**Abstract**

Type IV pili are expressed by a wide range of prokaryotes, including the opportunistic pathogen *Pseudomonas aeruginosa*. These flexible fibres mediate twitching motility, biofilm maturation, surface adhesion, and virulence. The pilus is composed mainly of major pilin subunits while the low abundance minor pilins FimU-PilVWXE, plus the putative adhesin PilY1, prime pilus assembly and are proposed to form the pilus tip. The minor pilins and PilY1 are encoded in an operon that is positively regulated by the FimS-AlgR two-component system. Independent of pilus assembly, PilY1 is proposed to be a mechanosensory component that - in conjunction with minor pilins - triggers up-regulation of acute virulence phenotypes upon surface attachment. Here, we investigated the link between the minor pilins and virulence. *pilW, pilX,* and *pilY1* mutants had reduced virulence towards *Caenorhabditis elegans* relative to wild type or a major pilin mutant, implying a role in pathogenicity that is independent of pilus assembly. We hypothesized that loss of specific minor pilins relieves feedback inhibition on FimS-AlgR, increasing transcription of the minor pilin operon and other members of the AlgR regulon.
Reporter assays confirmed that FimS-AlgR were required for the increased expression from the minor pilin operon promoter upon loss of select minor pilins. Overexpression of AlgR or its hyperactivation via point mutation reduced virulence, and the virulence defects of pilW, pilX, and pilY1 mutants were dependent on FimS-AlgR expression. We propose that PilY1 and the minor pilins inhibit their own expression, and that loss of these proteins leads to FimS-mediated activation of AlgR, and reduced expression of acute-phase virulence factors. This mechanism could contribute to adaptation of *P. aeruginosa* in chronic lung infections, as mutations in the minor pilin operon result in the loss of piliation and increased expression of AlgR-dependent virulence factors – such as alginate – that are characteristic of such infections.

**Author summary**

*Pseudomonas aeruginosa* causes dangerous infections, including chronic lung infections in cystic fibrosis patients. It uses many strategies to infect its hosts, including the use of grappling hook-like fibres called type IV pili. Among the components involved in assembly and function of the pilus are five proteins called minor pilins that - along with a larger protein called PilY1 - may help the pilus attach to surfaces. In a roundworm infection model, loss of PilY1 and specific minor pilins delayed killing, while loss of other pilus proteins did not. We traced this effect to increased activation of the FimS-AlgR regulatory system that inhibits expression of virulence factors used to initiate infections, while positively regulating chronic infection traits such as alginate production, a phenotype called mucoidy. A disruption in the appropriate timing of FimS-AlgR-dependent virulence factor expression when select minor pilins or PilY1 are missing may explain why those pilus-deficient mutants have reduced virulence compared with others whose products are not under FimS-AlgR control. Increased FimS-AlgR activity upon
loss of PilY1 and specific minor pilins could help explain the frequent co-occurrence of the non-piliated and mucoid phenotypes that are hallmarks of chronic *P. aeruginosa* lung infections.

**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]. Among its virulence factors are filamentous surface appendages called the type IV pili (T4P), sophisticated biological nanomachines that are broadly distributed among bacteria and archaea [2, 3]. In *P. aeruginosa*, T4P facilitate surface and host cell adhesion, colonization, biofilm maturation, virulence, and twitching, a form of surface-associated motility facilitated by cycles of extension, adhesion, and retraction of T4P fibres [3-11]. T4P are composed of hundreds to thousands of copies of small proteins called major pilins (PilA in *P. aeruginosa*) along with the low abundance minor pilins (MPs), FimU-PilVWXE [12-16]. The MPs are encoded in a polycistronic operon with the *pilY1* gene that codes for a large ~125 kDa non-pilin protein. This operon is positively regulated by the two-component system (TCS) FimS (AlgZ)-AlgR, where FimS is a predicted histidine sensor kinase and AlgR is a response regulator that promotes expression of genes important for biofilms and chronic cystic fibrosis (CF) lung infections [17-19]. The N-termini of immature pilins are cleaved and methylated at the cytoplasmic face of the inner membrane (IM) by the prepilin peptidase, PilD, while PilY1 may be processed by signal peptidase 1 [20-23]. Mature pilins are polymerized into a T4P fibre via an envelope-spanning assembly machinery, where individual PilA subunits are added or removed at the platform protein, PilC, via action of the ATPases PilB and PilT, respectively [2, 24].
The MPs and PilY1 are required for T4P function in several bacterial species, including *P. aeruginosa*, *Escherichia coli*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Myxococcus xanthus* [12-15, 25-28]. PilY1 and the MPs were originally proposed to oppose pilus retraction, as some surface pili remain in *pilY1* and MP mutants when retraction is blocked via deletion of *pilT* [21, 26, 27, 29, 30]. We recently showed that deletion of the minor pseudopilins of the Xcp type II secretion system in a *pilT* background lacking the T4P MPs abolished pilus assembly, suggesting that when MPs are missing, the minor pseudopilins can prime extension, but cannot counteract retraction [22]. We also demonstrated that PilY1 and the MPs are present in sheared pili, and that the loss of PilV, PilW, PilX, or PilY1 excludes the other three components from the pilus [22]. Thus, PilVWXY1 form a core assembly-initiation subcomplex, while FimU and PilE connect this complex to PilA. Initiation of assembly with subsequent addition of PilA subunits beneath would place the MPs at the pilus tip, with PilY1 – the largest component – at the distal position, supporting the hypothesis that PilY1 is a T4P-associated adhesin [29].

PilY1 and the MPs (and their regulators FimS-AlgR) are required for T4P biogenesis, and therefore T4P-mediated function [12-15, 17, 19]. However, recent studies hinted at more enigmatic roles of PilWXY1 in virulence. Bohn et al. [31] showed that in a non-piliated *P. aeruginosa* background, subsequent loss of *pilY1* reduced virulence in a *Caenorhabditis elegans* fast killing assay and in a murine airway infection model, and increased resistance to killing by neutrophils. Thus, PilY1 has a role in virulence that does not require functional pili. Other 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 [32-35]. Recently, Siryaporn et al. [36] showed that PilWXY1 were required for surface-activated virulence towards amoebae, while other non-piliated mutants had WT virulence. The N-terminal region of PilY1 has limited
sequence similarity to the eukaryotic von Willebrand factor A (VWFa) domain, which can be
deformed by shear forces [37]. In-frame deletion of this domain from PilY1 allowed normally
avirulent planktonic cells to kill amoebae [36]. PilY1 was therefore proposed to be a
mechanosensor, where deformation of its VWFa domain upon surface interaction induced
expression of virulence factors. One important caveat of that study was that an algR mutant
(which also lacks PilY1 and the MPs) had WT virulence towards amoebae [36].

Deformation of PilA subunits by tensile forces acting upon surface attached pili was also
proposed as a possible way to signal surface attachment. Detection of partly unfolded pilins by
the Pil-Chp chemotactic system could lead to increased cyclic adenosine monophosphate
(cAMP) synthesis via the CyaB adenylate cyclase [38, 39]. cAMP is bound by Vfr (virulence
factor regulator), a key transcription factor that promotes expression of virulence factors
involved in motility, attachment, and secretion [38-40]. fimS-algR transcription is activated by
Vfr, leading to increased transcription of fimU-pilVWXY1E [38]. PilVWXY1 were proposed to
repress their own expression in an AlgR-dependent manner, as the loss of pilV, pilW, pilX, or
pilY1 led to elevated expression of the MP operon and fimS-algR [21, 31, 36, 38]. The
mechanism of this putative feedback inhibition is largely uncharacterized, but has been
speculated to involve FimS [38].

Once expression of the MP operon is activated, extracellular PilY1 may sense surface
association via its VWFa domain and transduce this information through the T4P assembly
machinery [36, 38]. This signal is thought to activate an IM-localized diguanylate cyclase, SadC,
and increase levels of c-di-GMP, a second messenger that promotes expression of genes
associated with a biofilm lifestyle while repressing early-phase virulence traits such as swarming
motility [38, 41]. This model was supported by studies demonstrating that loss of pilW, pilX, or
in a high-c-di-GMP background resulted in hyper-swarming, indicative of reduced c-di-GMP [37, 42]. Rodesney et al. [43] showed that c-di-GMP levels increased in response to shear forces, and that functional T4P were required for this phenomenon, further supporting this hypothesis. However, unlike pilW, pilX, and pilY1 mutants, a sadC mutant had WT virulence towards amoebae, suggesting the PilWXY1-SadC pathway may be important for surface-sensing, but not necessarily for surface-activated virulence [36].

Although PilY1 and the MPs influence virulence, the underlying mechanism remains unclear [31-34, 36]. We hypothesized that a subset of these components represses FimS activity, such that loss of pilW, pilX, or pilY1 activates FimS-AlgR, shifting the bacteria to a less pathogenic phenotype typically associated with chronic infection. We found that pilW, pilX, and pilY1 mutants had attenuated virulence in C. elegans slow killing (SK) assays compared to WT or a pilA mutant, and this was was dependent on FimS-AlgR, as double mutants had wild type (WT) virulence. Hyperactivation (via phospho-mimetic point mutation) or overexpression of AlgR alone was sufficient to attenuate virulence. Together, these data are consistent with a model where loss of PilWXY1 relieves feedback inhibition on expression of the AlgR regulon, resulting in dysregulation of virulence factors that are important for C. elegans pathogenesis.

Results

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

Specific genes in the MP operon were reported to be important for virulence in amoebae, nematodes, and mouse models, but those studies were done using various strain backgrounds [31-34, 36]. We first sought to confirm these results in the C. elegans SK model, using two well-studied strains. SK assays were performed using PA14 with deletions of pilA, fimU, pilV, pilW,
pilX, pilY1, or pilE (Fig 1A). fimU and pilE mutants had WT virulence, while pilW, pilX, and pilY1 mutants had reduced virulence compared to WT. pilW, pilX, and pilY1 mutants were also less virulent than a pilA mutant, suggesting their reduced virulence was not due to loss of functional T4P. pilA and pilV mutants had intermediate virulence, where they were significantly less pathogenic than WT only in some trials. The twitching and virulence defects of pilW, pilX, and pilY1 mutants could be partially complemented by expression of the relevant gene in trans (Supplementary Fig S1 and S2). The 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 phenotypes were not strain-specific, we tested PAO1 transposon-insertion mutants of pilA, fimU, pilV, pilW, pilX, pilY1, and pilE in the SK assay (Fig 1B). Similar to the results in PA14, PilWXY1 were important for T4P-independent virulence. However, the fimU and pilV mutants were also less pathogenic than pilA; the PA14 and PAO1 MPs are divergent (61-75% amino acid similarity), so it is possible that FimU and PilV function slightly differently in PAO1 versus PA14 [44]. To focus on genes that were generally important for virulence of P. aeruginosa, we undertook studies of the mechanism responsible for loss of virulence in the pilW, pilX, and pilY1 mutants.

PilWXY1 promote virulence in a SadC-independent manner

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, indicative of low intracellular c-di-GMP [37, 38, 42]. Therefore, we hypothesized that biofilm defects of pilW, pilX, and pilY1 might impede their ability to colonize the C. elegans gut, leading to reduced virulence, and thus tested their ability to form biofilms in liquid SK medium. The
PA14 and PAO1 parent strains and their cognate pilA, fimU, pilV, pilW, pilX, pilY1, and pilE mutants formed negligible levels of biofilm in that medium (Supplementary Fig S3). Next, we performed swarming motility assays using 0.5% agar SK plates, as swarming is inversely correlated with biofilm formation [41, 45]. PA14 pilW, pilX, and pilY1 mutants had swarming defects (Fig 2), consistent with their reduced virulence in C. elegans, while all PAO1 strains had low swarming on this media (S4 Fig). These data are inconsistent with reports that PilWXY1 promote c-di-GMP production, and instead suggest that the absence of pilW, pilX, or pilY1 promotes c-di-GMP production, resulting in repression of swarming. 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 activity. However, there was no difference in virulence between PA14 and a sadC mutant (Supplementary Fig S5). Further, overexpression of SadC led to a hyper-biofilm phenotype in vitro but a slight reduction in virulence, demonstrating that the amount of biofilm formed does not correlate with virulence in C. elegans (Supplementary Fig S5). Although the exact mechanisms of P. aeruginosa pathogenesis in C. elegans are not fully understood, biofilms were suggested to be important for establishment of infection [46-48]. However, in these assay conditions, biofilms do not appear to be a major contributor to P. aeruginosa pathogenesis.

**PilVWXY1 repress expression of the minor pilin operon**

After ruling out involvement of the SadC pathway, we next explored the potential role of FimS-AlgR in PilWXY1-mediated virulence. Informed by previous work in our laboratory, showing that the sensor kinase PilS of the PilSR TCS interacts directly with PilA in the inner membrane to regulate major pilin levels [49], we hypothesized that FimS interacts with one or
more MPs, and that loss of that interaction could lead to phosphorylation of AlgR and
subsequent upregulation of the MP operon. Bacterial two-hybrid (BACTH) assays were used to
identify potential interactions between FimS and PilA, FimU, PilV, PilW, PilX, or PilE (Fig 3A).
Positive interactions were identified between FimS and each pilin; however, based on our
experience with PilS, binding of pilins is necessary but not sufficient for regulation.
To decipher which MPs might modulate expression of the operon, we monitored
expression from the fimU promoter using a luxCDABE reporter. Compared to WT PA14, there
was a ~25-fold increase in luminescence in pilV, pilW, pilX, and pilY1 mutants (Fig 3B). fimU
and pilA mutants had ~5-fold increased promoter activity, while a pilE mutant was comparable
to WT. fimS and algR mutants had low baseline luminescence, ~10-fold lower than 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
previously reported, the pilY1 algR double mutant had low luminescence (~10-fold lower than
WT), consistent with AlgR acting as a positive regulator of the MP operon [38]. Loss of fimS in
the pilY1 mutant background also abolished fimU promoter activity (~10-fold lower than WT),
supporting the idea that FimS may monitor PilVWX1 levels and activate AlgR when levels are
low. Based on these data, PilA, FimU, and PilE are unlikely to modulate FimS-AlgR activity
even though they can interact with FimS.

Hyperactivation of AlgR results in attenuated virulence
Thus far, our results supported the hypothesis that loss of PilWX1 relieves feedback
inhibition on FimS-AlgR, resulting in AlgR activation. Next, we aimed to test whether
hyperactivation of AlgR alone could decrease virulence. We constructed chromosomal algRDS4E
phospho-mimetic point mutants [50] in both the PA14 and PAO1 backgrounds. We also
constructed $\text{algR}_{\text{D54A}}$ point mutants, as AlgR phosphorylation is required for transcription of a
subset of genes in its regulon, including the MP operon [17, 50, 51]. SK assays were performed
for PA14 and PAO1 $\text{algR}_{\text{D54A}}$ and $\text{algR}_{\text{D54E}}$ mutants, along with PA14 and PAO1 $\text{fimS}$ and $\text{algR}$
deletion mutants. $\text{algR}_{\text{D54E}}$ mutants of both PA14 and PAO1 were less pathogenic than the
corresponding WT strains, while $\text{algR}$ deletion and $\text{algR}_{\text{D54A}}$ mutants had WT virulence (Fig 4).
$f\text{imS}$ mutants also had WT virulence. Loss of FimS-AlgR was shown previously to decrease
expression of the minor pilins and PilY1, and prevent pilus assembly [17, 38]. Because our data
show that loss of FimS-AlgR (and thus loss of minor pilin expression) had no impact on
virulence, we conclude that reduced virulence of $\text{pilW}$, $\text{pilX}$, and $\text{pilY1}$ mutants is not due to loss
of those gene products directly, but rather to the resulting activation of FimS-AlgR.

**Overexpression of AlgR results in attenuated virulence**

Increased transcription of $\text{fimS}$-$\text{algR}$ in a $\text{pilY1}$ mutant relative to WT has been reported
[36], suggesting that reduced virulence could arise through increased levels of FimS-AlgR
expression as well as activation of the TCS. Therefore, we asked whether increased AlgR levels
would attenuate virulence. When $\text{algR}$ was expressed in trans from a multicopy plasmid in PA14
$\text{algR}$, virulence was reduced compared to the vector-only control (Fig 5A). Because un-
phosphorylated AlgR can also affect transcription of a subset of genes [52, 53], we also tested
the same mutant complemented with $\text{algR}_{\text{D54A}}$. Complementation of the $\text{algR}$ mutant with
$\text{algR}_{\text{D54A}}$ resulted in a severe virulence defect relative to the vector-only control. Thus, AlgR
hyperactivation and overexpression independently diminish $\text{P. aeruginosa}$ virulence towards $\text{C. elegans}$. Lastly, as AlgR was reported to be a positive regulator of biofilm formation [54], we
performed biofilm assays for PA14 algR complemented with AlgR or AlgR_{D54A}. Expression of both AlgR and AlgR_{D54A} led to hyper-biofilm formation (Fig 5B), further emphasizing that the ability of a strain to form biofilms in SK medium does not correlate with virulence. Instead, we suggest that virulence factors that are repressed by FimS-AlgR are important for *C. elegans* slow killing, and thus an increase in AlgR levels and/or activity attenuates virulence.

The virulence defects of *pilW*, *pilX* and *pilY1* mutants are dependent on FimS-AlgR

To provide further support for this model, 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, and tested virulence of the double mutants (Fig 6). We also deleted *pilW*, *pilX*, and *pilY1* in the *algR_{D54A}* background, to test if AlgR activation 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 both FimS and AlgR are required for the reduction in virulence resulting from loss of PilWXY1. Although overexpression of AlgR_{D54A} in trans repressed virulence (Fig 5A), the chromosomal point mutation was sufficient to alleviate the virulence defect of *pilW*, *pilX*, and *pilY1* mutants, suggesting that AlgR phosphorylation at residue D54 is important for PilWXY1-mediated virulence.

Loss of FimS-AlgR rescues swarming motility defects of *pilW*, *pilX*, and *pilY1* mutants

After confirming that reduced virulence in PA14 *pilW*, *pilX*, and *pilY1* mutants was dependent on FimS-AlgR, we compared swarming motility of PA14 *pilW*, *pilX*, *pilY1*, *fimS*, *algR*, and *algR_{D54A}* single and double mutants (Fig 7). *pilW*, *pilX*, and *pilY1* mutants had
swarming defects, while swarming of \textit{fimS}, \textit{algR}, and \textit{algR}_{D54A} mutants was similar to WT PA14. Deletion of \textit{fimS} or \textit{algR} in the \textit{pilW}, \textit{pilX}, and \textit{pilY1} backgrounds restored swarming to the level of \textit{fimS} and \textit{algR} single mutants. Swarming of the \textit{pilW} \textit{algR}_{D54A}, \textit{pilX} \textit{algR}_{D54A}, and \textit{pilY1} \textit{algR}_{D54A} mutants resembled that of the \textit{algR}_{D54A} single mutant. These data mirror the SK assay results, in that the swarming defects of the \textit{pilW}, \textit{pilX}, and \textit{pilY1} mutants are dependent on FimS and AlgR. Taken together, our data support a model where PilWXY1 repress their own expression via FimS-AlgR, and loss of PilWXY1 relieves this inhibition, activating AlgR in a FimS-dependent manner. Activated AlgR then represses genes that are important for swarming and virulence towards \textit{C. elegans} under SK conditions.

\textbf{Discussion}

\textit{P. aeruginosa} uses T4P to attach to surfaces and host cells, during biofilm maturation, and to move across surfaces via twitching motility [2]. The MPs and PilY1 are important players in T4P biogenesis and function, but also in regulation of swarming motility, surface attachment, mechanosensation, and virulence [36-38, 42]. The MP operon is positively regulated by FimS-AlgR, a TCS implicated in regulation of chronic \textit{P. aeruginosa} lung infections [17-19]. Here, we explored the potential connection between loss of PilWXY1 (and thus, loss of T4P) and AlgR activation in virulence towards \textit{C. elegans}. We showed that \textit{pilW}, \textit{pilX}, and \textit{pilY1} mutants were less virulent than WT or a \textit{pilA} mutant – similar to the reduced virulence of these mutants towards amoebae [36]. These data support the idea that PilWXY1 modulate virulence independently of their role in T4P assembly. We confirmed previous reports [21, 31, 38] that in the absence of \textit{pilV}, \textit{pilW}, \textit{pilX}, or \textit{pilY1}, expression of the MP operon is significantly increased,
and that this requires FimS-AlgR. Either hyperactivation or overexpression of AlgR reduced virulence, while loss of \textit{fimS} or \textit{algR} in \textit{pilW}, \textit{pilX}, or \textit{pilY1} backgrounds reverted it to WT levels.

These data suggest that FimS acts as a molecular thermostat to monitor MP levels, and in their absence, activates AlgR to upregulate expression of the MP operon (Fig 8). A similar regulatory mechanism was recently described for the PilSR TCS, where PilS phosphorylates PilR when PilA levels are low, and dephosphorylates PilR when PilA levels are high [49]. It is not yet clear whether FimS responds to PilVWXYZ individually or as a group, as these four proteins are thought to form an inner membrane subcomplex that primes assembly of T4P [22, 55, 56]. When overexpressed individually \textit{in trans}, each of the MPs inhibited twitching motility in PAO1 [21], but since the others were still expressed from the chromosome, the exact nature of the signal detected by FimS remains to be determined.

Kuchma et al. [37, 42] reported that loss of \textit{pilW}, \textit{pilX}, or \textit{pilY1} increased swarming motility and decreased biofilm formation, both indicative of low c-di-GMP levels. As biofilms were proposed to contribute to \textit{P. aeruginosa} pathogenesis in \textit{C. elegans}, we investigated whether the reduction in virulence in the absence of PilWXY1 was linked to decreased biofilm via loss of SadC activation [46-48, 57]. In our hands, levels of \textit{sadC} had no impact on virulence even though they clearly modulated the amount of biofilm produced in SK media (Supplementary Fig S5). Irazoqui et al. [48] examined the \textit{C. elegans} gut during \textit{P. aeruginosa} infection, and described extracellular material that they suggested might indicate presence of a biofilm. Anti-biofilm compounds reduced \textit{P. aeruginosa} virulence towards \textit{C. elegans}, but a mechanism of action for those compounds has not been described [47]. Recently, the small RNA SrbA was shown to modulate both biofilm and virulence towards \textit{C. elegans}; however, deletion of \textit{srbA} led to altered transcription of at least 26 other genes that may also affect virulence [58].
Rather than using standard biofilm and swarming motility media, we performed these assays in SK media to more closely mimic the conditions that the bacteria are exposed to in the SK assay. To our knowledge, using SK media for swarming motility assays has only been attempted once [33], and it has not been used for biofilm formation and virulence, we suggest that acute-phase virulence factors may be more important for *C. elegans* pathogenesis in the SK model.

PilY1 and the MPs have been implicated in surface detection and activation of virulence [36, 38]. Because loss of PilY1 or any of the MPs prevents T4P assembly and function, it is important to distinguish phenotypes resulting from lack of those components specifically, rather than loss of piliation [22]. Luo et al. [38] suggested that association of PilY1 with surfaces transduces a signal through the T4P system to stimulate c-di-GMP production by SadC, while Rodesney et al. [43] showed that loss of *pilA, pilY1, or pilT* prevents surface-activated c-di-GMP production. Rodesney et al. [43] proposed that both PilY1 and functional T4P are required for mechanosensation; however, it is not possible to delete *pilY1* without ablating T4P assembly. Rather than PilY1 signalling through the T4P assembly machinery and SadC [38, 42], T4P-mediated surface attachment may transiently deplete PilVWXVY1 levels in the IM, resulting in increased FimS-AlgR activity and transition towards a sessile, biofilm lifestyle. Surface-activated increases in c-di-GMP levels are thought to occur within hours of attachment [38, 43]; this idea is consistent with our swarming data, where activation of FimS-AlgR in response to PilVWXVY1 depletion reduces swarming motility (Fig 2).

Whether the loss of *pilW, pilX, or pilY1* leads to increased amounts of AlgR, its increased phosphorylation via FimS, or both remains to be clarified. Okkotsu et al. [50] showed that AlgR and AlgR_{D54E} levels are comparable, suggesting that the loss of virulence we observed for PA14
algR<sub>D54E</sub> is attributable to the D54E phospho-mimetic mutation alone. While overexpression of AlgR<sub>D54A</sub> in trans reduced virulence (Fig 5A), the same mutation on the chromosome reverted virulence of pilW, pilX, and pilY1 mutants to WT levels (Fig 6). Therefore, we suspect that it is primarily AlgR phosphorylation (or lack of AlgR dephosphorylation) that is responsible for loss of virulence. However, it is possible that increased AlgR protein levels and phosphorylation both contribute. Kong et al. [54] showed that AlgR binds fimS-algR, suggesting that the TCS could positively regulate its own transcription in response to reduced PilWXY1 levels.

In addition to being essential for T4P function, FimS and AlgR control alginate production in the context of chronic CF infections, where algR transcription is high [18, 59]. Phosphorylation of AlgR increases binding affinity at some – but not all – of its target sequences [17, 50, 51, 53]. For example, AlgR<sub>D54N</sub> failed to support twitching motility, but did not affect alginate production [17, 51]. FimS is an unorthodox histidine kinase, with 4 transmembrane domains instead of the more typical 2, and lacks both a periplasmic sensing domain and the canonical motif involved in ATP coordination, which mediates auto-phosphorylation [19, 60]. Direct interaction and/or phospho-transfer between FimS and AlgR have not been reported. Rather, the idea that FimS acts as a kinase for AlgR comes from this and other studies demonstrating similar phenotypes for fimS, algR, and algR<sub>D54N</sub> mutants [17, 18, 61]. FimS and AlgR promote expression of genes important for production of alginate, biofilms, and c-di-GMP, and inhibit expression of virulence factors such as the T3SS, pyocyanin, and quorum sensing [54, 62-65]. Dynamic regulation of AlgR levels are important for pathogenesis, as either deletion or overexpression of algR attenuated virulence in a murine model of acute septicemia [66]. However, an algR mutant was also reported to have WT virulence towards amoebae [36]. The outcomes that result from interaction of P. aeruginosa with different hosts will depend on a
variety of factors including host defenses, site of infection, available nutrients, and virulence repertoire of a particular strain. However, our results suggest that changes in the specific repertoire of bacterial virulence factors, or the timing of their production, can tip the balance in the host’s favour.

The subset of AlgR-regulated virulence genes important for *C. elegans* pathogenesis is not clearly defined. Screening of a PA14 transposon library for loss of virulence implicated several genes encoding regulators rather than individual virulence factors, suggesting that *C. elegans* pathogenesis is multifactorial [33]. 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 gene [67]. We saw WT virulence for *algR* mutants, consistent with a role for AlgR in negative regulation of acute phase virulence factors. Factors under positive control of AlgR may be important during later stages of infection in more complex mammalian infection models [68, 69].

While important for the initial stages of infection, T4P are less critical in chronic CF lung infections and are often lost when the bacteria grow for extended periods as biofilms [5, 70, 71]. *P. aeruginosa* CF isolates frequently become mucoid via activation of AlgR, leading to reduced production of other virulence factors [59, 72, 73]. Although the two outcomes are not necessarily temporally or mechanistically linked, mutations that achieve both may be advantageous during chronic CF lung infections. Specifically, loss of PilWXY1 may be adaptive in the context of CF, leading to AlgR activation and loss of T4P function. It will be interesting to examine the genotypes of mucoid CF isolates for these types of mutations.
**Materials and methods**

**Bacterial strains and plasmids**

Strains and plasmids used in this work are listed in Supplementary Table S1. 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 [74]. 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 [75].

**Cloning procedures**

Vectors were constructed using standard cloning procedures, using the primers listed in Supplementary Table S2. Deletion constructs were designed to contain 500-1000 bp homology upstream and downstream the gene to be deleted. Deletion constructs for PA14 *fimU, pilV, pilW, pilX, pilY1, and pilE* were synthesized by Genscript in the pUC57Kan vector. pEX18Gm-*sadC* was created by amplifying the *sadC* deletion region from PA14 *sadC roeA* [41], followed by digestion and ligation into pEX18Gm. pEX18Gm-*algR*<sub>D54A</sub>, pEX18Gm-*algR*<sub>D54E</sub>, and pEX18Gm-*fimS* were made by overlap extension PCR [76]. pMS402-*PfimU* was created by amplifying and digesting the promoter region of the PA14 MP operon. Digested pBADGr was treated with alkaline phosphatase prior to ligation to avoid re-circularization of the vector. Constructs were verified by Sanger sequencing (MOBIX lab, McMaster, Hamilton, ON).
**Mutant generation by allelic exchange**

Allelic exchange was used to remove or alter specific genes [77]. 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.

**Twitching motility assays**

Twitching motility assays were performed as previously described [78], 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 30°C 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 twitching areas were measured using ImageJ. Twitching zones were normalized to WT (100%).

**Swarming motility assays**

SM assays were performed as previously described [79], with the following modifications. The concentration of agar in SK plates (50 mM NaCl, 0.5% agar, 0.35% peptone, 1 mM CaCl₂, 1 mM MgSO₄, 5 µg/ml cholesterol in 100% ethanol, 20 mM KH₂PO₄, and 5 mM
was reduced to 0.5% (w/v) to promote swarming. Plates were dried individually for 1.5 h at room temperature then 3.5 µl of an overnight culture was spotted onto the center of the duplicate plates. Plates were individually incubated upright at 37°C for 24 h, followed by incubation at 22°C for 24 h. Empty swarming motility plates were placed around the perimeter of arrayed experimental plates to reduce edge effects and promote formation of reproducible swarming zones. Experiments were repeated three times, 2 technical replicates per trial.

**Biofilm assays**

Biofilm assays were performed as previously described, with modifications [80]. *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 (50 mM NaCl, 0.35% peptone, 1 mM CaCl$_2$, 1 mM MgSO$_4$, 5 µg/ml cholesterol in EtOH, 20 mM KH$_2$PO$_4$, and 5 mM K$_2$HPO$_4$), 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.

*Caenorhabditis elegans slow killing assay*

SK assays were performed as described previously [81]. SK plates (0.35% peptone, 50 mM NaCl, 2% agar, 1 mM CaCl₂, 5 µg/ml cholesterol, 1 mM MgSO₄, 20 mM KH₂PO₄, 5 mM K₂HPO₄, 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 *E. coli* OP50 plates, washed twice in M9 buffer, and then >50 worms were seeded onto each bacterial lawn on individual 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 was 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.

*Luminescent reporter assay*

Luminescent reporter assays were performed as previously described, with minor modifications [49]. 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 with shaking 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.

**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 [82]. 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 [22, 49, 83], 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 to OD$_{600}$ = 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) (Difco). 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. BTH 101 expressing pUT18C and pKT25 empty vectors was used as a negative control, and BTH 101 expressing pUT18C-*fimS* and pKT25-*fimS* was used as a positive control [49].
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718 **Figure captions**

719 **Fig 1.** PilWXY1 contribute to T4P-independent virulence.
720 (A) SK assays for PA14 pilA, fimU, pilV, pilW, pilX, pilY1, and pilE mutants. Synchronized L4
721 worms were seeded onto SK plates and scored for death every 24 h, then plotted as “percent
722 survival” over the course of the assay. “Day” represents the number of days after L4 on which
723 the plates were scored. 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. (B) (A) SK assays for PAO1 $pilA$, $fimU$, $pilV$, $pilW$, $pilX$, $pilY1$, and $pilE$ mutants. 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. In (A) and (B), asterisks indicate strains that were consistently less virulent than a $pilA$ mutant in all trials by Gehan-Breslow-Wilcoxon test at $p = 0.05$, $n = 3$ trials.

**Fig 2.** PA14 $pilW$, $pilX$, and $pilY1$ mutants have swarming motility defects.

SM assays for PA14 $pilA$, $fimU$, $pilV$, $pilW$, $pilX$, $pilY1$, 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. Representative images are shown, $n = 3$ trials.

**Fig 3.** PilVWXYZ repress their 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. Assays were set up in technical triplicate, and 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 trials.

**Fig 4. AlgR hyperactivation reduces virulence.**

(A) SK assays for PA14 fimS, algR, algR<sub>D54A</sub>, and algR<sub>D54E</sub> mutants. The fimS, algR, and algR<sub>D54A</sub> mutants had WT virulence, while the algR<sub>D54E</sub> mutant was less virulent than WT. (B) SK assays for PAO1 algR, algR<sub>D54A</sub>, and algR<sub>D54E</sub> mutants. The algR and algR<sub>D54A</sub> mutants had WT virulence, while the algR<sub>D54E</sub> mutant was less virulent than WT. For (A) and (B), asterisks indicate strains that were less virulent than WT in all trials, by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3 trials.

**Fig 5. 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<sub>D54A</sub> reduced virulence at 0.05% arabinose. Asterisks indicate strains that were consistently less virulent than PA14 + pBADGr in all trials by Gehan-Breslow-Wilcoxon test at p = 0.05, n = 3 trials. (B) Biofilm assays for algR deletion and overexpression strains. 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. Loss of algR had no effect on biofilm formation. When grown at 0.05% arabinose, overexpression of pBADGr-algR or pBADGr-algR<sub>D54A</sub> increased biofilm formation, p < 0.001. Significance was determined by one-way ANOVA followed by Dunnett post-test relative to WT, n = 3 trials.
Fig 6. The virulence defect of pilW, pilX, and pilY1 mutants is dependent on FimS-AlgR.

SK assays for pilW, pilX, pilY1, fimS, algR, and algR<sub>D54A</sub> single and double mutants. fimS, algR, and algR<sub>D54A</sub> mutants have WT virulence. pilW, pilX, and pilY1 have reduced virulence relative to WT, fimS, algR, and algR<sub>D54A</sub> mutants. Combination of pilW, pilX, or pilY1 mutations with fimS, algR, or algR<sub>D54A</sub> mutations results in virulence equivalent to fimS, algR, and algR<sub>D54A</sub> single mutants, respectively. 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.

Fig 7. The swarming motility defect of pilW, pilX, and pilY1 mutants is dependent on FimS-AlgR.

SM assays for pilW, pilX, pilY1, fimS, algR, and algR<sub>D54A</sub> 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<sub>D54A</sub> mutants had WT swarming. Double mutants swarmed similar to fimS, algR, and algR<sub>D54A</sub> single mutants. Representative images are shown, n = 3.

Fig 8. Model for regulation of the MP operon via FimS-AlgR.

When PilVWXYZ1 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 PilVWXYZ1 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.

Supporting information

**S1 Fig.** Twitching motility 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.

**S2 Fig.** Virulence 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.

**S3 Fig.** PA14 and PAO1 produce low levels of biofilm in liquid slow killing media.
Biofilm assays for (A) PA14 and (B) PAO1 pilA, fimU, pilV, pilW, pilX, pilY1, and pilE mutants. 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.
S4 Fig. PAO1 does not swarm optimally on 0.5% agar slow killing plates.

SM assays for PAO1 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. All strains formed very small smarming zones, and the patterns were not reproducible between strains. Representative images are shown, n = 3.

S5 Fig. 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.
A

Growth (OD₆₀₀)

Biofilm (Absorbance₆₀₀)

Arabinose

PA14 + pB
sadC + pB
sadC + sadC
PA14 + pB
sadC + pB
sadC + sadC

***

B

No arabinose

- PA14 + pB
- sadC + pB
- sadC + sadC *

0.05% arabinose

- PA14 + pB
- sadC + pB
- sadC + sadC *