *sadC* deletion from PA14 *sadC roeA* (78). The *sadC* polymerase chain reaction (PCR) fragment was digested with SacI/BamHI then ligated into pEX18Gm. *algR*$_{D54A}$ and *algR*$_{D54E}$ mating constructs were made by overlap extension PCR to contain ~500 bp homology upstream and downstream from the PA14 *algR* genes, bases 160-162 (D54). Primers algR(D54A)F/algR(D54A)R and algR(D54E)F/algR(D54E)R were used to mutate D54 to alanine or glutamic acid, respectively; algRD54-500F and algRD54+500R were used as outside primers in

combination with either set of inside primers. PCR fragments were digested with HindIII/XbaI and ligated into pEX18Gm. pEX18Gm-*fimS* was creating by amplifying ~500 bp upstream and downstream of PA14 *fimS* with primers fimS(-500)F/fimS(-500)R, and with inner primers with partial homology to each other, fimS(18)F/fimS(15)R. This facilitated the use of overlap extension PCR to ligate these two fragments (116). *fimS* was cloned into pEX18Gm using KpnI/HindIII. pBADGr-*algR* and pBADGr-*algR*$_{D54A}$ were generated by amplifying the *algR* gene from PA14 and PA14 *algR*$_{D54A}$, respectively, with primers algR CF and algR CR. Fragments were digested with NcoI/HindIII then ligated into pBADGr. The PA14 *pilX* gene was amplified using pilX CF/pilX CR primers, digested with EcoRI/HindIII, then ligated into pBADGr. PA14 *pilY1* was cloned into pBADGr by amplification with pilY1 CF/pilY1 CR primers, digestion with EcoRI/SmaI, and ligation into pBADGr. pMS402-P*fimU* was created by amplifying the promoter region of the PA14 MP operon with primers PfimU F and PfimU R and digesting with BamHI. Digested pBADGr was treated with alkaline phosphatase prior to ligation to avoid re-circularization of the vector. To create pKT25-*fimS* (T25 fused to the FimS N-terminus), primers FimS Bac-F/FimS Bac-R were used to amplify PAO1 *fimS*. *fimS* was digested with XbaI/EcoRI and ligated into pKT25. To create T18 N-terminal fusions to PilA, PilV, PilW, and PilX, primers pilAB2HFor/pilA2B2HRev, pilVB2HFor/pilVB2HRev, pilWB2HFor/pilWMCS2Rev, and pilXB2HFor/pilXMCS2Rev were used to amplify each gene, respectively. *pilA* was digested with XbaI/EcoRI, while *pilV,*

pilW, and *pilX* were digested with XbaI/KpnI. Each was then ligated into pUT18C. Constructs were verified by Sanger sequencing (MOBIX lab, McMaster, Hamilton, ON).

Table 2. Primers used in this study.

Primer name	**Sequence**
pilA(-1100)F	TCGAGGATCCGCATCACGATCTTCTC
pilA(52)R	TACCTGCAGTCGCAACCACGATCATCAG
pilA(62)F	ACCTGCAGATATGCCTGCCCTGACTGCA
pilA(+1056)R	CTGGAAGCTTCCGGCGGAATCAACG
pilX CF	GTCGAATTCATGACCCTGCGCCATACCTCTC
pilX CR	GACAAGCTTTCAGTTGGTATACAGGCGTGCA
pilY1 CF	GTCAGAATTCTGGAGCCAGCGCATGATC
pilY1 CR	CTATCCCGGGTCATTTCTCCTCGACGAC
sadC(-500)F	GATTGAATTCGAGCTCGAACACGGTGACGATCCCG
sadC(+558)R	CTAATCTAGAGGATCCCAGTCCGGCTCGTAGCGC
algRD54-500F	CGGCTCTAGATGAGCAGTATCGTCTTGGCGATCG
algRD54+500R	GATTAAGCTTGCACGAAGCGCTCGCCGAAC
algR(D54A)F	ATCGTCCTGCTGGCTATCCGCATGCC
algR(D54A)R	GGCATGCGGATAGCCAGCAGGACGAT
algR(D54E)F	ATCGTCCTGCTGGAAATCCGCATGCCC
algR(D54E)R	GGGCATGCGGATTTCCAGCAGGACGAT
fimS(-500)F	GACTGGTACCGTTCATGTGCACGTCTTCCAG
fimS(+500)R	GCCGAAGCTTTGTGGTCGGCAATGAAGAAG
fimS(18)F	GTACAACCATGGTAAGTTCCTTGAATCGGATAGGC
fimS(15)R	GAACTTACCATGGTTGTACATGCAGGAAGCCTGA
algR CF	GTAACCATGGCTCATGCAGGAAGCCTGAGCTTATG
algR CR	CAGTAAGCTTTCAGAGCTGATGCATCAGACGCCTG
PfimU F	GTTAGGATCCGCTCTCTTACCTGTGCTCCA
PfimU R	GCATGGATCCGCAGTACTCCACAAGGAAAAG
FimS Bac-F	CATTCTAGACATGCCTATCCGATTCAAG
FimS Bac-R	CCTGAATTCTCAGGCTTCCTGCATGAGTCG
pilAB2HFor	GCATCTAGACTTTACCTTGATCGAACTGATGATCGTG GTTG

pilA2B2HRev	CATGAATTCTTAGTTATCACAACCTTTCGGAGTGAAC ATCGG
pilVB2HFor	GCATCTAGACTTCAGCATGATCGAAGTGCTGGTCG
pilVB2HRev	CATGGTACCTCATGGCTCGACCCTGAGG
pilWB2HFor	GCATCTAGACCTGTCCATGATCGAACTACTGGTGGCC
pilWMCS2Rev	AAGGTACCTCATGGCACGAGATTCCTGAGTGTCTGG
pilXB2HFor	GTATCTAGACGCCACGCTGGTCATCGCC
pilXMCS2Rev	AAGGTACCTCAGTTGGTATAGAGACGGGCGAGAA

2.3. Mutant generation by allelic exchange

To remove or alter specific genes, the allelic exchange method was employed (117). pEX18Gm suicide plasmid derivatives (see Cloning procedures and Table 1) were used to create all mutants in this work. After heat-shock transformation into *E. coli* SM10 cells, pEX18Gm constructs were conjugated into corresponding PA14 or PAO1 parent strains. Cells were then transferred to *Pseudomonas* isolation agar (PIA) Gm100 plates and incubated for 18 h at 37°C, to select for integration of pEX18Gm derivatives into the chromosome. Colonies were streaked onto LB/sucrose and incubated at 30°C for 18 h to select against merodiploids. Resultant colonies were patched onto LB and LB Gm30 to identify gentamicin-sensitive colonies. Regions flanking the desired mutations were amplified and sequenced to confirm success.

2.4. Twitching motility assay

TM assays were performed as previously described (118), with the following modifications. Individual colonies were stab-inoculated in triplicate into 1% agar LB solidified in plasma-treated tissue culture-grade plates (Thermo Fisher) and incubated at 30ºC for 48 h. Agar was carefully removed and plates were stained with 1% crystal violet for 5 min. Unbound dye was removed by rinsing with water, then stained TM areas were measured using ImageJ. Twitching zones were normalized to WT (100%).

2.5. Swarming motility assay

SM assays were performed as previously described (119), with the following modifications. SK plates were modified to contain 0.5% agar to promote swarming (0.3% NaCl, 0.5% agar, 0.35% peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 μg/ml cholesterol in 100% ethanol, 20 mM KH_2PO_4, and 5 mM K_2HPO_4). Plates were dried for 1.5 h at room temperature, unstacked, then 3.5 μl of an overnight culture was spotted onto the center of the plates, in duplicate. Plates were incubated upright and unstacked at 37°C for 24 h, followed by incubation at 22°C for 24 h. Empty SM plates were placed on the perimeter of the array to avoid edge effects and promote uniform SM zones. Experiments were repeated three times, 2 technical replicates per trial.

2.6. Biofilm assay

Biofilm levels were used as a proxy for c-di-GMP levels, and to determine if biofilm formation correlated with virulence. Biofilm assays were performed as previously described, with modifications (120). *P. aeruginosa* cultures were grown for 16 h at 37°C, diluted 1:200 in fresh LB, and grown to OD_{600} ~ 0.1. Cultures were then diluted 1:500 in liquid SK media (0.3% NaCl, 0.35% peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 μg/ml cholesterol in EtOH, 20 mM KH_2PO_4, and 5 mM K_2HPO_4), then 96-well plates were inoculated with 150 μl each strain, in triplicate. Sterility controls (liquid SK media) were included throughout the plate to check for contamination. Plates were covered with peg lids (Nunc) then wrapped in parafilm and incubated at 37°C for 24 h, shaken at 200 rpm. After incubation, the OD_{600} of the plate was measured to check for uniform growth and lack of contamination. Peg lids were washed for 10 min in 200 μl/well 1X phosphate-buffered saline (PBS), then stained with 200 μl/well 0.1% (w/v) crystal violet for 15 min. Unbound crystal violet was removed by washing lids in 70 ml distilled water 5 times at 10 min intervals. Crystal violet was solubilized from lids in 200 μl/well 33.3% acetic acid, then the absorbance at 600 nm was measured. Optical density and absorbance at 600 nm were plotted for growth and biofilm formation, respectively, then analyzed by one-way ANOVA followed by Dunnett post-test to compare each mutant to the WT control, $p = 0.05$. Error bars indicate

standard error of the mean. Representative wells of acetic acid-solubilized crystal violet were imaged.

2.7. Caenorhabditis elegans slow killing assay

SK assays were performed as described previously (104). SK plates (0.35% peptone, 0.3% NaCl, 2% agar, 1 mM $CaCl_2$, 5 μg/ml cholesterol, 1 mM $MgSO_4$, 20 mM KH_2PO_4, 5 mM K_2HPO_4, 100 μM FUDR were seeded with 100 μl of an overnight culture and incubated overnight at 37°C. The following day, plates were enriched with 1 ml of an overnight culture concentrated to 100 μl. Synchronized L4 worms were collected from OP50 plates, washed twice in M9 buffer, and then >50 worms were seeded onto each bacterial lawn on the SK plates. SK plates were incubated at 25°C and scored for dead worms every 24 h. Worms were considered dead when they did not respond to touch, and were removed from the plate. OP50 SK plates were included as a negative control for virulence. Percent survival was plotted as a function of time. Survival curves were plotted on GraphPad Prism 5.00 for Windows, then compared using the Gehan-Breslow-Wilcoxon test, $p = 0.05$. Each assay was performed at least 3 times, and differences were only considered significant if they were reproducible in all trials. Representative trials are shown.

2.8. Luminescent reporter assay

Luminescent reporter assays were used to measure transcription of the MP operon, and were performed as previously described, with minor modifications (90). Various strains harbouring the pMS402-P*fimU* plasmid, encoding the luciferase genes under control of the *fimU* promoter, were grown for 16 h at 37°C in LB Kan150, then diluted 1:50 in fresh LB Kan150. Subsequently, 100 µl of each culture was added to white-walled, clear-bottom 96-well plates (Corning) in triplicate, and incubated at 37°C in a Synergy 4 microtiter plate reader (BioTek). Luminescence readings were taken every 15 min for 5 h, and normalized to growth (OD_{600}) at each time point. Readings that exceeded the limit of detection (>4 000 000 luminescence units) were discarded. At least 3 individual trials were performed. Error bars indicate standard error of the mean.

2.9. Bacterial two-hybrid β-galactosidase activity assay

To test for interactions between FimS and individual pilins, bacterial two-hybrid (BACTH) assays were performed as previously described (121). pUT18C and pKT25 derivatives, encoding the T18 and T25 domains of the *Bordetella pertussis* CyaA adenylate cyclase fused to the N-terminus of FimS, PilA, FimU, PilV, PilW, PilX, or PilE (23, 90, 112), were co-transformed into *E. coli* BTH 101 to screen for pairwise interactions. Each interaction was tested in both orientations, with FimS in pUT18C and each pilin in pKT25, or FimS in pKT25

and each pilin in pUT18C. Single colonies were inoculated in 5 ml LB Amp100 Kan50 and grown overnight. The following day, 100 µl was inoculated into 5 ml fresh media and grown toOD_{600} = 0.6, then 5 µl was spotted onto MacConkey plates (1.5% agar, 100µg/ml ampicillin, 50µg/ml kanamycin, 1% (w/v) maltose, 0.5mM isopropyl b-D-thiogalactopyranoside). Plates were incubated at 30°C for 24 h. An interaction was considered positive when colonies appeared pink, as a result of fermentation of maltose.

CHAPTER 3. RESULTS

3.1. PilWXY1 are important for T4P-independent virulence in PA14 and PAO1

A subset of genes in the MP operon that are important for virulence in amoebae, nematodes, and mouse models were previously identified (61, 65-68). Siryaporn et al. (68) examined the virulence of PA14 *fimU*, *pilW*, *pilX*, *pilY1*, *pilE*, and other non-piliated deletion mutants towards amoebae, and found that only *pilW*, *pilX*, and *pilY1* mutants were avirulent. First, we sought to test if these results were reproducible in our *C. elegans* SK model. SK assays were performed for PA14 with deletions of *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, or *pilE* (Fig. 4A). The *fimU* and *pilE* mutants had WT virulence, while the *pilW*, *pilX*, and *pilY1* mutants had reduced virulence compared to WT. The *pilW*, *pilX*, and *pilY1* mutants were also less virulent than the *pilA* mutant, suggesting their reduced virulence was not due to loss of functional T4P. The *pilA* and *pilV* mutants had intermediate virulence, where they were significantly less virulent than WT only in some trials. The TM and virulence defects of *pilW*, *pilX*, and *pilY1* mutants could be partially complemented *in trans* with pBADGr-*pilW*, pBADGr-*pilX*, and pBADGr-*pilY1*, respectively (Fig. 5,6). The correct stoichiometry of PilY1 and the MPs is important for optimal T4P function, which may explain the lack of full complementation (21).

To verify that these virulence phenotypes were not strain-specific, we also tested PAO1 transposon mutants of *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* in

the SK assay (Fig. 4B). Similar to the results in PA14, PilWXY1 were important for T4P-independent virulence of PAO1. The *fimU* and *pilV* mutants were also less virulent than *pilA*; however, the PA14 and PAO1 MPs are divergent (61-75% amino acid similarity), so it is possible that FimU and PilV function is slightly different in PAO1 versus PA14 (108). To focus on genes that were generally important for virulence of *P. aeruginosa*, we undertook studies of the mechanism underlying loss of virulence in the *pilW*, *pilX*, and *pilY1* mutants.

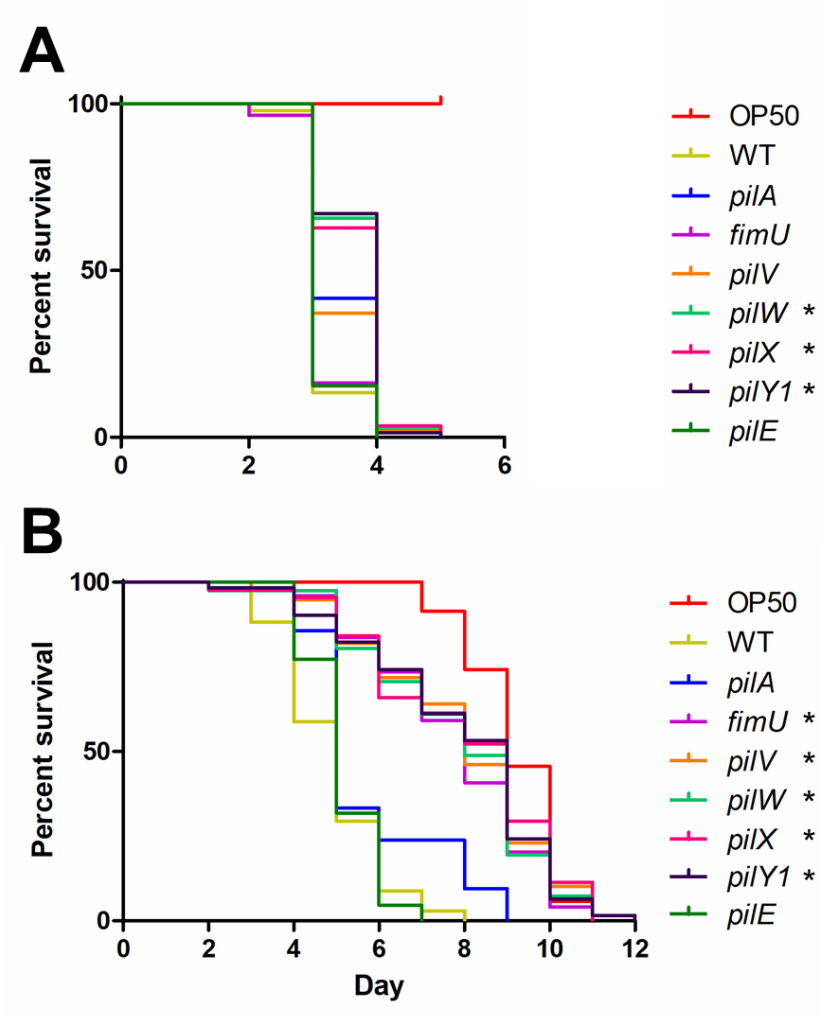

Figure 4. PilWXY1 contribute to T4P-independent virulence. SK assays for PA14 (**A**) and PAO1 (**B**) *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. Synchronized L4 worms were seeded onto SK plates and scored for death every 24 h, then plotted as "percent survival" over the course of the assay. PA14 *fimU* and *pilE* mutants had similar virulence to WT, *pilA* and *pilV* mutants were slightly less virulent than WT in some trials, and *pilW*, *pilX*, and *pilY1* mutants were less virulent than all other strains tested. The PAO1 *pilE* mutant had similar virulence to WT, the *pilA* mutant was slightly less virulent in some trials, and *fimU*, *pilV*, *pilW*, *pilX*, and *pilY1* mutants were much less virulent. Asterisks indicate strains that were consistently less virulent than a *pilA* mutant by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3.

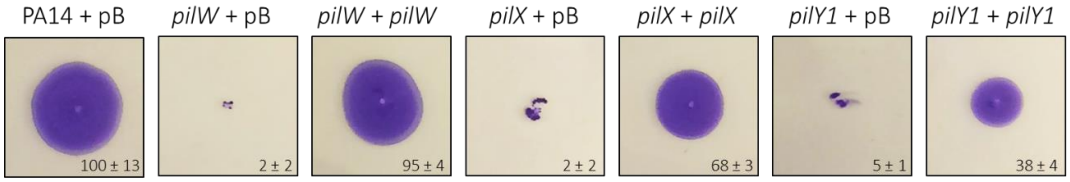

Figure 5. TM of *pilW*, *pilX*, and *pilY1* mutants can be complemented *in trans*. Colonies were stab-inoculated into 1% agar LB plates, in triplicate. Plates were stained with crystal violet after 48 h at 30°C. Complementation of PA14 *pilW*, *pilX*, and *pilY1* mutants with pBADGr-*pilW*, pBADGr-*pilX*, or pBADGr-*pilY1*, respectively, led to increased TM relative to complementation with pBADGr alone. Numbers indicate percent twitching area relative to WT, n = 3.

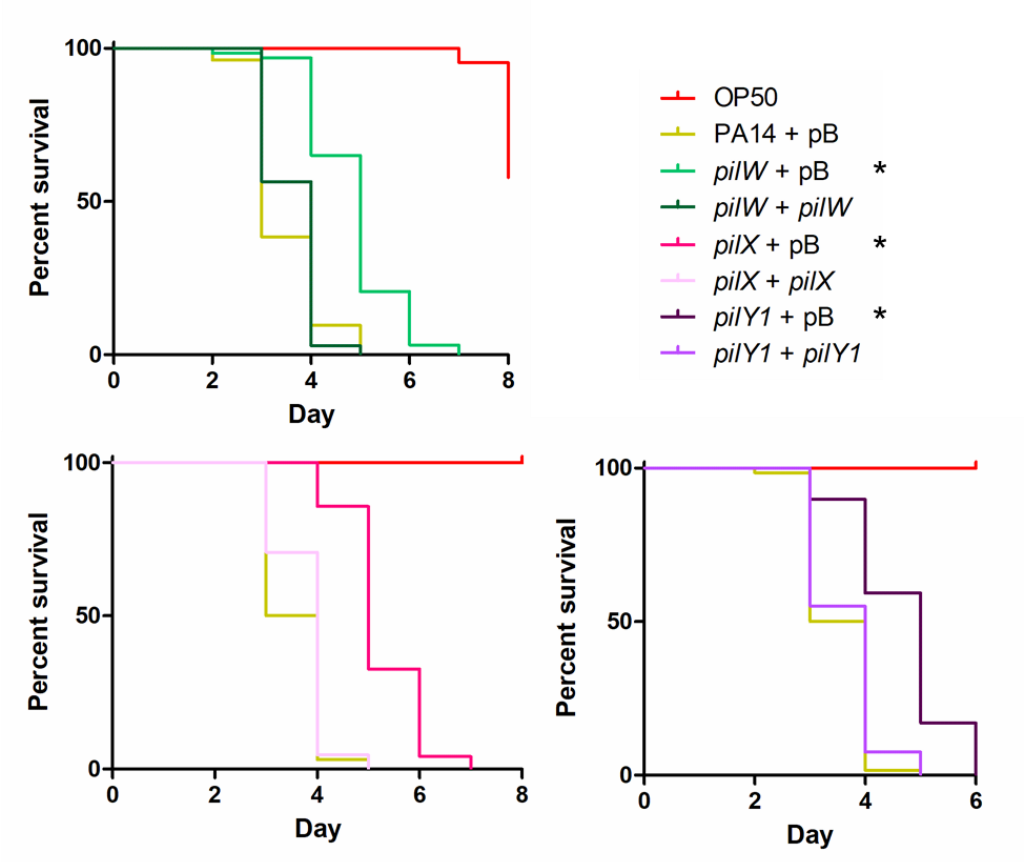

Figure 6. SK of *pilW*, *pilX*, and *pilY1* mutants can be complemented *in trans*. Complementation of PA14 *pilW*, *pilX*, and *pilY1* mutants with pBADGr-*pilW*, pBADGr-*pilX*, or pBADGr-*pilY1*, respectively, restored virulence to near-WT levels. Asterisks indicate strains that were consistently less virulent than WT by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3. Individual graphs represent separate trials.

3.2. Loss of PilWXY1 results in reduced swarming motility

PilWXY1 were previously implicated in promoting c-di-GMP production via SadC, such that loss of *pilW*, *pilX*, or *pilY1* resulted in a hyper-swarming, biofilm-deficient phenotype under laboratory growth conditions (62, 74, 77). Therefore, we hypothesized that biofilm defects of *pilW*, *pilX*, and *pilY1* could

reduce their ability to colonize the *C. elegans* gut, leading to reduced virulence. To test this hypothesis, we first tested biofilm formation in liquid SK media, to mimic conditions of the SK assay. However, the PA14 and PAO1 WT, *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants formed poor biofilms in this media (Fig. 7). As an alternative, we performed SM assays on 0.5% agar SK plates, as swarming is a c-di-GMP-regulated behaviour that is inversely correlated with biofilm formation (78, 79). Only PA14 *pilW*, *pilX*, and *pilY1* mutants had SM defects (Fig. 8A), consistent with their reduced virulence in *C. elegans*. The pattern was less clear for PAO1, which does not swarm optimally on this media (Fig. 8B). However, the mutants with reduced virulence in *C. elegans* (PAO1 *fimU*, *pilV*, *pilW*, *pilX*, and *pilY1*) swarmed less than *pilA* and *pilE* mutants. These data are inconsistent with reports of PilWXY1 promoting c-di-GMP production, and instead suggest that loss of *pilW*, *pilX*, or *pilY1* promotes c-di-GMP production, resulting in swarming repression.

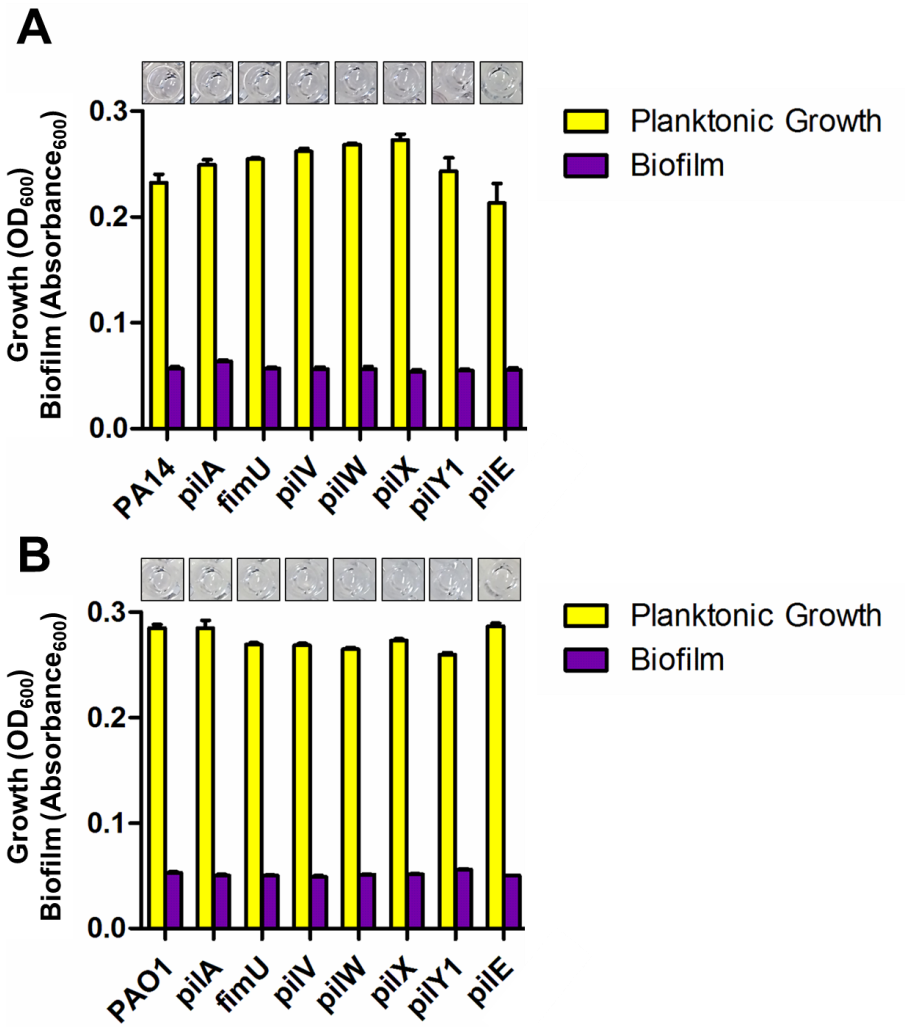

Figure 7. PA14 and PAO1 produce poor biofilms in liquid SK media. Biofilm assays for PA14 (**A**) and PAO1 (**B**) *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. Microtiter plate biofilm assays were performed in liquid SK media over 24 h, in triplicate. Biofilms were stained with 1% crystal violet then solubilized in acetic acid. Very little biofilm formation was detectable in liquid SK media for any strains. There were no differences in biofilm formation as determined by one-way ANOVA followed by Dunnett post-test relative to WT at $p = 0.05$, $n = 3$.

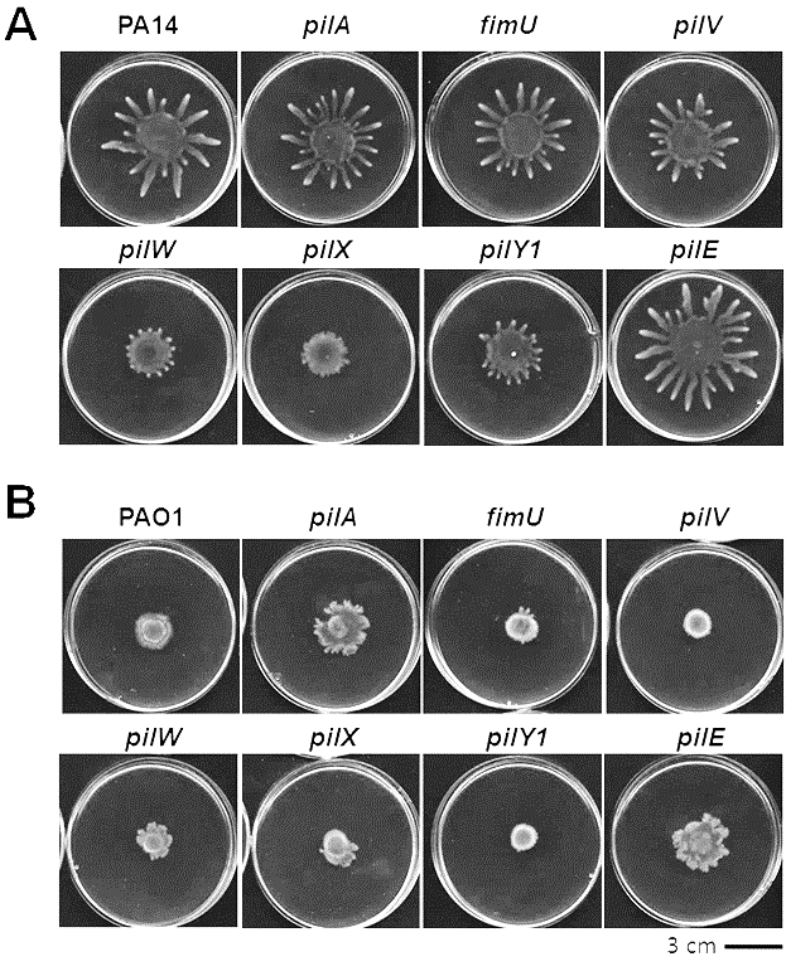

Figure 8. PA14 *pilW*, *pilX*, and *pilY1* mutants have SM defects. SM assays for PA14 (**A**) and PAO1 (**B**) *pilA, fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, and *pilE* mutants. SM assays were performed in duplicate on 0.5% agar SK plates, and imaged after 24 h incubation at 37°C and 24 h at 22°C. PA14 *pilW*, *pilX*, and *pilY1* mutants had reduced SM relative to WT. PAO1 *fimU*, *pilV*, *pilW*, *pilX*, and *pilY1* mutants had SM defects relative to *pilA* and *pilE*. Representative images are shown, n = 3.

3.3. SadC is not required for virulence

Despite the lack of biofilm formation in liquid SK media, we next investigated whether SadC-mediated biofilm formation affected virulence towards

C. elegans, as would be expected if the loss of virulence in *pilW*, *pilX*, and *pilY1* mutants was due to dysregulation of SadC. As previously reported, overexpression of SadC (PA14 *sadC* + pBADGr-*sadC*) led to a dramatic increase in biofilm formation (78) (Fig. 9A). Due to the low baseline level of biofilms in WT PA14, no further reduction in biofilm formation was seen upon deletion of *sadC* (PA14 *sadC* + pBADGr). SK assays were then performed to determine if loss of *sadC* led to reduced virulence (Fig. 9B). Unexpectedly, there was no difference in virulence between WT and the *sadC* mutant. Further, overexpression of SadC led to a hyperbiofilm phenotype but a slight reduction in virulence, demonstrating that the level of biofilm formed does not correlate with virulence in *C. elegans*. Although the exact mechanisms of *P. aeruginosa* pathogenesis in SK of *C. elegans* are not fully understood, biofilms were thought to be important for the establishment of an infection (102, 122, 123). Our data do not support this hypothesis, and instead suggest that biofilms are not a major contributor to *P. aeruginosa* pathogenesis in *C. elegans*. Though PilWXY1 were reported to regulate c-di-GMP production via SadC (62, 77), this is unlikely to be the primary mechanism behind the reduced virulence of these mutants.

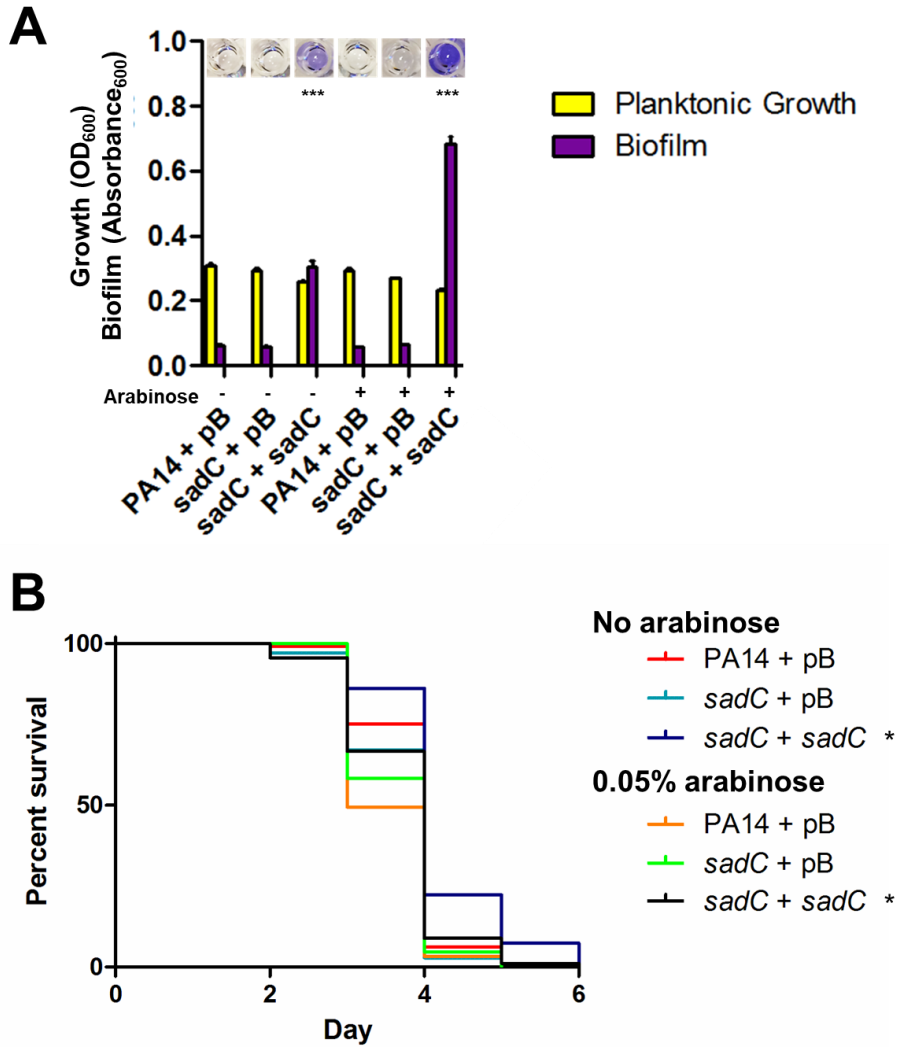

Figure 9. SadC promotes biofilm formation but is not required for virulence. (**A**) Biofilm assays for *sadC* deletion and overexpression strains. PA14 *sadC* biofilm levels were similar to WT. Expression of SadC *in trans* from a multicopy plasmid led to increased biofilm formation relative to WT at 0% (due to leaky promoter) and 0.05% arabinose, $p < 0.001$. Significance was determined by one-way ANOVA followed by Dunnett post-test relative to PA14 + pBADGr, n = 3. (**B**) SK assays for *sadC* deletion and overexpression strains. Overexpression of SadC led to a subtle but reproducible loss of virulence relative to WT at 0% and 0.05% arabinose. A *sadC* mutant had WT virulence. Asterisks indicate strains that were consistently less virulent than PA14 + pBADGr by Gehan-Breslow-Wilcoxon test at $p = 0.05$, n = 3.

3.4. PilVWXY1 repress expression of the minor pilin operon via FimS-AlgR

After ruling out the SadC pathway, we next explored the potential involvement of FimS-AlgR in PilWXY1-mediated virulence. Informed by previous work in our laboratory that showed that the sensor kinase PilS of the PilSR TCS interacts with PilA to regulate major pilin levels in the IM (90), we hypothesized that FimS detects the loss of PilW, PilX, and/or PilY1, leading to phosphorylation of AlgR and subsequent upregulation of the MP operon and changes in expression of other genes in the AlgR regulon. BACTH assays were performed to identify potential interactions between FimS and PilA, FimU, PilV, PilW, PilX, or PilE (Fig. 10A). Positive interactions were identified between FimS and each pilin; however, based on our experience with PilS where binding of pilins is necessary but not sufficient for regulation, the ability to bind FimS does not necessarily indicate a productive interaction. Previous studies showed that loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to increased expression of the MP operon (21, 61, 74). Therefore, we monitored expression of the MP operon using a pMS402-P*fimU* luminescent reporter, with the *luxCDABE* genes under control of the *fimU* promoter. Compared to WT PA14, there was a dramatic increase in luminescence in *pilV*, *pilW*, *pilX*, and *pilY1* mutants (Fig. 10B). *fimU* and *pilA* mutants had slightly elevated promoter activity, while *pilE*, *fimS*, and *algR* mutants had low baseline luminescence, comparable to WT. To determine whether the increased promoter activity in *pilV*, *pilW*, *pilX*, and *pilY1* mutants was

dependent on FimS-AlgR, either *fimS* or *algR* was deleted in the *pilY1* mutant background. As predicted, the *pilY1 algR* double mutant had negligible luminescence, consistent with AlgR acting as a positive regulator of the MP operon. Loss of *fimS* in the *pilY1* mutant background also abolished *fimU* promoter activity, supporting the idea that FimS may monitor PilVWXY1 levels and phosphorylate AlgR when PilVWXY1 levels are low. Although PilA, FimU, and PilE can interact with FimS in the BACTH assay, they likely do not act as a signal to modulate FimS-AlgR activity.

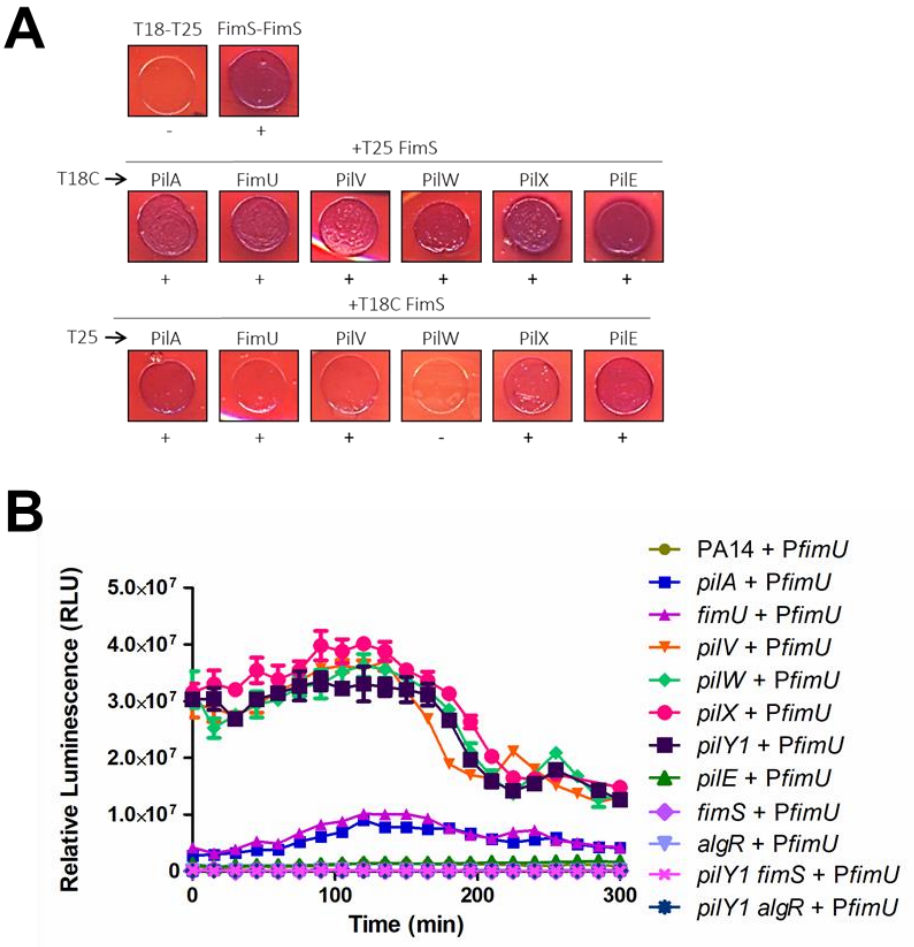

Figure 10. PilVWXY1 repress their own expression via FimS-AlgR. (**A**) BACTH assays for FimS, PilA, and MPs. Protein fusions with T18 and T25 fragments of the CyaA adenylate cyclase were screened for interactions on MacConkey plates. FimS interacted with PilA, FimU, PilV, PilW, PilX, and PilE in at least one orientation. Positive (+) or negative (-) interactions are indicated below each image, n = 3. (**B**) *fimU* promoter activity in PA14 *pilA*, *fimU*, *pilV*, *pilW*, *pilX*, *pilY1*, *pilE*, *fimS*, *algR*, *pilY1 fimS*, or *pilY1 algR* mutants. pMS402-P*fimU*, containing the *fimU* promoter upstream of the *lux* genes, was introduced into strains of interest. Measurements were taken every 15 min over 5 h. Loss of *pilV*, *pilW*, *pilX*, or *pilY1* led to highly elevated *fimU* promoter activity. *pilA* and *fimU* mutants had slightly increased promoter activity relative to WT. Loss of *fimS* or *algR* reverted *fimU* promoter activity in the *pilY1* mutant to baseline. n = 3.

3.5. Hyperactivation of AlgR results in attenuated virulence

Thus far, our results were consistent with the hypothesis that loss of PilWXY1 relieves the feedback inhibition on FimS-AlgR, resulting in activation of AlgR. Next, we aimed to test whether hyperactivation of AlgR alone led to decreased virulence in SK assays. To this end, we constructed chromosomal $algR_{D54E}$ phospho-mimetic point mutants (50) in the PA14 and PAO1 backgrounds. We also constructed $algR_{D54A}$ point mutants, lacking the site of phosphorylation, as AlgR phosphorylation is required for transcription of a subset of genes in its regulon, including the MP operon (48, 50, 86). Past studies have used a D54N mutation to prevent AlgR phosphorylation; however, asparagine can undergo spontaneous deamidation to aspartic acid (124), so we opted for a D54A variant to avoid this complication. SK assays were performed for $algR_{D54A}$ and $algR_{D54E}$ mutants, along with PA14 *fimS* and *algR* deletion mutants, and a PAO1

algR deletion mutant. *algR*$_{D54E}$ mutants of both PA14 and PAO1 had attenuated virulence relative to the corresponding WT strain, while *algR*, and *algR*$_{D54A}$ mutants had WT virulence (Fig. 11). The PA14 *fimS* mutant also had WT virulence. Previous studies demonstrated reduced transcription of the MP operon and loss of T4P function upon loss or inactivation of FimS-AlgR (48, 74). Our results suggest that the reduced virulence of *pilW*, *pilX*, and *pilY1* mutants is not specifically due to lack of their products, but to the resulting activation of FimS-AlgR.

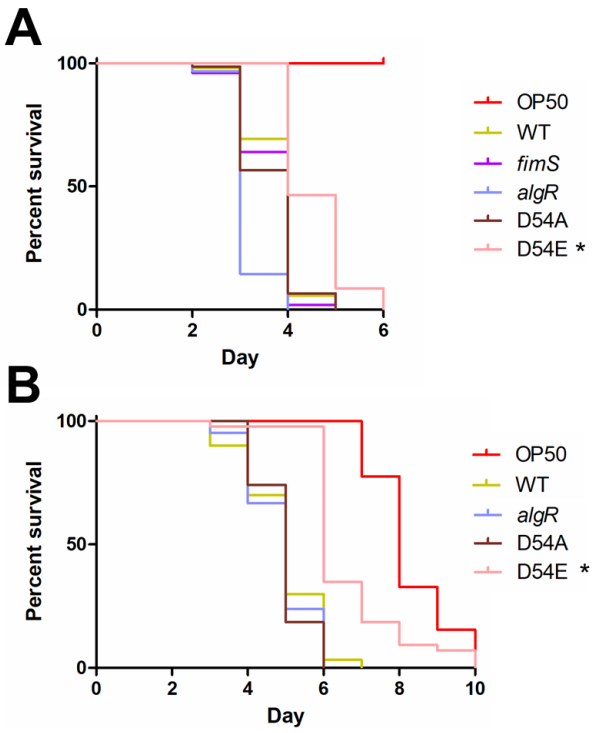

Figure 11. AlgR hyperactivation leads to reduced virulence. SK assays for PA14 (**A**) and PAO1 (**B**) *fimS*, *algR*, *algR*$_{D54A}$, and *algR*$_{D54E}$ mutants. The *fimS*, *algR*, and *algR*$_{D54A}$ mutants had WT virulence, while the *algR*$_{D54E}$ mutants were less virulent than WT. Asterisks indicate strains that were consistently less virulent than WT by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3.

3.6. Overexpression of AlgR results in attenuated virulence

An increase in *fimS-algR* transcription relative to WT was previously reported in a *pilY1* mutant (68); therefore we asked whether increased AlgR levels would lead to attenuated virulence in the *C. elegans* model. A decrease in virulence for PA14 *algR* + pBADGr-*algR* relative to PA14 *algR* + pBADGr was seen only upon addition of 0.05% arabinose (Fig. 12A). One consequence of AlgR overexpression is that the global levels of phosphorylated AlgR may rise, but un-phosphorylated AlgR can also affect transcription of a subset of genes (87, 100). Therefore, we tested the virulence of a PA14 *algR* mutant complemented with pBADGr-*algR*$_{D54A}$. At 0.05% arabinose, PA14 *algR* + pBADGr-*algR*$_{D54A}$ had a severe virulence defect relative to PA14 + pBADGr, though it was still significantly more virulent than PA14 *algR* complemented with WT *algR*. Thus, AlgR hyperactivation and overexpression can independently impair *P. aeruginosa* virulence towards *C. elegans*. Lastly, we performed biofilm assays for PA14 + pBADGr, and PA14 *algR* complemented with pBADGr, pBADGr-*algR*, or pBADGr-*algR*$_{D54A}$, as AlgR was reported to be a positive regulator of biofilm formation (96). At 0.05% arabinose, either AlgR or AlgR$_{D54A}$ expression led to hyper-biofilm formation, further emphasizing our finding that biofilm formation does not correlate with virulence in *C. elegans* (Fig. 12B). Instead, it is likely that virulence factors repressed by FimS-AlgR contribute to *C. elegans* pathogenesis, so that an increase in the levels or activity of AlgR attenuates virulence.

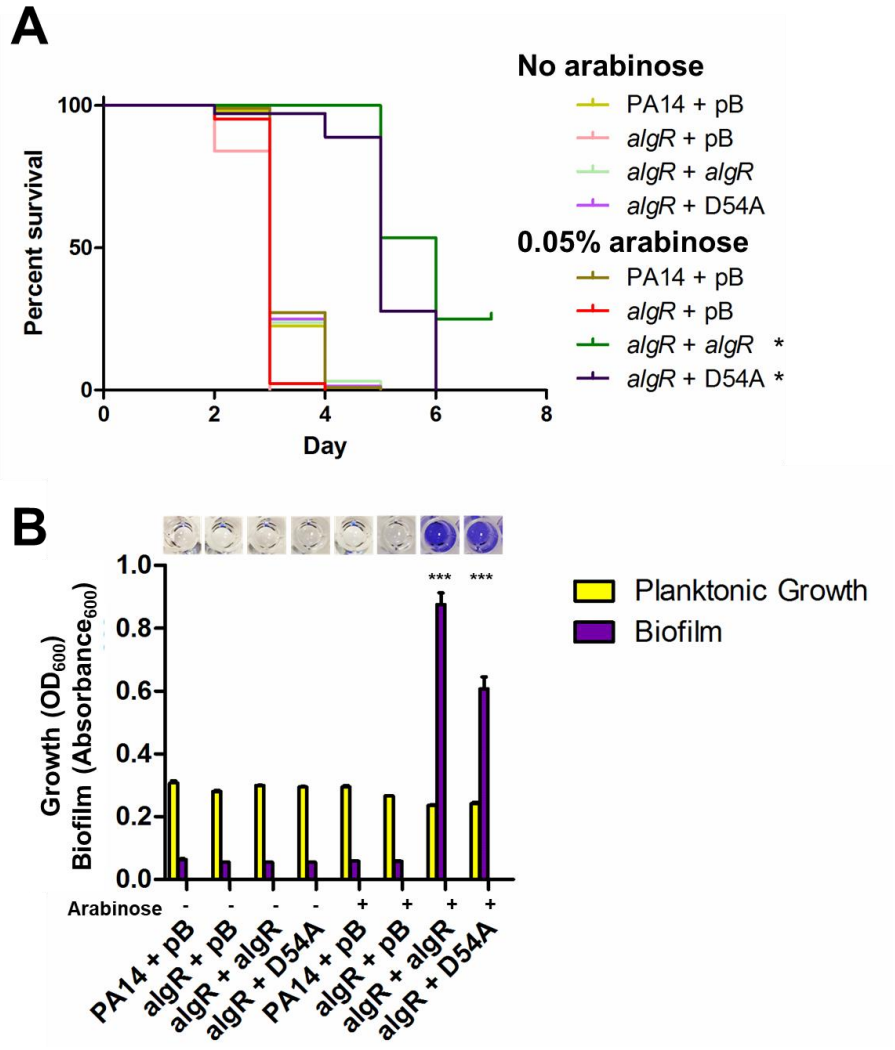

Figure 12. AlgR promotes biofilm formation and represses virulence. (**A**) SK assays for *algR* deletion and overexpression strains. Loss of *algR* had no effect on SK, while overexpression of pBADGr-*algR* or pBADGr-*algR*$_{D54A}$ reduced virulence at 0.05% arabinose. Asterisks indicate strains that were consistently less virulent than PA14 + pBADGr by Gehan-Breslow-Wilcoxon test at $p = 0.05$, $n = 3$. (**B**) Biofilm assays for *algR* deletion and overexpression strains. Loss of *algR* had no effect on biofilm formation. At 0.05% arabinose, overexpression of pBADGr-*algR* or pBADGr-*algR*$_{D54A}$ increased biofilm formation, $p < 0.001$. Significance was determined by one-way ANOVA followed by Dunnett post-test relative to WT, $n = 3$.

3.7. The virulence defect of pilW, pilX and pilY1 mutants is dependent on FimS-AlgR

After demonstrating that overexpression or hyperactivation of AlgR impaired virulence, we asked whether the virulence defects of PA14 *pilW*, *pilX*, and *pilY1* mutants were dependent on FimS-AlgR. We deleted *fimS* or *algR* in the *pilW*, *pilX*, and *pilY1* backgrounds, creating *pilW fimS*, *pilW algR*, *pilX fimS*, *pilX algR*, *pilY1 fimS*, and *pilY1 algR* double mutants, and tested if loss of *fimS* or *algR* rescued virulence (Fig. 13). We also deleted *pilW*, *pilX*, and *pilY1* in the *algR*$_{D54A}$ background, creating *pilW algR*$_{D54A}$, *pilX algR*$_{D54A}$, and *pilY1 algR*$_{D54A}$ double mutants, to assess if AlgR phosphorylation, via FimS or another sensor kinase, was required for the loss of virulence in *pilW*, *pilX*, and *pilY1* mutants. In all cases the double mutants had WT virulence, equivalent to that of the *fimS*, *algR*, or *algR*$_{D54A}$ single mutants. These results demonstrate that FimS and AlgR are both required for reduced virulence resulting from loss of PilWXY1. Although overexpression of AlgR$_{D54A}$ repressed virulence, the D54A point mutation was sufficient to suppress the virulence defect of *pilW*, *pilX*, and *pilY1* mutants, suggesting that AlgR phosphorylation at residue D54 is required for PilWXY1-mediated virulence.

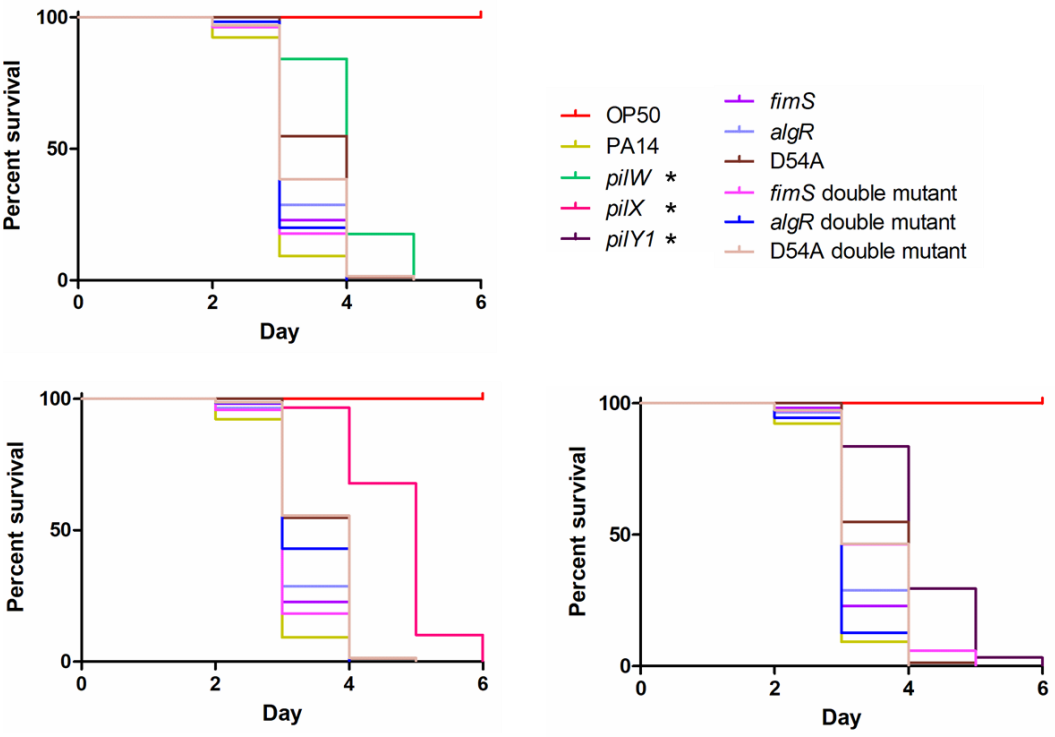

Figure 13. The virulence defect of *pilW*, *pilX*, and *pilY1* mutants is dependent on FimS-AlgR. SK assays for *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and $algR_{D54A}$ single and double mutants. *fimS*, *algR*, and $algR_{D54A}$ mutants have WT virulence. *pilW*, *pilX*, and *pilY1* have reduced virulence relative to WT, *fimS*, *algR*, and $algR_{D54A}$ mutants. Combination of *pilW*, *pilX*, or *pilY1* mutations with *fimS*, *algR*, or $algR_{D54A}$ mutations results in virulence equivalent to *fimS*, *algR*, and $algR_{D54A}$ single mutants, respectively. "*fimS* double mutant" represents *pilW fimS*, *pilX fimS*, and *pilY1 fimS* mutants, "*algR* double mutant" represents *pilW algR*, *pilX algR*, and *pilY1 algR* mutants, and "D54A double mutant" represents *pilW* $algR_{D54A}$, *pilX* $algR_{D54A}$, and *pilY1* $algR_{D54A}$ mutants. All graphs represent 1 trial, separated into 3 graphs for clarity. Asterisks indicate strains that were consistently less virulent than PA14 by Gehan-Breslow-Wilcoxon test at $p = 0.05$, $n = 3$.

3.8. Loss of FimS/AlgR rescues swarming motility defects of pilW, pilX, and pilY1 mutants

After showing that loss of virulence in PA14 *pilW*, *pilX*, and *pilY1* mutants was dependent on FimS-AlgR, we next compared the SM of PA14 *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and *algR*$_{D54A}$ single and double mutants (Fig. 14). *pilW*, *pilX*, and *pilY1* single mutants had SM defects relative to WT, while swarming of *fimS*, *algR*, and *algR*$_{D54A}$ mutants was similar to WT PA14. Deletion of *fimS* or *algR* in the *pilW*, *pilX*, and *pilY1* backgrounds restored SM to the level of *fimS* and *algR* single mutants. Swarming of the *pilW algR*$_{D54A}$, *pilX algR*$_{D54A}$, and *pilY1 algR*$_{D54A}$ mutants resembled the *algR*$_{D54A}$ single mutant. These data mirror the SK assay results in that FimS and AlgR are required for the SM defects of the *pilW*, *pilX*, and *pilY1* mutants. Taken together, our data supports the hypothesis that PilWXY1 repress their own expression via FimS-AlgR, and that loss of PilWXY1 relieves this inhibition, resulting in activation of AlgR in a FimS-dependent manner. Activation of AlgR may lead to repression of genes that are important for SM and virulence towards *C. elegans*.

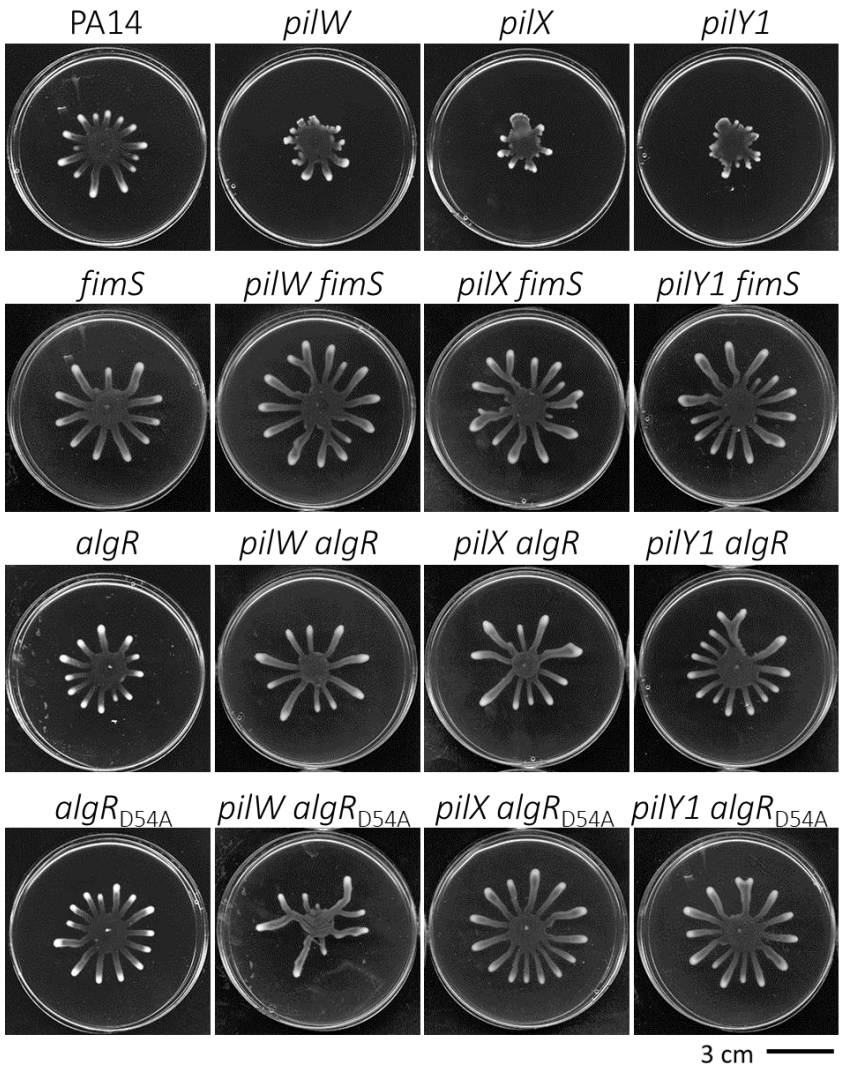

Figure 14. The SM defect of *pilW*, *pilX*, and *pilY1* mutants is dependent on FimS-AlgR. SM assays for *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and *algR*$_{D54A}$ single and double mutants. The reduction in SM of *pilW*, *pilX*, and *pilY1* mutants was dependent on FimS-AlgR. *pilW*, *pilX*, and *pilY1* had reduced SM relative to WT. *fimS*, *algR*, and *algR*$_{D54A}$ mutants had WT swarming. Double mutants swarmed similar to *fimS*, *algR*, and *algR*$_{D54A}$ single mutants. Representative images are shown, n = 3.

CHAPTER 4. DISCUSSION

P. aeruginosa uses its T4P to attach to surfaces and host cells, during biofilm maturation, and to move across surfaces via TM (9). These versatile appendages are important for virulence and host colonization (25-28). The MPs and PilY1 are thought to be involved in the detection of surfaces and subsequent initiation of a surface-triggered virulence program (68, 74). Despite being important for the initial stages of infection, T4P are less critical for chronic CF lung infections where the bacteria grow as biofilms, and are often lost due to selective pressures (25, 36, 39). Incidentally, *P. aeruginosa* CF isolates frequently become mucoid via activation of AlgU and downstream activation of AlgR, leading to reduced production of virulence factors (47, 83, 84). Interestingly, alginate itself has been implicated in the repression of a subset of these virulence factors, independent of AlgU – although it was not specifically addressed in that study, our data lead us to suspect that AlgR may be involved in that phenotype (125). Although loss of T4P function and increased alginate production are not necessarily temporally or mechanistically linked, we argue that mutations that achieve both outcomes may be advantageous during chronic CF lung infections. Here, we explored the connection between loss of PilWXY1 (and thus, loss of T4P function) and AlgR activation in the context of virulence towards *C. elegans*.

The MPs and PilY1 are important players in T4P biogenesis and function, as demonstrated in many multiple bacterial species including *P. aeruginosa*, *E.*

coli, *N. meningitidis*, *N. gonorrhoeae*, and *M. xanthus* (15-18, 51-54). More recently, PilWXY1 were implicated in SM repression, surface attachment, mechanosensation, and virulence (62, 68, 74, 77). We showed that PA14 and PAO1 *pilW*, *pilX*, and *pilY1* mutants were less virulent than WT and a *pilA* mutant – a result that echoes the reduced virulence of PA14 *pilW*, *pilX*, and *pilY1* mutants in amoebae (68). These data support the idea that in *P. aeruginosa*, PilWXY1 serve a function in virulence that is independent of their role in T4P assembly. MP and *pilY1* mutants have been previously identified in large-scale screens for factors affecting *C. elegans* virulence (65-67). We confirmed previous reports (21, 61, 74) that in the absence of *pilV*, *pilW*, *pilX*, or *pilY1*, promoter activity of the MP operon increased dramatically. In past studies, intermediate phenotypes have been described for *pilV* mutants (23, 77). For example, Kuchma et al. (77) showed that loss of *pilV* in a PA14 *bifA* mutant weakly suppressed the *bifA* mutant's hyper-biofilm formation (similar to a *bifA pilA* mutant), but strongly suppressed its SM defect (similar to *bifA pilW*, *bifA pilX*, and *bifA pilY1* mutants). As increased *fimU* promoter activity was dependent on FimS-AlgR, we hypothesized that this TCS is activated upon depletion of PilWXY1, leading to reduced virulence. Indeed, both hyperactivation and overexpression of AlgR alone led to decreased virulence towards *C. elegans*, while loss of *fimS* or *algR* in the *pilW*, *pilX*, or *pilY1* backgrounds reverted virulence to WT levels. These data support a model wherein FimS acts as a molecular thermostat to monitor and regulate PilWXY1 levels, and loss of any of these components leads to FimS-mediated

phosphorylation of AlgR to upregulate expression of the MP operon (Fig. 15). A similar regulatory mechanism was recently described for the PilSR system, where PilS phosphorylates PilR when PilA levels are low, and dephosphorylates PilR when PilA levels are high (90). Whether loss of *pilW*, *pilX*, or *pilY1* leads to increased levels of AlgR or its increased phosphorylation has not yet been clarified. Okkotsu et al. (50) showed that levels of AlgR versus $AlgR_{D54E}$ are comparable, suggesting the loss of virulence we observed for PA14 $algR_{D54E}$ is attributable to the D54E phospho-mimetic mutation alone. However, phosphorylation was not absolutely required for loss of virulence if AlgR was overexpressed, as PA14 *algR* + pBADGr-$algR_{D54A}$ had reduced virulence relative to PA14 + pBADGr. Since a D54A mutation of the AlgR phosphorylation site reverts virulence of *pilW*, *pilX*, and *pilY1* mutants to WT, we suspect that it is primarily AlgR phosphorylation, or loss of AlgR dephosphorylation, that is responsible for loss of virulence in these mutants. Though FimS lacks obvious phosphatase and ATP-coordination motifs, it is possible that these functions are carried out in a novel manner, or via trans-phosphorylation by another sensor kinase, as demonstrated between LadS/GacS (88, 89, 91). Alternatively, it is possible that both AlgR protein levels and phosphorylation contribute to this phenotype. Kong et al. (96) used chromatin immunoprecipitation followed by DNA sequencing to show that AlgR can bind to the *fimS-algR* operon; it is possible that upon AlgR phosphorylation in response to reduced PilWXY1 levels, there is increased AlgR-mediated *fimS-algR* transcription. However, further work

is required to fully elucidate the mechanism of PilWXY1-mediated AlgR activation.

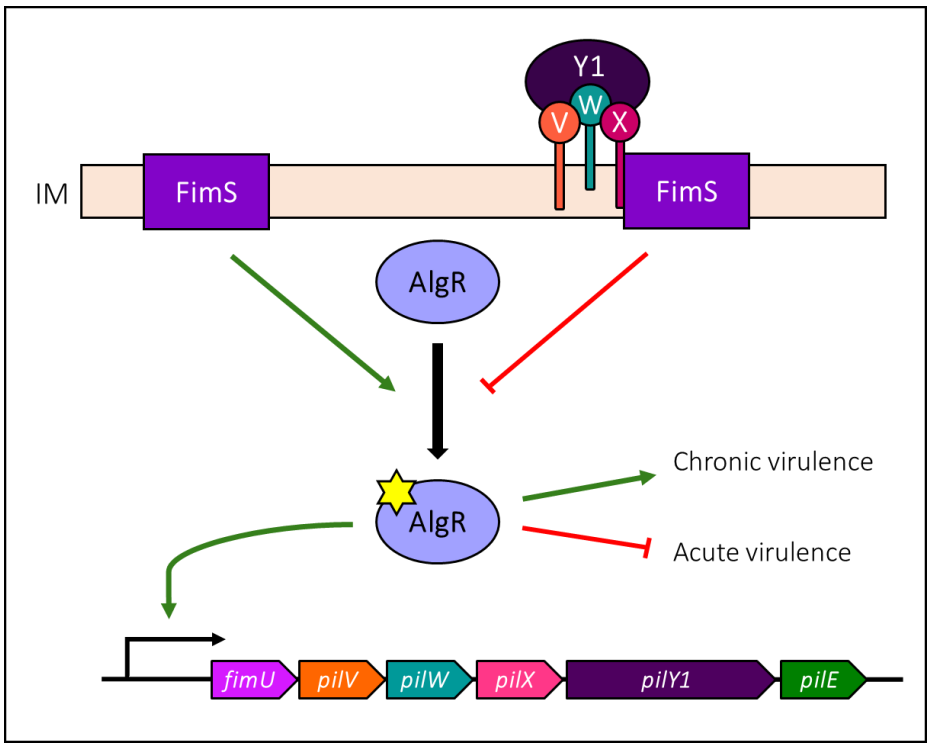

Figure 15. Model for regulation of the MP operon via FimS-AlgR. When PilVWXY1 are absent, FimS promotes phosphorylation of AlgR (phosphate indicated by yellow star). Phospho-AlgR binds the *fimU* promoter to promote expression of *pilY1* and the MP. Phospho-AlgR promotes expression of genes associated with chronic infections, and represses those associated with acute infections. As PilVWXY1 accumulate in the IM, they are detected by FimS, leading to reduced AlgR phosphorylation. Abbreviations: PilV, V (orange); PilW, W (cyan); PilX, X (pink); PilY1, Y1 (dark purple); IM, inner membrane.

Kuchma et al. (62, 77) demonstrated that PilWXY1 were involved in SM repression via SadC, where loss of *pilW*, *pilX*, or *pilY1* led to increased SM, indicative of lower c-di-GMP. Low c-di-GMP leads to reduced biofilm formation; it has been proposed that biofilms contribute to *P. aeruginosa* pathogenesis in *C. elegans*, so we also investigated whether PilWXY1-mediated virulence was linked to SadC (102, 122, 123, 126). However, loss of *sadC* had no impact on virulence. These data are in agreement with Siryaporn et al. (68), who showed that a PA14 *sadC* mutant had WT virulence towards amoebae. Further, we showed that overexpression of SadC led to a slight virulence defect, despite its hyper-biofilm phenotype. Evidence that biofilms contribute to *P. aeruginosa* pathogenesis towards *C. elegans* is indirect. Irazoqui et al. (102) used transmission electron microscopy to examine the *C. elegans* gut during *P. aeruginosa* infection, and described an extracellular material that they suggested may be indicative of a biofilm. Anti-biofilm compounds reduce *P. aeruginosa* virulence towards *C. elegans*, but a mechanism of action for these compounds has not been described (123). Recently, the small RNA SrbA was found to be important for biofilms and virulence towards *C. elegans*; however, this evidence is correlational, as the deletion of *srbA* led to altered transcription of at least 26 other genes that may also affect virulence (127). Biofilms are important for infections in many hosts; however, it is not yet known whether SK is representative of an acute or chronic *P. aeruginosa* infection (8, 66). We demonstrated that biofilms are not directly correlated with virulence, suggesting

this sessile mode of growth may not be relevant in the SK model. Instead, virulence factors that are important in the initial stages of colonization and infection may play a greater role in *C. elegans* pathogenesis.

Presently, the identity of the subset of virulence genes that are repressed by AlgR and important for *C. elegans* pathogenesis is unknown. A genome-wide SK screen of a PA14 transposon library revealed several genes that contributed to SK, including those involved in quorum sensing, O-antigen biosynthesis, attachment, motility (T4P and flagella), amino acid metabolism, efflux, and cell wall metabolism (66). The authors noted that rather than individual virulence factors, many virulence regulators were identified in that screen, suggesting that *C. elegans* pathogenesis is multifactorial. In support of this hypothesis, a study of 18 WT *P. aeruginosa* strains revealed no correlation between virulence and the presence of any specific virulence genes (128). The results emphasize that there is likely no specific virulence factor that is responsible for virulence towards *C. elegans*. Rather, loss of regulatory elements can impair the bacteria's ability to modulate its virulence repertoire, impairing pathogenesis. Regulators that contribute to virulence include GacA/S, Vfr, LasR, RhlR, and FleQ (129). The T3SS is thought to be important in the initial stages of infection (130), yet there are contradictory reports regarding whether the T3SS contributes to *C. elegans* pathogenesis; PA14 T3SS mutants have WT virulence, while PAO1 T3SS mutants have attenuated virulence (Kilmury et al., in preparation) (66, 67, 131). In

our experiments, PA14 kills twice as fast as PAO1. It is possible that there are other fast-acting virulence factors that make T3SS less critical in PA14, or that PAO1 and PA14 kill *C. elegans* via different mechanisms. However, it is unlikely that any one virulence factor regulated by AlgR is responsible for the virulence defect upon FimS-AlgR activation; rather, it may be a subset of virulence genes including the T3SS. Though some virulence factors have historically been considered "acute" or "chronic", we emphasize that dysregulation of virulence factor expression can be detrimental, regardless of the nature of the infection. Here, we did not see reduced virulence upon loss of AlgR – however, the short time scale and relative simplicity of the *C. elegans* infection model may mask the importance of virulence factors that are required during later stages of colonization and infection in mammalian models, such as biofilm development (132, 133). Both the loss and overexpression of AlgR led to attenuated virulence in a mouse model, supporting the notion that dysregulation of this key virulence factor may impair the ability of the bacteria to control the timing of virulence factor expression, ultimately disrupting pathogenesis (101).

Although most mutants tested did not produce detectable biofilms in liquid SK media, we showed that PA14 *pilW*, *pilX*, and *pilY1* mutants had SM defects relative to WT, in disagreement with past studies (62, 74, 77). However, ours is not the first study to report SM defects upon loss of T4P genes, including *pilY1* (119, 134, 135). Though PilWXY1 are thought to promote c-di-GMP production

via SadC, our data suggests that they may indirectly repress c-di-GMP production via feedback inhibition on FimS-AlgR. Though it is well established that AlgR promotes biofilm formation, there are also conflicting data in the literature regarding the SM of *algR* and *fimS* mutants (86, 95). For example, Luo et al. and Okkotsu et al. showed that *fimS* mutants of PA14 and PAO1, respectively, both swarm (50, 74). PAO1 and PA14 *algR* mutants have SM defects, which could be complemented by the MP operon *in trans*, while Okkotsu et al. showed that a PAO1 *algR* mutant can swarm, but an $AlgR_{D54N}$ variant cannot (50, 95, 134, 135). It is likely that there are additional factors that determine whether the PilWXY1-SadC or PilWXY1-AlgR pathway dominates, with regard to c-di-GMP levels, swarming, and virulence. It should also be noted that local concentrations of intracellular c-di-GMP can have differential effects on c-di-GMP-related phenotypes, including biofilms (136). For example, expression of a SadC variant lacking its transmembrane domains prevented SadC-mediated biofilm formation and repression of TM, demonstrating that its localization to the IM is critical (137).

In the broader picture of *P. aeruginosa* virulence, both AlgR and SadC are implicated in modulation of the RsmAYZ regulatory system (97, 100, 138). RsmA is a post-transcriptional regulator that negatively affects genes associated with biofilm lifestyles, while activating virulence genes important in early stages of infection (139-141). The small RNAs RsmY and RsmZ sequester RsmA,

resulting in expression of those genes repressed by RsmA (142, 143). When RsmA is inhibited, c-di-GMP levels increase, leading to increased biofilms and loss of SM (138, 144, 145). *rsmYZ* levels increase when c-di-GMP levels are high, suggesting a positive feedback loop (146). AlgR positively regulates both *rsmYZ* and *rsmA*, where binding to the *rsmA* promoter does not require AlgR phosphorylation (97, 100). AlgR directly regulates c-di-GMP production via MucR, but also indirectly controls c-di-GMP production via SadC, as *sadC* is post-transcriptionally repressed by RsmA (96, 138). Based on our swarming and virulence data, strains with increased AlgR levels or AlgR activity (PA14 *pilW*, *pilX*, *pilY1*, and *algR*$_{D54E}$ mutants, and PA14 + pBADGr-*algR*) have "high c-di-GMP" phenotypes, where swarming and virulence are reduced, and biofilms are increased.

PilY1 and the MPs are thought to be important for the initial stages of colonization via surface detection and activation of virulence (68, 74). Our data partly support this hypothesis. However, it is important to consider that loss of PilY1 or any of the MPs impairs T4P assembly and function; thus, it is difficult to attribute specific phenotypes to the loss of the MPs, rather than loss of piliation (23). Luo et al. (74) suggested that surface association of PilY1 triggers a signal through the T4P system to stimulate c-di-GMP production through SadC, while Rodesney et al. (80) showed that loss of *pilA*, *pilY1*, or *pilT* prevents surface-activated c-di-GMP production. Rodesney et al. (80) proposed that both PilY1 and

functional T4P are required for mechanosensation; however, it is not possible to assess the effects of deleting *pilY1* without perturbing T4P function. Rather than PilY1 signalling through the T4P assembly machinery and SadC (74, 77), surface attachment may lead to depletion of PilVWXY1 levels in the IM, leading to increased FimS-AlgR activity and a transition towards a sessile, biofilm lifestyle. These hypotheses are not necessarily mutually exclusive. The surface-activated increase in c-di-GMP levels via PilY1-SadC is thought to occur within hours of surface attachment (74, 80), whereas FimS-AlgR activation in response to PilVWXY1-depletion in the IM may occur on a different timescale. Additionally, there may be other factors involved in repressing transcription of the MP operon once a biofilm is established, enabling FimS-AlgR activation without a corresponding increase in PilVWXY1 levels. Ultimately, the progression from planktonic growth to sessility is a complex, tightly regulated process. It is conceivable that activating FimS-AlgR too early, ie. via reduced levels of the MPs, perturbs this process.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Future directions

Our BACTH data indicate a physical interaction between FimS and each of PilA, FimU, PilV, PilW, and PilX. In agreement with past studies (21, 61, 74), our luminescent reporter assay data suggests that FimS responds to loss of PilVWXY1 by activating transcription of the MP operon via AlgR. Since only PilVWXY1 control their own expression, it is likely that FimS-PilA, FimS-FimU, and FimS-PilE interactions are not productive. Unproductive interactions have been reported previously, where the sensor kinase PilS interacts with PilA, FimU, and PilE in BACTH assays, but only PilA and PilE levels influenced PilS activity (90). Importantly, it is unclear whether FimS responds to PilVWXY1 as a complex or individually, as these four proteins are thought to associate before priming of T4P (23, 55, 56). To address this, we can delete the entire MP operon to activate *fimU* promoter activity, then replace each gene individually or in combination to determine which of them can suppress promoter activity. Mutational analyses of the MPs and FimS may help to clarify which residues are important for functional interactions.

In our work, we did not compare AlgR levels between WT, MP, and *pilY1* mutants directly. It has been previously shown by microarray analysis that *fimS* and *algR* levels increase upon loss of *pilY1*; we could use a real time quantitative PCR strategy to compare *fimS-algR* levels in PA14, *pilA*, *fimU*, *pilV*, *pilW*, *pilX*,

pilY1 and *pilE* mutants to determine if *pilW*, *pilX*, and *pilY1* mutants have higher -*fimS-algR* than the others. This experiment would help solidify the link between loss of PilWXY1 and activation of the FimS-AlgR TCS. A recent study by Pritchett et al. (81) showed that there are two *algU* binding sites within *fimS*. Although these may play a greater role in mucoid backgrounds due to increased AlgU binding, it is possible that AlgR levels are negatively affected in our *fimS* deletion mutants. A $FimS_{H175E}$ kinase-dead mutant has been previously used to assess potential polar effects – this strategy could be adopted in our work (81, 100). Real time quantitative PCR could also be used to address whether *algR* levels are decreased in our *fimS* deletion mutants.

Our work has been performed solely in the non-mucoid strains, PA14 and PAO1. In the future, it may be useful to reproduce these experiments in a mucoid background to determine if loss of *pilW*, *pilX*, or *pilY1* leads to reduced virulence when AlgR levels are already drastically higher. We could also sequence the MP region of CF *P. aeruginosa* isolates to screen for nonsense or frameshift mutations, which could provide evidence for loss of PilWXY1 as an adaptive benefit in the context of CF, due to AlgR activation and loss of T4P function.

5.2. Conclusions

P. aeruginosa pathogenesis is a complicated process, involving many interconnected virulence pathways. The T4P is an important virulence factor, and

is involved in detection of and attachment to surfaces, movement across surfaces via TM, and biofilm development to remain surface-associated. Here, we described a mechanism by which the T4P proteins PilWXY1 modulate virulence, independent of their role in T4P biogenesis. We propose that the sensor kinase FimS responds to low PilWXY1 levels resulting in increased AlgR activity, which leads to expression of genes implicated in chronic infections, and repression of genes associated with acute infections. Understanding the mechanism of PilWXY1-mediated virulence enhances our understanding of the global *P. aeruginosa* network, and may aid in the targeted development of novel anti-virulence compounds.

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