# Type IV pilins regulate their own expression via direct intramembrane interactions with the sensor kinase PilS

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**Type IV pili are important virulence factors for many pathogens, including *Pseudomonas aeruginosa*. Transcription of the major pilin gene—*pilA*—is controlled by the PilS-PilR two-component system in response to unknown signals. The absence of a periplasmic sensing domain suggested that PilS may sense an intramembrane signal, possibly PilA. We suggest that direct interactions between PilA and PilS in the inner membrane reduce *pilA* transcription when PilA levels are high. Overexpression *in trans* of PilA proteins with diverse and/or truncated C termini decreased native *pilA* transcription, suggesting that the highly conserved N terminus of PilA was the regulatory signal. Point mutations in PilA or PilS that disrupted their interaction prevented autoregulation of *pilA* transcription. A subset of PilA point mutants retained the ability to interact with PilS but could no longer decrease *pilA* transcription, suggesting that interaction between the pilin and sensor kinase is necessary but not sufficient for *pilA* autoregulation. Furthermore, PilS's phosphatase motif was required for the autoregulation of *pilA* transcription, suggesting that under conditions where PilA is abundant, the PilA–PilS interaction promotes PilR dephosphorylation and thus down-regulation of further *pilA* transcription. These data reveal a clever bacterial inventory control strategy in which the major subunit of an important *P. aeruginosa* virulence factor controls its own expression.**

type IV pili | two-component system | transcriptional regulation | *Pseudomonas aeruginosa* | *Geobacter sulfurreducens*

A wide variety of bacteria, including the opportunistic pathogen *Pseudomonas aeruginosa*, use type IV Pili (T4P) for attachment to surfaces and host tissues, biofilm formation, DNA uptake, and twitching motility (1–4). T4P are retractile surface appendages comprised predominantly of thousands of subunits of the major pilin protein, PilA (5), which is rapidly polymerized and depolymerized by a complex assembly machine. Also incorporated into T4P are small amounts of the minor pilins FimU, PilV, PilW, PilX, and PilE (*P. aeruginosa* nomenclature), which prime pilus assembly (6, 7).

PilA and the minor pilins share a similar lollipop-like topology, with a highly conserved, hydrophobic N-terminal α-helix packed against a variable C-terminal antiparallel β-sheet (8, 9). The first ∼24 residues of mature pilins anchor the subunits in the inner membrane until they are polymerized by the assembly machinery (10). When pili are retracted, subunits are disassembled from the base of the pilus and returned to the inner membrane for reuse in subsequent cycles of extension. In *P. aeruginosa*, high levels of PilA in the inner membrane lead to decreased *pilA* transcription, whereas depletion of PilA inner membrane pools significantly elevates *pilA* transcription (11, 12). These data suggest that *pilA* expression is responsive to levels of intracellular PilA, although the sensory mechanism is unknown.

*pilA* transcription in *P. aeruginosa* and many other species is dependent on the PilS-PilR two-component regulatory system (13–16). Two-component systems (TCSs) allow bacteria to rapidly detect and adapt to changes in their environment (17). The sensor kinase (SK) detects physical or chemical signals, typically via an extracytoplasmic domain flanked by two transmembrane (TM) segments (17). On detection of an activating signal, the protein undergoes autophosphorylation on a conserved His residue in the cytoplasmic kinase domain. The phosphate is transferred to a cytoplasmic response regulator, which regulates gene expression in response to the stimulus (18).

In the PilS-PilR TCS, PilR is the cytoplasmic response regulator that activates *pilA* transcription (19). Its cognate SK PilS is atypical, with six TM segments connected by very short loops and no obvious external signal input domain (20). When PilS is absent, *pilA* transcription is significantly reduced, whereas loss of PilR abrogates *pilA* transcription (13). Interestingly, overexpression of full-length PilS also decreases pilin expression, whereas expression of its cytoplasmic kinase domain alone leads to constitutive *pilA* transcription, a common result of decoupling sensor kinases from their signal input domains (19, 21, 22).

Recently, a conserved E/DxxN/T phosphatase motif adjacent to the phosphorylated His was identified in the HisKA family of SKs to which PilS belongs, indicating that many SKs can have dual kinase and phosphatase activities to fine tune regulation (23). PilS has a canonical ExxN motif at position 320–323, beside the H319 phosphorylation site. Coupled with the observation that PilS overexpression reduces *pilA* transcription, the presence of this motif suggests that PilS could have intrinsic phosphatase activity on phospho-PilR.

Among the most significant challenges in the TCS field is identification of the specific signal(s) to which SKs respond. The observation that *pilA* transcription is inversely correlated with levels of PilA in the inner membrane, coupled with the unusual six TM topology of PilS, suggested that it recognizes an intramembrane signal, possibly PilA itself. Here, we show that direct intramembrane interactions between the conserved, hydrophobic N terminus of PilA and one or more TM of PilS down-regulate *pilA* transcription, and define the sequence elements on PilA involved in interaction and regulation. Based on analysis of PilS mutants, we propose that PilA–PilS interactions likely maintain PilS in a phosphatase state when membrane pools of PilA are high, providing a sensitive feedback mechanism for pilin inventory control.

## Significance

**Although two-component systems are a ubiquitous means of rapid bacterial adaptation to changing environments, identification of the specific signals detected by sensor kinases can be challenging. Also, little is known about the diverse, poorly characterized family of sensor kinases that detect intramembrane signals. We show that the major type IV pilin, PilA, is an inhibitory intramembrane ligand for the PilS sensor kinase that controls *pilA* expression and we characterize the mechanism of signal transduction. Because the conserved N-terminal domain of PilA alone can repress *pilA* expression, peptides corresponding to this short region could have potential as therapeutic agents to suppress type IV Pili (T4P) biogenesis.**

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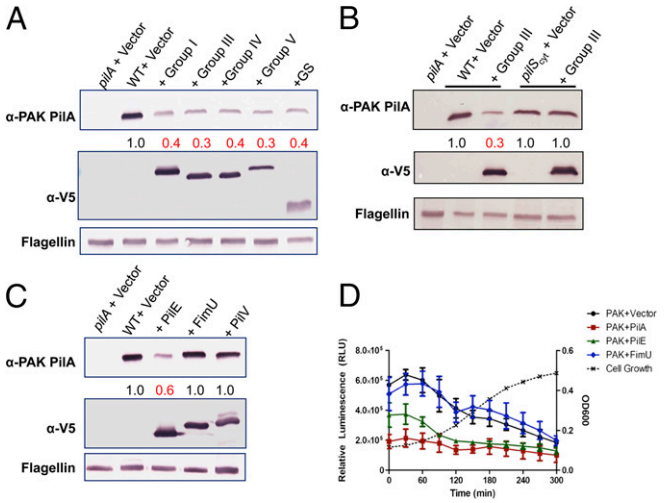


**Fig. 1.** Overexpression of heterologous pilins reduces native PilA levels. (*A*) V5 epitope tagged major pilins from PA1244 (group I), PA14 (group III), PA5196 (group IV), PA1457 (group V), and the *G. sulfurreducens* PilA were overexpressed in the PAK (group II) strain. Heterologous PilAs overexpressed to similar extents (as demonstrated by α-V5 Western blot) reduced native PilA levels. (*B*) In the absence of the TM segments of PilS ($pilS_{cyt}$), overexpression of group III PilA no longer reduces native pilin levels. (*C*) Of the minor pilins—FimU, PilV, and PilE—only PilE caused a reduction in native pilin levels. For all blots, the flagellin band was used as a loading control. Numbers represent relative expression of native PilA in recombinant strains compared the empty vector control, as measured by densitometry and a one-way ANOVA statistical test ($n = 3$). (*D*) PilA and minor pilins FimU and PilE were overexpressed in PAK+pMS402 p*pilA*, and relative luminescence as a function of *pilA* promoter activity was recorded. Mean and SEM of three independent experiments are shown.

## Results

**Overexpression of Heterologous Pilins Reduces PilA Levels by Decreasing *pilA* Transcription.** *P. aeruginosa* strains carry one of five *pilA* alleles (groups I–V) (24), encoding pilins that vary in size and sequence of the C-terminal domain (9), plus the presence or absence of posttranslational modifications (25, 26) and accessory proteins (25, 27). However, the first ∼24 amino acids of mature pilins are highly conserved (Fig. S1), whereas the C-terminal domains are diverse (24). In contrast, PilS in *P. aeruginosa* strains is invariant (28), regardless of the strain's pilin type. These data—plus PilS's lack of an extramembranous signal recognition domain—suggested that the highly conserved hydrophobic N terminus of PilA could be the PilS ligand. To test whether diverse pilins could suppress PilA expression in a heterologous strain, V5-epitope tagged pilins of groups I, III, IV, and V were overexpressed in strain PAK (group II). Intracellular levels of native PAK PilA were monitored using PAK PilA-specific antisera. Overexpression of each of the heterologous pilins from an inducible vector reduced native PilA levels by >50% (Fig. 1*A*).

*Geobacter sulfurreducens* (GS), which regulates *pilA* transcription using a similar PilSR TCS (29), has unusually short type IV pilins that retain the characteristic conserved hydrophobic N-terminal α-helix, but lack a globular C-terminal domain. This architecture is thought to promote efficient electron transfer through pilus "nanowires" (30). The first 24 residues of the mature GS pilin are 92% identical to those of *P. aeruginosa* PilA (Fig. S1). When the GS pilin was overexpressed in *P. aeruginosa*, native pilin expression was reduced to the same extent as with heterologous *P. aeruginosa* pilins (Fig. 1*A*), showing that the N-terminal segment of a pilin is sufficient for repression of PilA expression. This autoregulatory phenotype is dependent on the TM segments of PilS. The chromosomal copy of *pilS* was replaced with a version encoding an N-terminal truncation lacking the TM segments: residues 3–176 ($PilS_{cyt}$). Although this form of PilS supported near-WT levels of PilA expression, overexpression of the heterologous group III PilA in this background failed to reduce native PilA levels (Fig. 1*B*).

Although overexpression of heterologous pilins in the presence of WT PilS reduced native PilA levels, T4P function was unaffected if the heterologous subunits were competent for assembly. GS can assemble *P. aeruginosa* pilins (31), but the reverse was not true, even when pilus retraction was blocked to maximize the capture of assembled pili (Fig. S2*A*). When the assembly-incompetent GS pilins were expressed from an inducible vector in *P. aeruginosa*, twitching motility decreased in an inducer concentration-dependent manner (Fig. S2*B*).

To address whether overexpression of other pilin-like proteins with divergent N termini could impact PilA levels, each of the minor pilins—FimU, PilV, PilW, PilX, or PilE—was overexpressed in strain PAK. However, the levels of PilW and PilX were lower than those of other minor pilins and they were therefore excluded. Of the three remaining minor pilins, only PilE reduced native PilA levels, to ∼60% of the vector control. Neither FimU nor PilV had an effect on PilA levels when overexpressed (Fig. 1*C*). Thus, specific sequences in the pilin N terminus are required for *pilA* regulation.

To determine if PilA autoregulation occurred at the level of transcription, the *pilA* promoter was cloned upstream of a luciferase (*lux*) reporter (pMS402-p*pilA*). *pilA* promoter activity was monitored in PAK carrying the pBADGr vector or pBADGr-*pilA*, pBADGr-*pilE*—both of which reduced native pilin levels when overexpressed—or pBADGr-*fimU*, which had no effect on PilA levels (Fig. 1*D*). In agreement with the Western blot data in Fig. 1*C*, overexpression of FimU had no impact on *pilA* transcription. However, *pilA* transcription was significantly decreased in both PilA and PilE overexpression strains (Fig. 1*D*). Due to the inherent leakiness of the pBADGr promoter, differences in *pilA* transcription as a result of protein overexpression can be seen even at $t = 0$, when expression from pBADGr is first induced with arabinose. Together, these data suggest that the conserved N termini of PilA and PilE, but not FimU, contain the appropriate sequence information needed for down-regulation of *pilA* transcription.

**PilA and PilS Interact Directly in the Inner Membrane.** We next tested our hypothesis that direct PilA–PilS interaction led to decreased *pilA* transcription, using a bacterial adenylate cyclase two-hybrid

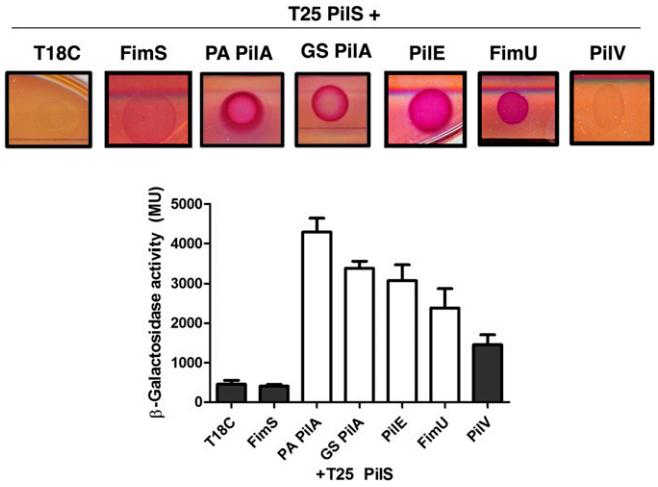


**Fig. 2.** PilA and select minor pilins interact with PilS. T25-PilS interacts with PilA from both *P. aeruginosa* and *G. sulfurreducens*, PilE, and to a lesser extent, FimU, each tagged with the T18 domain of adenylate cyclase. PilV did not interact with PilS in either the McConkey agar plate assay or the β-galactosidase activity assay (gray bars). β-Galactosidase activity resulting from protein–protein interactions was measured by ONPG-hydrolysis as described in *Materials and Methods*. FimS was used as a membrane-bound negative control for PilS interactions.

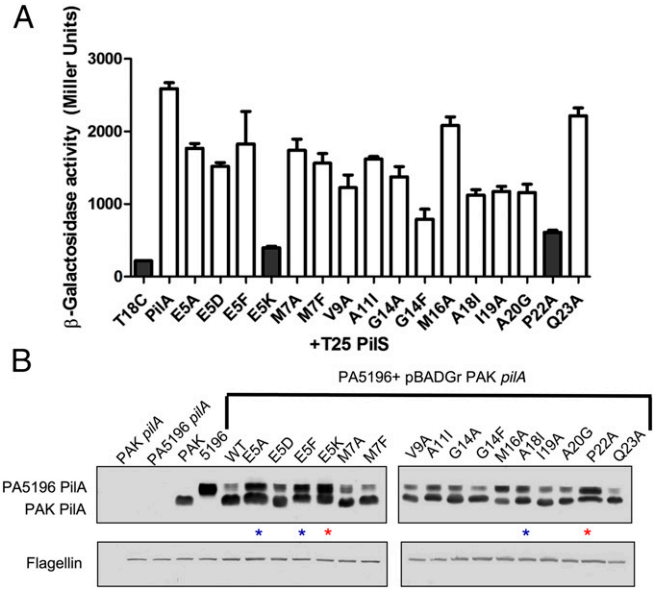


**Fig. 3.** PilA residues E5 and P22 are important for the PilA–PilS interaction and PilA autoregulation. Point mutations in the conserved N terminus of PAK PilA were generated using site-directed mutagenesis. (*A*) T18-PilA fusions coexpressed with T25-PilS in the β-galactosidase liquid assay were tested for interaction. PilA E5K and P22A fail to interact with PilS (gray bars, $P < 0.01$). (*B*) The same set of PAK PilA point mutants were overexpressed in strain PA5196 (group IV), and both PAK mutant and native PA5196 PilA levels were detected by Western blot with α-PAK and α-PA5196 PilA-specific antibodies. Overexpression of PAK WT and most mutant PilA derivatives decreased PA5196 PilA levels, whereas E5A, E5F, and M16A interacted with PilS, but their overexpression resulted in near-WT levels of native PilA (blue stars), and E5K and P22A transformants did not interact with PilS and also had near-WT levels of PA5196 PilA (red stars).

(BACTH) assay (32). T18-pilin fusions of PAK PilA, *G. sulfurreducens* PilA, and minor pilins PilE, FimU, and PilV were coexpressed with T25-PilS in *Escherichia coli* BTH 101, and potential interactions were detected by monitoring β-galactosidase activity. PilA from *P. aeruginosa* and *G. sulfurreducens*, as well as the minor pilin, PilE, interacted with PilS (Fig. 2; $P < 0.01$), correlating with their ability to reduce *pilA* transcription when overexpressed in *P. aeruginosa*. Unexpectedly, FimU, which had no effect on *pilA* transcription, interacted with PilS, suggesting that interaction and regulation are separable phenotypes. Based on the results of a one-way ANOVA analysis ($P > 0.05$) and a negative result in the McConkey plate assay, PilV did not interact with PilS (Fig. 2, gray bars).

**E5 and P22 of PilA Are Important for Interaction and Regulation.** The ability of PilA, PilE, and FimU to interact with PilS, even though FimU did not repress *pilA* transcription, implied that specific pilin N-terminal residues were important for discrimination of the regulatory signal. To dissect the residues required for both the PilA–PilS interaction and subsequent regulation of *pilA* transcription, a series of point mutations was generated in the PAK T18-PilA fusion and the inducible pBADGr-*pilA* construct, respectively. We targeted residues E5, A20, and P22, important for a number of T4P-related functions (8, 15, 33–36), plus additional residues conserved between PilA and PilE, but not FimU (Fig. S1), suggesting that they could be regulatory positions. In general, the native residue was substituted to Ala, but E5A, E5D, E5F, and E5K substitutions were also made to establish which R-group characteristics were important at this position. The stability of each mutant T18-PilA fusion was verified by α-PilA Western blot (Fig. S3). Although its expression is slightly decreased compared with the other T18-PilA fusions, T18-PilA E5K is present in high enough abundance to support interaction with WT T25-PilA (Fig. S3*B*). Only substitutions at E5 or P22 disrupted the PilA–PilS interaction in the BACTH assay (Fig. 3*A*). Interestingly, only PilE and FimU have a P22 residue, supporting a role for a kinked N-terminal helix in pilin–PilS interactions. A positive charge at position E5 was nonpermissive, as E5K, but not E5A, E5F, or E5D, abolished the PilA–PilS interaction (Fig. 3*A*).

The effects of PilA point mutant overexpression were also tested directly in *P. aeruginosa*. To more easily differentiate between plasmid and chromosomally encoded pilins, PAK PilA point mutants were overexpressed in strain PA5196 (group IV). Because PA5196 pilins are glycosylated with D-arabinofuranose (25, 26), they are of larger mass than those of PAK, allowing the two to be readily separated on 18% SDS/PAGE. Coincubation of Western blots with non–cross-reactive α-PAK PilA and α-PA5196 PilA antibodies allowed for the simultaneous detection of both pilins. PAK PilA point mutants were all stably expressed in PA5196 (Fig. 3*B*, lower band). Overexpression of most PAK PilA derivatives reduced native pilin levels to the same extent as the WT pilin, whereas PAK PilA E5K and P22A failed to reduce PA5196 PilA levels (Fig. 3*B*, upper band), consistent with their inability to interact with PilS. Notably, PAK PilA E5A and E5F could interact with PilS (Fig. 3*A*) but had no effect on PA5196 PilA expression. The M16A and A18I point mutants had intermediate phenotypes, in that their overexpression reduced levels of PA5196 PilA, but to a lesser extent than the WT PAK pilin. Together, the data suggest that the PilA–PilS interaction is necessary but not sufficient for modulating PilA expression levels and that specific intermolecular contacts that depend on PilA N-terminal conformation (P22) and charge (E5) are required for proper signal transduction.

PilS and its homologs in other species contain a conserved, positively charged arginine residue in the first predicted TM (PilS R24 in *P. aeruginosa*) that we hypothesized might interact with PilA E5. R24 is not required for PilS activity, but an R24E substitution leads to loss of *pilA* autoregulation (Fig. 4*A*). As predicted, PilS R24E failed to interact with PilA (Fig. 4*B*). Interestingly, charge-swapped PilA E5K and PilS R24E variants also failed to interact (Fig. 4*B*), suggesting that the R24 has another role: potentially formation of a salt bridge with a second conserved charged residue in TM3 of PilS, D86. Charge alteration at either R24 or D86 disrupted the PilA–PilS interaction, but PilA interaction was restored with a PilS R24E D86K double charge-swapped mutant, suggesting that the charged TM residues control PilS conformation and thus its ability to interact with pilins (Fig. S4).

**A PilS Phosphatase Motif Is Required for PilA Autoregulation.** In earlier studies (21), PilS was predicted to have kinase and potentially phosphatase activities. We identified a canonical ExxN phosphatase motif (23) adjacent to the H319 residue that is the site of PilS phosphorylation and engineered a PAK chromosomal point mutant expressing PilS N323A, a substitution shown

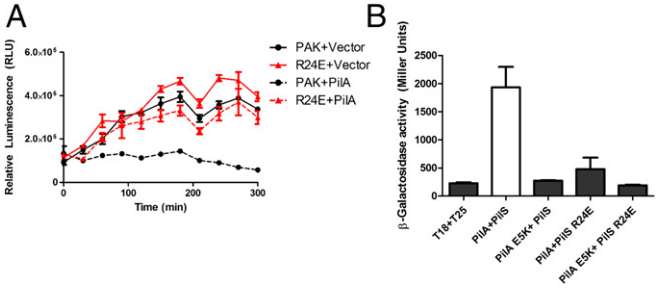


**Fig. 4.** PilS R24E neither interacts with PilA E5K nor autoregulates *pilA* transcription in vivo. (*A*) The conserved R24 residue of PilS was substituted with a glutamic acid. When expressed from the chromosome, PilS R24E activated *pilA* transcription in the *lux-pilA* assay, but was no longer responsive to PilA overexpression. (*B*) In the T25-PilS fusion, the R24E PilS variant failed to interact with T18-WT PilA or T18-PilA E5K (gray bars).

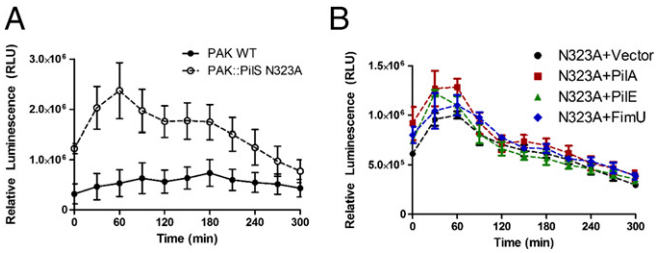


**Fig. 5.** A conserved PilS phosphatase motif is required for PilA autoregulation. A chromosomal PilS N323A substitution was made to disrupt the putative PilS phosphatase motif. (*A*) The PilS N323A mutant has significantly higher than WT levels of *pilA* transcription. (*B*) When PilA, PilE, or FimU are overexpressed in the PilS N3232A background, *pilA* promoter activity is comparable to the empty vector control. Error bars represent the SE calculated from three independent experiments.

previously to disrupt SK phosphatase, but not kinase, activity (23). WT PAK and the PilS N323A mutant were each transformed with the pMS402-p*pilA lux* reporter to test the effect of this mutation on *pilA* transcription. The PilS N323A mutant had an approximately threefold increase in basal *pilA* transcription compared with WT (Fig. 5*A*). These data suggest that PilS N323A could lack phosphatase activity or have increased kinase activity.

To distinguish between these possibilities, the PilS N323A mutant was cotransformed with pMS402-p*pilA* and empty pBADGr vector, or pBADGr-*pilA, -pilE*, or *-fimU*, and the ability of the pilins to modulate *pilA* transcription was tested. Unlike in the WT background (Fig. 2*C*), overexpression of PilA or PilE failed to reduce *pilA* transcription in the PilS N323A point mutant (Fig. 5*B*), even though the pilins interact with PilS N323A (Fig. S5). Together, these data suggest that PilA–PilS interactions likely down-regulate *pilA* transcription by promoting PilS phosphatase activity on PilR.

## Discussion

TCSs are widely used by bacteria for rapid adaptation to changes in their intra- and extracellular environments. However, the signals to which most SKs respond remain unknown (17). In this work, we provide evidence that PilA is a protein ligand for the SK, PilS, which controls pilin expression. We propose an autoregulatory model for the control of *pilA* transcription that is dependent on intrinsic PilS phosphatase activity (Fig. 6).

When levels of intracellular PilA are low, *pilA* transcription is significantly increased (11). In our model, depletion of PilA in the inner membrane leads to fewer PilA–PilS interactions. In the absence of such interactions, PilS adopts a kinase conformation and phosphorylates PilR, activating *pilA* transcription until intracellular PilA inventory increases. Conversely, when PilA levels are high, *pilA* transcription is dramatically reduced (11). Changes in PilA protein levels under the same conditions are more modest due to inherent pilin stability (11). It is also possible that PilS-PilR could impact PilA levels by regulating the expression of additional factors that indirectly impact *pilA* expression, contributing to the difference in magnitude between transcription and protein levels.

We propose a model where PilA–PilS interaction promotes a PilS phosphatase conformation, preventing further *pilA* transcription until intracellular pilin inventories become depleted. Although PilA interacts with PilS N323A (Fig. S5), the SK presumably no longer dephosphorylates PilR, and *pilA* transcription is elevated even when intracellular PilA levels are high. Similarly, our model explains why overexpression of only the cytoplasmic portion of PilS does not impair *pilA* transcription: lack of inhibitory signal input via interaction of PilA with the TM segments of PilS likely prevents propagation of the conformational changes that favor phosphatase activity. This hypothesis is supported by lack of reduction in native pilin levels when heterologous pilins are overexpressed in a strain that expresses a TM-less form of PilS, indicating that PilA autoregulation specifically requires the TM segments of PilS (Fig. 1*B*).

Although an activating signal for *pilA* transcription has not been identified, this work suggests that PilA is an inhibitory signal for its own transcription. The opposite paradigm was identified in *Streptococcus bovis*, where the kinase activity of the eight-TM SK BovK is partially controlled by its product, the lantibiotic HJ50, via interactions at the periplasm–inner membrane interface (37). Unlike PilA, HJ50 acts as an inducer of its own expression when it interacts with BovK, which, like PilS, lacks an extracytoplasmic signal detection domain (37).

Autoregulation in TCSs has also been demonstrated in some gram-negative systems. The most relevant example is the PhoPQ TCS of *E. coli*, which controls the transcription of a diverse set of genes in response to low magnesium (38–40), low pH, or exposure to antimicrobial peptides (38–40). MgrB is a small membrane peptide whose expression is positively regulated by PhoPQ. When

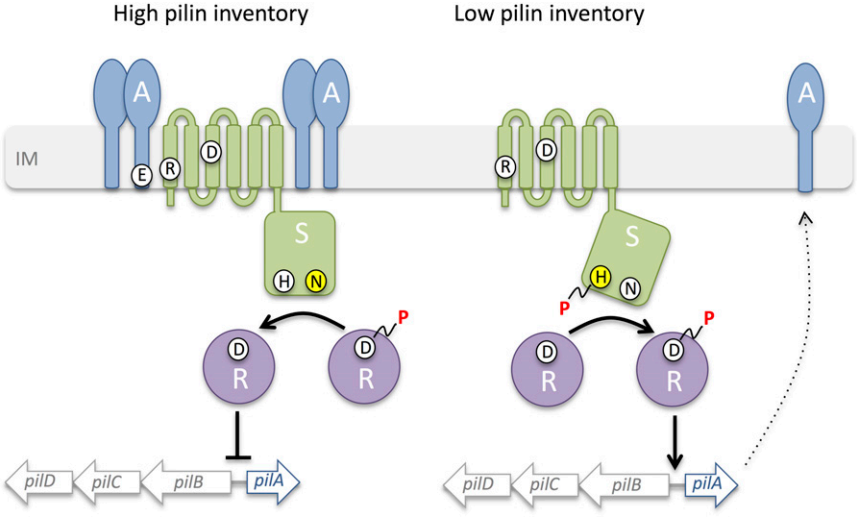


**Fig. 6.** Model of *pilA* transcriptional autoregulation. (*Left*) When PilA levels in the inner membrane are high, PilA-PilS interactions occur more frequently and PilS dimers bound to PilA adopt a phosphatase-active conformation, deactivating PilR and thus reducing *pilA* transcription. (*Right*) When inner membrane pools of PilA are low, PilA–PilS interactions are less frequent. PilS remains in a kinase state and continues to up-regulate *pilA* transcription until PilA levels rise.

MgrB is highly expressed, transcription of the entire PhoPQ regulon is decreased, whereas the regulon is up-regulated in *mgrB* mutants (41). MgrB interacts directly with PhoQ (41). Interestingly, autoregulation appears to be the only function of MgrB, whereas PilA has both autoregulatory and structural roles.

Intramembrane-signal sensing SKs have been most widely studied in gram-positive bacteria, where they often sense perturbations in the membrane itself (42, 43). Only a few gram-negative SKs that rely on intramembrane interactions for signal transduction have been identified and most require accessory proteins for signal transduction. For example, *E. coli* UhpB is a predicted eight-TM SK that modulates expression of a sugar phosphate transport protein, in response to extracellular glucose-6-phosphate (44). Unlike PilS, UhpB cannot detect its signal directly. Instead, UhpC, a single TM inner membrane protein, binds glucose-6-phosphate and interacts with UhpB in the inner membrane to drive downstream transcriptional activation (44). Thus, PilS represents one of the first gram-negative SKs that directly detect its ligand, PilA, in the inner membrane without the use of accessory proteins.

The N-terminal amino acid sequence of PilA is highly conserved, even among distantly related species (35, 45, 46), whereas the C-terminal domains of pilins, both major and minor, can be extremely divergent (24). Heterologous *P. aeruginosa* pilins, plus the naturally truncated pilin from *G. sulfurreducens*, interacted with PilS (Fig. 2) and reduced chromosomal PilA expression in *P. aeruginosa* (Fig. 1*A*), suggesting that the N terminus of PilA mediates both the interactions and their regulatory consequences. However, the pilin–PilS interaction alone is not sufficient for regulation, as specific point mutants of PilA (E5A or E5F) or minor pilins (FimU) interacted with PilS (Figs. 2 and 3) but failed to decrease PilA expression (Figs. 1*C* and 3). It is possible, although less likely, that these pilins interact with PilS at a different site than the WT pilin, leading to their inability to reduce *pilA* transcription. Although PilS–minor pilin interactions are probably physiologically irrelevant due to the low abundance of minor pilins in vivo (6, 47), the effects of their overexpression on *pilA* transcription gave insight into sequence specificity of PilS interaction and regulation. PilE and FimU each share 14 of 24 N-terminal residues with PilA, but the pattern of conservation is different (Fig. S1), implicating both overall similarity and the presence of specific residues in regulation. For example, P22 creates a kink in the N terminus and controls pilin angle in the membrane (10). The P22A mutation abolished PilA–PilS interactions and PilA autoregulation, and PilV, which lacks a proline at position 22, failed to interact stably with PilS. Very low levels of β-galactosidase activity in this sample could be indicative of a weak or transient interaction, although the plate-based assay supports lack of interaction (Fig. 2).

Also important for the PilA–PilS interaction and autoregulation of *pilA* transcription was the highly conserved E5 residue, present in PilA, PilE, and FimU—all of which interact with PilS—but also in PilV, which did not (Fig. 2). Thus, multiple contacts are important for the pilin–PilS interaction. Rare charged residues in TM segments are typically buried within multi-TM proteins or involved in protein–protein interactions (48, 49). PilA E5K or PilS R24E substitutions disrupted PilA–PilS interactions and dysregulated PilA expression, but the PilA E5K and PilS R24E charged-swapped pair failed to interact (Fig. 4*B*). Instead, interaction with WT PilA was restored when PilS R24E and D86K substitutions were combined (Fig. 4*B*), suggesting that these residues form a salt bridge that stabilizes a PilS conformation amenable to PilA binding.

Identification of the signals detected by SKs remains a significant challenge. Although we show here that PilA is an inhibitory signal for PilS, there may also be an activating signal, as hypothesized previously (15, 21). Recent studies showed that T4P and a number of other *P. aeruginosa* virulence factors are significantly up-regulated on surface contact, through increases in intracellular cAMP production (50, 51). Increased cAMP production leads to activation of *pilSR* expression by the cAMP-binding regulatory protein Vfr (52), which could account for increased *pilA* transcription on surface interaction. Alternatively, cAMP itself, or other molecules associated with the surface-responsive Chp chemotaxis system (50), could activate PilS and thus increase PilA levels.

Each pilus contains thousands of subunits whose synthesis consumes cellular resources, but the subunits can be recycled back to the inner membrane on disassembly to mitigate demand (53). We suggest that the inventory control mechanism identified here is used by T4P-expressing bacteria to regulate expression of major pilins in response to their levels in the inner membrane. The ability of the naturally truncated GS pilin to impair expression of the heterologous *P. aeruginosa* pilin gene suggests that it may be possible to design PilA N-terminal–mimetic peptides with the potential to block T4P expression and function.

## Materials and Methods

**Bacterial Strains and Plasmids.** Bacterial strains and plasmids used in this study are summarized in Tables S1 and S2. All vectors were constructed using standard cloning techniques and introduced into *E. coli* and *P. aeruginosa* using heat shock and electroporation, respectively. All *E. coli* and *P. aeruginosa* strains were grown in Lennox broth (LB) media (Bioshop) or LB 1.5% (wt/vol) agar plates in the presence of appropriate antibiotics. Antibiotic concentrations for *E. coli* strains were as follows: gentamycin, 15 μg/mL; ampicillin, 100 μg/mL; or kanamycin, 50 μg/mL. For the comparable *P. aeruginosa* strains, the following were used: gentamycin, 30 μg/mL; or kanamycin, 150 μg/mL. Deletion and Flp recombinase target (FRT) sequence insertion mutants were made as described in ref. 54.

**Site-Directed Mutagenesis.** Site-directed mutagenesis of PAK *pilA* was performed using the QuikChange protocol (Agilent Technologies) Following PCR amplification, reactions were then treated with 1 μL 10 U/μL FastDigest DpnI (Fermentas) for 2 h in a 37 °C water bath and transformed into chemically competent DH5α cells. Nucleotide substitutions were verified by DNA sequencing [McMaster Institute for Molecular Biology and Biotechnology (MOBIX)].

**Lux-*pilA* Luminescent Reporter Assay.** *pilA* transcription in various *P. aeruginosa* strains was measured in 96-well plate liquid cultures similar to the method described in ref. 55. Briefly, bacterial strains carrying the pMS402+p*pilA* plasmid were grown for 16 h at 37 °C in 5 mL LB-Kan, 150 μg/mL Cultures were then diluted 1:20 in fresh media and grown to a standardized $OD_{600}$ = 0.15, and the $OD_{600}$ and relative luminescence of each culture were measured at 15-min intervals using a Synergy 4 microtiter plate reader (BioTek) for 5h. See *SI Materials and Methods* for additional details.

**Twitching Motility Assay.** Twitching motility assays were performed as described previously (24). Briefly, 1% LB agar plates were stab inoculated to the plate–agar interface with a single *P. aeruginosa* colony and incubated at 37 °C for 24 h. The agar was then removed, and plates were stained with 1% (wt/vol) crystal violet for 20 min and washed with water to remove excess dye. Twitching zone diameter was measured in ImageJ (imagej.nih.gov/ij/; National Institutes of Health), and statistical significance was determined using a one-way ANOVA test.

**Sheared Surface Protein Analysis.** Sheared surface protein preparations were conducted as described in ref. 56. Additional details are available in *SI Materials and Methods*.

**Western Blot Analysis.** See *SI Materials and Methods* for details on protein sample preparation. Protein samples were separated on 15% SDS/PAGE and transferred to nitrocellulose membrane. Membranes were then blocked in 5% (wt/vol) milk solution in PBS. α-5196 PilA and α-PAK PilA rabbit polyclonal antibodies (Cedarlane Laboratories) were used at 1:7,500 dilutions. α-V5 monoclonal primary antibody and alkaline phosphatase-conjugated goat α-rabbit secondary antibody (both Sigma-Aldrich) were used at 1:3,000 dilutions. Blots were developed using manufacturers' instructions. Blots were scanned and densitometry was performed using ImageJ and data from at least three independent experiments. One-way ANOVA analysis was used to determine significance of native pilin decreases in Graphpad Prism 5.01.

**Bacterial Two-Hybrid β-Galactosidase Activity Assay.** Chemically competent *E. coli* BTH 101 cells were cotransformed with derivatives of the pUT18C and pKT25 plasmids—expressing the T18 and T25 domains of adenylate cyclase, respectively—and interactions were determined using a 96-well β-galactosidase assay as described in ref. 57 or on MacConkey agar supplemented

with 1% maltose. A one-way ANOVA statistical analysis was performed on β-galactosidase assay results of four independent experiments with a Dunnett posttest to determine significance. Additional details can be found in *SI Materials and Methods*.


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