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A bacterial sense of touch: T4P retraction motor as a means of surface sensing by *Pseudomonas aeruginosa* PA14

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ABSTRACT Most microbial cells found in nature exist in matrix-covered, surface-attached communities known as biofilms. This mode of growth is initiated by the ability of the microbe to sense a surface on which to grow. The opportunistic pathogen *Pseudomonas aeruginosa* (*Pa*) PA14 utilizes a single polar flagellum and type 4 pili (T4P) to sense surfaces. For *Pa*, T4P-dependent "twitching" motility is characterized by effectively pulling the cell across a surface through a complex process of cooperative binding, pulling, and unbinding. T4P retraction is powered by hexameric ATPases. *Pa* cells that have engaged a surface increase production of the second messenger cyclic AMP (cAMP) over multiple generations via the Pil-Chp system. This rise in cAMP allows cells and their progeny to become better adapted for surface attachment and activates virulence pathways through the cAMP-binding transcription factor Vfr. While many studies have focused on mechanisms of T4P twitching and regulation of T4P production and function by the Pil-Chp system, the mechanism by which *Pa* senses and relays a surface-engagement signal to the cell is still an open question. Here we review the current state of the surface sensing literature for *Pa*, with a focus on T4P, and propose an integrated model of surface sensing whereby the retraction motor PilT senses and relays the signal to the Pil-Chp system via PilJ to drive cAMP production and adaptation to a surface lifestyle.

KEYWORDS surface sensing, type 4 pili, biofilm, PilT, motor, Pil-Chp, *Pseudomonas aeruginosa*

> "All models are wrong, but some are useful"
>
> *-George Box*

Bacteria exist as either free-swimming planktonic cells or as communities of surface-attached cells encased in an extracellular matrix known as a "biofilm" (1). Of the estimated 1.2×10^{30} microbial cells on planet Earth, up to 80% of them may be in a biofilm (2). The first step in the transition from the planktonic lifestyle to the biofilm mode of growth is detecting surface engagement and transmitting this signal to the cell, which we refer to here as "surface sensing" (1, 3–5). *Pseudomonas aeruginosa* (*Pa*) is an opportunistic pathogen that is able to cause biofilm infections in persons suffering from burn wounds, cystic fibrosis, and acute leukemia (6). Like many other bacteria, *Pa* uses its two motility appendages, flagella and type 4 pili (T4P), to sense and traverse surfaces (Fig. 1) (3, 7). *Pa* possesses a single polar flagellum that rotates and propels the cell forward in both a liquid environment (swimming) and across surfaces (swarming) (8, 9). Stator proteins incorporated into the flagellum machinery utilize the cell's proton motive force (PMF) to power rotation (10–12). T4P are hairlike appendages that extend from the cell's poles. Polymerization of the pilin monomer leads to extension of the pilus fiber, and depolymerization leads to retraction (13, 14). In *Pa* PA14, T4P extension is powered by a single motor, PilB, and retraction is powered by primary and accessory retraction

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motors, PilT and PilU, respectively. All three of these motor ATPases form hexamers and are powered by ATP hydrolysis (14–19).

The motility appendages necessary for surface sensing are present in many strains of *Pa*, but the extent to which each of these appendages contributes to surface adaptation and biofilm formation varies widely between backgrounds (20–22). Various studies have investigated the different mechanisms of surface sensing and their impact among *Pa* strains. For example, while both *Pa* strains PA14 and PAO1 encode the Wsp and Pil-Chp systems, Pil-Chp and cyclic AMP (cAMP) appear to be more critical for early T4P-driven surface sensing and attachment in the PA14 background, whereas the Wsp system and cyclic di-guanosine monophosphate (c-di-GMP) are more important in the PAO1 background (20, 21). Furthermore, extracellular components that can affect biofilm formation, such as exopolysaccharides (EPSs), also vary between *Pa* backgrounds. PAO1 contains the genes necessary for Pel, Psl, and alginate production. In contrast, the PA14 strain lacks the biosynthetic machinery to produce Psl but retains proteins that interact with Psl such as the adhesin CdrA and T4P, as well as the glycosyl hydrolase responsible to Psl degradation, PslG. Furthermore, as the mechanism of T4P-mediated surface sensing is still unclear despite recent efforts, we focus on this aspect of surface sensing rather than a comprehensive review of all surface sensing pathways. For these reasons, this review will focus on studies using *P. aeruginosa* PA14 as a model organism with a focus on T4P-mediated surface sensing.

After surface contact by *Pa* PA14, one of the first cellular changes to occur is the increase of the nucleotide second messenger 3′5′-cAMP (23, 24). In *Pa*, this molecule is required for further surface adaptation as well as acute virulence (25–27). Robust cAMP production by *Pa* is dependent on functional T4P and the presence of a surface (24, 28, 29). Although the precise relationship between cAMP and c-di-GMP signaling is complex

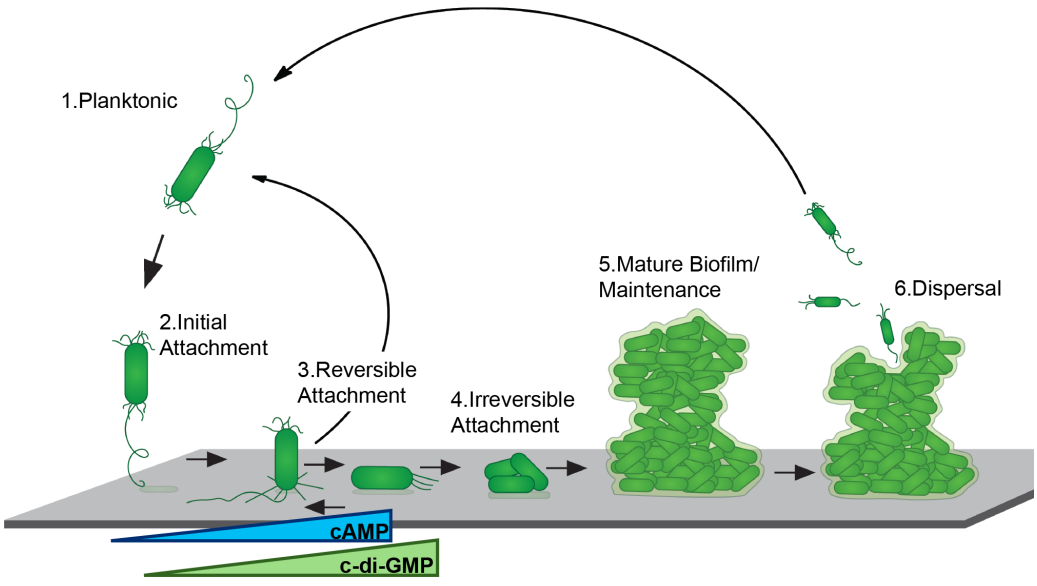

FIG 1 Diagram of the biofilm cycle of *Pseudomonas aeruginosa* PA14. Biofilm formation by *Pseudomonas aeruginosa* PA14 (green) begins with free-swimming planktonic cells (1) making initial surface attachment through its single polar flagellum (2). After initial surface contact, bacteria can either continue to explore the surface using their motility appendages or return to the planktonic population in a process known as reversible attachment (3). As cells continue to explore the surface using T4P, they become better surface adapted in a process that is mediated by cAMP level (blue triangle). Surface contact also stimulates c-di-GMP production (green triangle). Both second messengers oscillate at the single-cell level over multiple generations. How the levels of these two messengers are related to each other remains an area of active research. Once cells are surface adapted, they commit to the biofilm lifestyle and become irreversibly attached (4). As biofilm cells continue to grow on a surface, they produce matrix components necessary for the formation of a mature biofilm (5). When conditions become unfavorable for the biofilm, cells can either passively or actively disperse into the planktonic population (6). cAMP, cyclic AMP; c-di-GMP, cyclic di-guanosine monophosphate.

and not fully understood, this T4P- and surface-mediated increase in cAMP leads to a subsequent increase in c-di-GMP level that is necessary for production of a mature biofilm (5). The biofilm can be maintained, which is an active process. Alternatively, if conditions become unfavorable, cells can disperse from the biofilm and return to the planktonic state (5, 30–34). It is important to note that when we say surface sensing, we are focusing on the first few steps of surface engagement that lead to an increase in both nucleotide second messengers. Here we review data regarding surface sensing mediated by T4P for *Pa* PA14 and propose a new model whereby cells sense and transmit this surface engagement signal via the T4P retraction motor PilT.

SURFACE SENSING AND ITS CONNECTION TO VIRULENCE

The surface-mediated increase in cAMP to initiate biofilm formation cascade also contributes to activation of virulence genes (25, 27, 35–37). Most cellular changes that occur in *Pa* upon cAMP increase are due to its cAMP-binding protein and virulence factor regulator called Vfr (35, 38). Vfr bound to cAMP directly activates many genes necessary for type 2 secretion systems and type 3 secretion systems, including expression of the *exsA* gene (37). Vfr also plays a critical role in the feed-forward loop for cAMP production, which is discussed in detail below. These cAMP-regulated genes are active during acute *Pa* infections. Thus, studying surface sensing will help us integrate two key aspects of bacterial biology: biofilm formation and host-microbe interactions.

SURFACE SENSING BY MOTILITY APPENDAGES

As cells near a surface, motility appendages can contact the surface, leading to a dramatic change in the forces experienced by the cell envelope as well as the motors powering T4P retraction and stators powering flagellar rotation (39, 40). Here the mechanical force experienced by the cell can serve as the signal of surface engagement for the cell, a process known as mechanosensing in bacteria. This aspect of surface sensing focuses on the mechanical forces exerted on the cell due to the presence of attachment to a substrate that is not encountered in a planktonic environment. For a full explanation on the difference between surface sensing and "mechanosensing," please see a recent review from our team (4). Below, we highlight the different forces experienced by *Pa* due to motility appendages interacting with the surface and the resulting cellular changes that occur.

Forces experienced by *Pa* cells upon surface contact: the flagellum

Planktonic *Pa* cells experience force due to flagellar rotation in a liquid medium. Swimming cells in a liquid with a viscosity similar to water will experience a drag force of ~0.5 to 2.0 pN (39, 41). Flagellar rotation creates a force that causes the cell body to counter-rotate and generates a resistive torque of ~1,600 pN (39). The *Pa* flagellar motor has a torque output of 2 pN·µm (42). When the load on the flagellum increases either due to changes in viscosity or engagement of the cell body or flagellum with a surface, the flagellar machinery is able to undergo remodeling to recruit more stator units (43–47). Stators only conduct protons upon incorporation into the flagellar motor, and the ability to conduct protons is crucial to mechanosensitive stator recruitment, as mutant stators that no longer allow the flow of protons are not recruited to the flagellar motor (47).

Based on work in *Escherichia coli*, under low loads, the flagellum can contain 4–6 stator units, but this number can increase to up to 11 in *E. coli* and up to 16 units under high load in some organisms (48–51). When the flagellum is experiencing a high load, each stator unit can contribute ~10 pN of force toward flagellar rotation (47). Stator units bind the peptidoglycan (PG) layer as well as FliG of the C ring of the flagellar base to convert the energy supplied by PMF into flagellar torque. It is thought that under high loads, the stators undergo a catch-bond regime, which enhances binding between stators and the flagellar machinery (52); that is, binding of the stator to the motor "increases" with increasing load on the flagellum.

Pa contains two sets of stators that power flagellar rotation. MotAB stators are dominant in low load environments and are produced at ~40× higher levels than the second stator set, MotCD (53). The MotCD stator is required for high-load flagellar rotation like that experienced during swarming motility (8, 10, 54). Interestingly, based on single motor measurements, both stator sets exert approximately the same amount of torque, but flagella composed of MotCD stators are 10× more likely to be active and rotating at any given instance of time (53).

For *Pa*, initial surface contact can be mediated by the flagellum and is accompanied by a sharp increase in the flagellar load from ~5 to >150 pN·nm·s/revolution (39, 42, 55). The change in forces due to flagellar-mediated surface contact is not unique to *Pa*, and many different organisms rely on their flagella to sense a surface (56–58). While it is likely that the flagellum contributes to surface sensing in *Pa* (12, 42, 55, 59, 60), a detailed discussion of the mechanism(s) of flagellar-mediated surface sensing and links to T4P sensing is beyond the scope of this review.

Forces experienced by *Pa* cells upon surface contact: the T4P

Under planktonic conditions, the T4P extends and retracts freely and consequently, the force required to retract the pilus through a liquid environment is thought to be negligible (39). Although it was previously thought that surface contact by T4P stimulated retraction, it has since been shown that the rate of pilus extension and retraction is not significantly different for surface-associated versus liquid-suspended cells (14, 61). After surface attachment by *Pa*, the T4P mediates two types of surface motility: upright walking and directional crawling, the latter of which is also known as twitching (3, 62–64). T4P retraction motors make up some of the strongest motors found in nature (19, 65). Some microbes are able to retract a single pilus fiber with a force upward of 100 pN, and some T4P can form bundles and cooperate during retraction to generate forces in the 1 nN range (19, 65, 66). *Pa* shows a more modest average retractive force of ~30 pN per pilus (67). A single T4P, however, can attain a maximal adhesive force of up to 750 pN as measured by atomic force microscopy, and surfaces covered with the Pel exopolysaccharide have been shown to enhance T4P surface attachment (67, 68).

Many organisms contain T4P with multiple PilT and PilU retraction motors. Recent publications have shown that these retraction motors lack specificity and are able to interact with T4P from different organisms to promote retraction (69, 70). For example, for *Vibrio cholerae*, it was demonstrated that retraction motors from *Acinetobacter baylyi*, *Pseudomonas stutzeri*, *E. coli*, and *P. aeruginosa* were able to promote competence pili retraction. This finding is in contrast to the extension ATPase, which displays specificity to their endogenous type 4 pili system (69, 70).

Twitching motility by *Pa* PA14 normally occurs through constant slow movement (~0.3 µm/s) punctuated with periods of rapid movement (1 µm/s). This increased speed occurs when multiple T4P engage the surface, and the release of one pilus results in a "slingshot" effect, propelling the cell forward faster than pilus retraction allows (71). In other bacteria such as *Neisseria*, this framework is similar to the "tug of war" picture previously proposed (72). The increased retraction speed can also be due in part to the elastic, spring-like nature of T4P filaments that are able to stretch 3× their original length when under tension, as shown for *Neisseria* pili (73).

The retractive force of T4P in *Pa* is thought to originate from the primary and accessory retraction motors PilT and PilU, respectively (15, 19, 25, 69, 74–76). However, it is worth noting that others have hypothesized that a large fraction of the retractive force is stored in the pilus fiber itself as there have been examples of low force retractions in the absence of any retraction motor (77, 78). PilT is able to interface with the pilus machinery directly, and it is thought that when under sufficient load, PilU will bind PilT, and the coordinated ATP hydrolysis of both motors is used to depolymerize the bound pilus fiber leading to pilus retraction and T4P-mediated cell movement across a surface (14, 15, 17, 69). While others have shown via two-hybrid experiments that PilU and PilC can interact, only in the presence of PilT can PilU interact with the rest of the pilus

machinery to promote retraction in both *V. cholerae* and *P. aeruginosa* (69, 79). These data indicate that PilU requires PilT to functionally engage the pilus machinery.

Forces experienced by *Pa* cells upon surface contact: the cell body

In addition to the appendages described above, the cell body itself can bind to the surface with marked adhesive force. Note that the cell body of *Pa* PA14 without T4P and flagella can bind hydrophobic surfaces with adhesion forces up to 1,000 pN (68). In addition, van der Waals forces can also have sizable contributions, depending on the material that makes up the attachment surface, and the degree of conformal contact between the bacterial cell surface and the solid surface (80). Adhesion of the cell body to a surface can be modified by several factors including extracellular matrix components that are deposited on the surface or localized to the outer membrane of the cell body. *Pa* produces the large adhesive protein CdrA as well as chaperone-usher pili that line the body of the cell and aid in cell-cell adhesion as well as cell-surface binding (81, 82). In addition, the CdrA adhesin can bind to the EPS Psl, further enhancing surface attachment. Since many of these adhesive systems are regulated by c-di-GMP, their expression varies across the early stages of biofilm formation, making it difficult to determine the exact level of adhesion of a given *Pa* cell contacting a surface at any given time (81, 82). How these cell-surface adhesins impact surface sensing through the motility appendages is largely unexplored (67, 68, 83).

CASCADE OF SECOND MESSENGER SIGNALING UPON SURFACE ENGAGEMENT

The forces experienced by the cell upon surface contact can serve as a signal to initiate cellular changes necessary for the biofilm mode of growth. One of the first changes to occur for Pa is an increase in the nucleotide second messengers c-di-GMP and cAMP (24). Both *E. coli* and *Pa* have been shown to increase c-di-GMP within seconds of surface contact (84, 85).

How does surface contact by *Pa* PA14 result in an increase in second messenger production? As outlined above, for *Pa* PA14, cAMP is thought to be the first second messenger upregulated upon surface engagement, a process that requires functional T4P, the Pil-Chp system, and the adenylate cyclases CyaA and CyaB (Fig. 2) (24, 35, 37, 86). This upregulation of cAMP results in an increase of T4P levels after surface engagement via the increased expression of the genes required for pilus synthesis, generating a positive feedback loop in the global regulatory circuit for cAMP/T4P production (24). (Below, we will discuss the regulation of cAMP production by *Pa* PA14 and the possible mechanism whereby T4P surface engagement triggers cAMP signaling). Once cAMP levels are induced, Vfr bound to cAMP transcribes the two-component system, FimS-AlgR, which directly regulates the T4P minor pilin operon (24). The minor pilins are thought to serve as an initiation complex for pilus assembly and are required for T4P function (87, 88). In the minor pilin operon, there is also a gene that codes for the T4P tip-associated protein PilY1, which we discuss in the next paragraph.

The activity of the diguanylate cyclase SadC has been shown to be regulated by both T4P and flagella for *Pa* PA14 (12, 89). Data from our group and others support a model whereby conformational changes of the protein at the tip of T4P, PilY1, due to the mechanical tension generated by pilus retraction and surface adhesion, activate a signaling pathway along the alignment complex of T4P, which modulates the activity of the diguanylate cyclase SadC (4, 12, 89, 90). The alignment complex of T4P, composed of PilMNOP, connects PilY1 surface signaling, via the PilQ secretin with TsaP, to the inner membrane (IM)-localized SadC via PilO (89, 91). Interactions between PilO and SadC have a complex influence on SadC's diguanylate cyclase activity. Our recent cell tracking work indicates that the evolution of intracellular c-di-GMP levels during biofilm formation is not reducible to a simple monotonic increase but rather exhibits oscillations over many generations. In fact, our data show that the binding strength between PilO-SadC impacts the frequency and amplitude of c-di-GMP oscillations in lineages of *Pa* PA14 cells

Figure 2

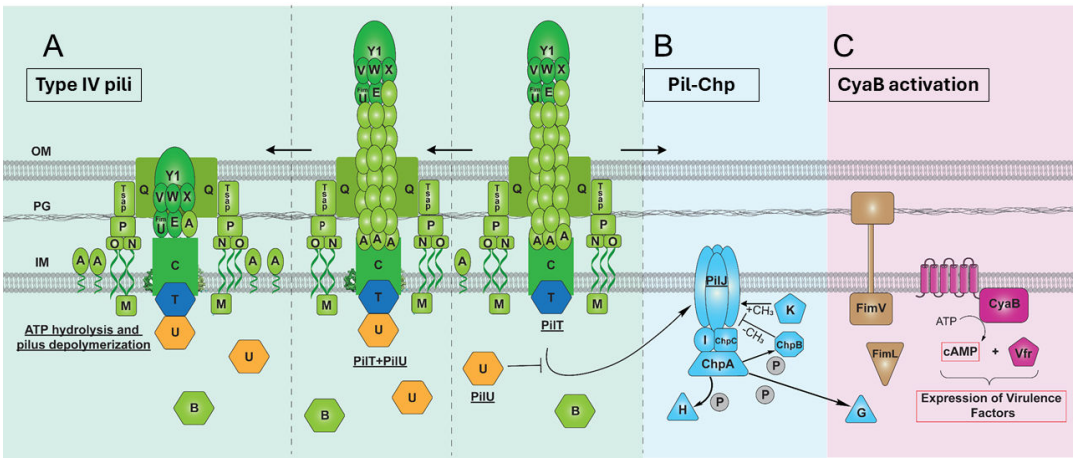

FIG 2 New proposed model for surface sensing by *P. aeruginosa*. (A) After surface binding by extended T4P, retraction of pili begins with the retraction motor PilT binding PilC. The accessory retraction motor PilU binds PilT and aids in depolymerization of the pilus fiber via conformational changes in PilC. However, if PilU is absent during retraction, we hypothesize that PilT alone is unable to exert sufficient force to power retraction, and the PilT motor will stall, potentially entering a conformational state due to improper ATP binding and/or hydrolysis and/or ADP release. We believe that PilT in this stalled conformation binds PilJ of the Pil-Chp complex to transduce the surface signal from T4P to Pil-Chp (B). After PilJ activation, the signal is transmitted to the kinase ChpA, which leads to the phosphorylation of several response regulators including PilG. PilG, along with FimV and FimL, then activates the adenylate cyclase CyaB, leading to an increase in cAMP (C). All depicted proteins are products of *pil* genes unless otherwise stated. Note that not all known interactions among pili proteins can be shown in this figure. Abbreviations: IM, inner membrane; OM, outer membrane; PG, peptidoglycan layer.

attaching to a surface, which in turn directly impact surface commitment in the form of irreversible attachment (92).

Once released from its interaction with PilO, SadC is free to interact with other membrane components and produce c-di-GMP. When c-di-GMP increases within the cell, the protein FlgZ can bind this second messenger, and the FlgZ•c-di-GMP complex will remove MotCD stator units from the flagellar machinery (12). This process not only shuts down flagellar rotation under high loads but also allows MotC to interact with SadC. This MotC-SadC interaction has been shown to have a stimulatory effect on diguanylate cyclase activity, leading to a positive feedback loop of c-di-GMP production and flagellar motility repression (12). Thus, protein-protein interactions with a component of the T4P (PilO) and the flagellar machinery (MotC) can modulate the activity of SadC (12, 89). Furthermore, another surface sensing system in *Pa*, the Gac-Rsm system, has been shown to regulate SadC at the level of translation. The protein RsmA prevents translation of the *sadC* transcript until activation of the Gac-Rsm system upon surface contact. Activation leads to production of sRNA that inhibits RsmA, leading to translation and an increase in SadC levels upon surface contact (93). The c-di-GMP produced by SadC as well as other DGCs in turn influence pili via FimX/FimW and flagellar function via FlgZ. These data suggest that SadC could serve as a "hub" to integrate information for the two principal *Pa* motility machines when they engage the surface.

cAMP REGULATION BY *Pa*

For *Pa* PA14, there are 40 DGCs and PDEs that regulate c-di-GMP levels in the cell, but cAMP production and degradation use only three enzymes (37, 94). There are three potential adenylate cyclases (ACs) encoded in the *Pa* genome, but only two of them, CyaA and CyaB, appear to contribute to intracellular levels of cAMP. ExoY, the third AC, is a type 3 secretion system effector toxin and is only active after being injected into another cell. A single phosphodiesterase, CpdA, degrades cAMP (26, 37, 95).

CyaA is a cytoplasmic adenylate cyclase, but its regulation remains unknown. CyaB has been shown to contribute to the majority of cAMP produced under all tested

conditions in *Pa* (24, 36, 37, 96). CyaB is a class IIIb AC which is composed of a MASE2 regulatory domain fused to its catalytic domain. The MASE2 domain localizes CyaB to the membrane in *Pa* PA14 and is thought to aid its localization to the cell's poles. Like other class IIIb ACs, CyaB has been shown to be stimulated by HCO_3^- and has an optimum pH of 7.5 when tested *in vitro* (97). Other chemical stimuli such as low calcium have been shown to stimulate cAMP in *Pa* by increasing the expression of the *cyaB* gene (37).

Surface-dependent cAMP is produced mainly by CyaB with some contribution by CyaA (24). The cellular regulation of CyaB via its MASE2 domain has been shown to be controlled by components of the Pil-Chp system (Fig. 2B), as detailed below (24, 36, 86, 96).

All known cellular changes due to cAMP are dependent on the cAMP-binding protein Vfr, which regulates genes responsible for virulence as well as surface adaptation (35, 38). The *cpdA* promoter, which controls the expression of the cAMP phosphodiesterase encoded by *cpdA*, is part of the Vfr regulon and is positively regulated under high levels of cAMP to restore the levels of this signal back to baseline after induction (26). A second protein is also found in *Pa*, CbpA, which localizes to the flagellated pole upon cAMP binding, but its cellular function remains a mystery (98).

The relationship between cAMP levels and T4P activity appears to be complex and not reducible to simply direct correlation or anticorrelation. At the single-cell level within lineages (family trees) of *Pa*, there are out-of-phase oscillations between levels of cAMP and T4P activity that persist over multiple generations, with oscillation periods that span several division cycles. Surface adaptation via cAMP-dependent T4P production leads to a memory of surface contact that allows cells and their progeny to remain surface adapted for several generations. This surface adaptation decreases the longer cells remain away from a surface (23). Single *Pa* cells that remain on a surface display oscillations of cAMP across several generations (23). How these oscillations of cAMP, with the recently documented oscillations of c-di-GMP, relate to each other in controlling single-cell surface behavior remains an active area of study.

Interestingly, there are mutations in the flagellum that also stimulate the production of cAMP. Mutating the regulator of flagellar number, FleN, creates a cell with multiple polar flagella that bundle together and are unable to rotate. Researchers hypothesize that the multiple polar flagella impart a high load on the flagellar machinery, which likely also happens during initial surface attachment by the polar flagellum of *Pa*. The increase in load has been shown to increase the amount of intracellular cAMP ~3-fold and is dependent on the structural hub protein FimV in the PAK strain of *Pa* (59). This finding indicates that there are likely multiple mechanisms to regulate cAMP via the other surface sensing appendages in *Pa*.

REGULATION OF CyaB ACTIVITY BY Pil-Chp

Several genetic screens have indicated that members of the Pil-Chp system are involved in CyaB regulation, and subsequent studies have uncovered the mechanisms of signal transduction and activation. Three proteins, FimV, PilG, and FimL, must form a complex to activate CyaB via its MASE2 domain (24, 86, 96). FimL is thought to connect FimV to PilG to form the activation complex (Fig. 2C) (36, 96). Localization of PilG and FimL to the poles of the cell is dependent on the polar hub protein FimV (36). The FimV protein contains a PG-binding LysM motif as well as a large cytoplasmic domain with a conserved tetratricopeptide repeat motif that is used to bind FimL (99). The LysM motif preferentially binds PG with fewer stem peptides, a form of PG that is enriched at the poles of the cells, thus providing an explanation for the polar localization of FimV (100–103). Besides activating the AC CyaB, FimV has other roles including T4P assembly and polar localization, stopping flagellar rotation, and polar localization of other proteins like PilS (59, 100, 104).

PilG, which is a response regulator (RR) of the kinase ChpA, is also required for CyaB activation. Phosphorylation of PilG by ChpA is required for AC activation as well as twitching motility, but not binding to FimL or polar localization (36). PilG is part

of the larger Pil-Chp complex that is genetically and functionally similar to chemoreceptors described in other Gram-negative organisms (Fig. 2B). This Che-like system contains a membrane-bound, methyl-accepting chemotaxis protein (MCP), PilJ, with a periplasmic ligand-binding domain (29, 105–107). The periplasmic domain has been implicated in directional twitching toward chemoattractants such as phosphatidylethanolamine, mucin, BSA, and, recently, phenol soluble modulins produced by *Staphylococcus aureus* (107–110). Interestingly, addition of these chemoattractants also stimulates cAMP production via CyaB (111). While the ligand-binding domain of PilJ is required for directional twitching, it is *not* required for surface-dependent cAMP production (112).

PilJ, like other MCPs, is predicted to be methylated and demethylated by two proteins, PilK and ChpB, respectively, to tune sensitivity of the receptor (24). There are two predicted adaptor proteins for this system, PilI and ChpC, which likely link PilJ to ChpA (29, 105, 106). PilI is required for twitching motility, but ChpC is required only for directional twitching (111). The kinase ChpA has a second response regulator in addition to PilG, PilH. Until recently the function of these two RRs was unknown. It has now been demonstrated that the phosphorylation state of these two proteins controls the directional twitching of *Pa* by biasing the polar localization of the T4P extension motor PilB, thereby allowing *Pa* to efficiently colonize a new surface by controlling reversal frequencies of twitching cells (36, 113, 114).

Surface-dependent activation of Pil-Chp, and subsequently CyaB, also requires functional T4P, but the mechanism by which T4P senses a surface and transmits this information to Pil-Chp is still an open question (24, 86, 115). Below, we address several models proposed to explain T4P-mediated surface sensing by *Pa*, and in light of new genetic, structural, and comparative genomics data, we present a new model of T4P-mediated surface sensing by *Pa* PA14.

T4P REGULATION OF Pil-Chp

Multiple studies have established the requirement for functional T4P as well as the Pil-Chp system for surface-dependent cAMP production, but the mechanism whereby T4P mediates surface sensing via Pil-Chp is still an open question (24, 96, 115). As the statistician George Box stated, "All models are wrong but some are useful." This quote nicely sums up the current state of surface sensing. Indeed, we would argue that how the T4P senses and transduces surface engagement is one of the key knowledge gaps regarding surface sensing by *Pa*.

A previous study proposed a possible mechanism of surface sensing by T4P via the pilin monomer (115). This study demonstrated that PilA as well as its motor proteins were required for the surface-dependent cAMP response. Furthermore, a novel interaction between PilA and PilJ was described using the bacterial adenylate cyclase two-hybrid (B2H) system. The authors speculated that the pilin monomer enters a force-induced conformational change, as seen in *Neisseria*, when there is sufficient tension on the pilus fiber that allows for binding with PilJ, leading to cAMP production. To test whether tension on pili without retraction motors could stimulate cAMP production, the *Pa* Δ*pilTU* mutant was allowed to attach to a flow cell and then subjected to increased medium flow causing a shear force of ~10 pN per cell. Despite the tension on pili due to the shear force, the extent of cAMP production was identical to Δ*pilTU* strain under the no-flow condition, indicating that the motor-mediated retraction is necessary for surface signaling and not just tension on pili (115). Subsequent studies have demonstrated a correlation between the flux of pilins in and out of the IM on surfaces of different stiffnesses and the extent of surface-dependent cAMP production. A model was developed based on the flux of PilA in the inner membrane that accurately predicted the cAMP response of T4P motor mutants with altered extension and retraction dynamics (116). While this T4P-based model of surface sensing by *Pa* explains observed data at the time, it is at odds with key observations made subsequently.

A recent study revealed a lack of correlation between the strength of binding between PilA and PilJ and levels of cAMP when grown on a surface (117). We suggest

that if PilA interaction with PilJ mediates the surface signal, then we would predict that stronger binding between PilA-PilJ would result in more cAMP production for surface-grown cells, but this correlation is not observed for several PilA mutants. Moreover, deletion of the accessory retraction motor, PilU, increases the cAMP response. PilU is the only component of the T4P machine that increases intracellular cAMP upon its deletion (23, 24, 117, 118). As mentioned earlier, PilU can only interact with the T4P machinery to promote retraction via the primary retraction motor PilT (69, 79). The overproduction of cAMP in this background is dependent on the presence of PilT as the double Δ*pilT*Δ*pilU* mutant does not increase cAMP when grown on a surface (23, 117, 118). PilU has been shown to contribute to retraction of T4P by increasing the maximum force of retraction for *V. cholerae* (69). If conformationally stretched pili were key to surface signaling, we would expect a reduction in stretched pili when we eliminate one of the two motors contributing to the retractive force, resulting in less cAMP, which is not consistent with recent data, although other factors could be impacting pilus tension. Along a different line of evidence, expression of a functionally inactive PilU, PilU-K136A, in a Δ*pilU* background phenocopies the Δ*pilU* mutant strain in terms of T4P-related phenotypes, such as twitching and phage susceptibility, but reduces surface-dependent cAMP production (118). In both the Δ*pilU* mutant and a Δ*pilU* mutant over-expressing the PilU-K136A protein, T4P are only able to retract with enough force to retract pili that are bound to phage, but are unable to generate enough force to overcome the adhesion between the surface and cell body needed for twitching motility. A similar phenotype is observed with PilT-K136A and functional PilU for both *P. aeruginosa* and *V. cholerae*. For these mutants, low load retraction events are powered by PilU ATPase activity using the catalytically dead PilT to interface with the platform protein PilC. This low-level retraction force can drive DNA uptake for the competence pili of *V. cholerae* (69, 75) and confer susceptibility of phages for *Pa* (82). In these mutant backgrounds, the T4P tension is below its maximum due to the lack of functional PilU and/or PilT, but these strains exhibit disparate cAMP responses when grown on a surface that cannot be explained by the pilin signaling model. Finally, this PilA-PilJ model necessitates interaction between a conformationally altered PilA buried in the T4P machine with PilJ in the inner membrane, a model at odds with cryoET structures of the T4P machine (119). Alternatively, the force-induced conformational change in PilA would need to be maintained long enough for the T4P to disassemble and pilin to diffuse into the membrane in its altered conformation to engage PilJ. Neither of these scenarios seem likely.

Due to the lack of unambiguous evidence supporting a PilA-PilJ signaling model (and multiple lines of evidence arguing against this model), as well as accumulation of recent data showing that manipulating the accessory retraction motor can affect levels of cAMP, we hypothesized that the primary retraction motor PilT may be required for sensing and relaying a surface signal to the Pil-Chp system.

A PROPOSED MODEL OF PilT-MEDIATED SURFACE SENSING SIGNAL TRANSDUCTION

The PilT retraction motor drives depolymerization of extended T4P using the power generated by ATP hydrolysis (Fig. 3A) (15, 17). Assignment to the type 4a pili (T4aP), which includes the T4P of *Pa*, requires a homolog of the *pilT* gene to be, present in the genome. The other retraction motor, encoded by the *pilU* gene, is considered accessory and not all microbes with a T4aP have a *pilU* gene in their genome (120).

For *Pa*, the N-terminus of the PilT monomer contains a PAS-like domain that is connected to the C-terminal NTPase domain by a flexible linker (Fig. 3B). There are several conserved motifs in the C-terminal NTPase domain including a Walker-A motif responsible for ATP binding, a Walker-B motif responsible for ATP hydrolysis, and Asp and His boxes that coordinate the terminal phosphate group of ATP (15). The last conserved motif of PilT is the surface exposed, amphiphilic AIRNLIRE motif. Residues in the AIRNLIRE motif are required for the *in vivo* function of PilT, and mutating these residues result in

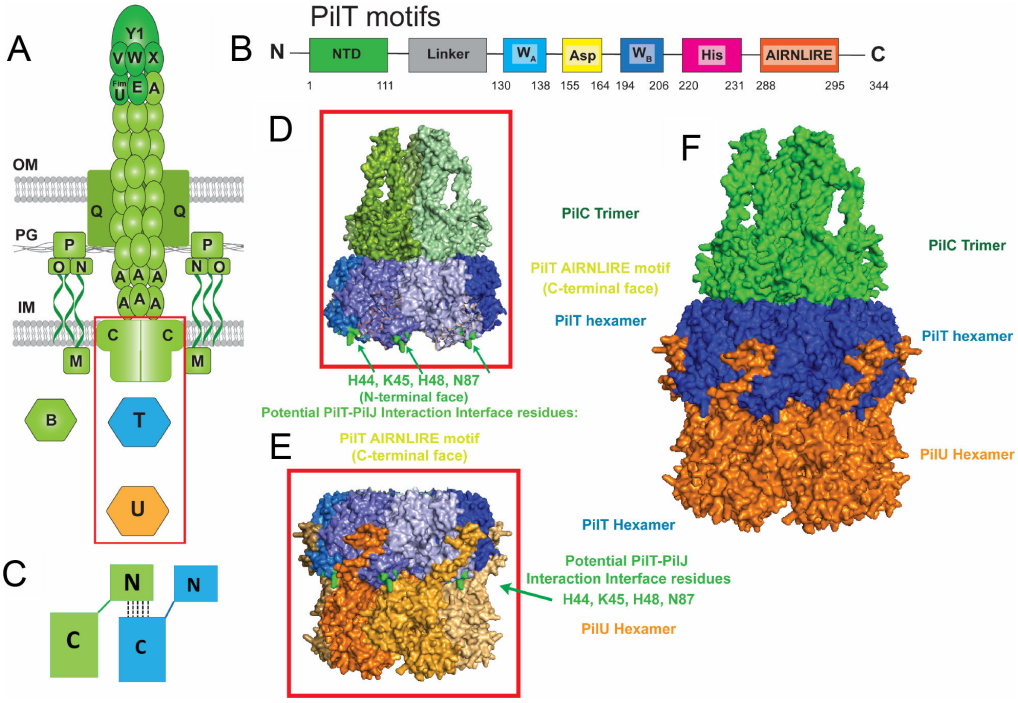

FIG 3 Predicting the interface between the C-terminal face of the PilT hexamer and the PilC hexamer. (A) An illustration of the T4P of *P. aeruginosa* with the motor proteins highlighted. (B) A linear representation of the PilT protein with known motifs labeled and the beginning and ending aa of each motif indicated. This depiction is based on a previous publication (121). (C) A representative image of how two PilT monomers oligomerize. (D) The AlphaFold-Multimer program was used to predict the structure of PilT hexamer (blue) bound to the PilC trimer (green) with the model giving the best score shown. The residues comprising the PilT-PilJ interface are highlighted in green. (E) AlphaFold-Multimer was used to predict the structure of PilT hexamer bound to the hexamer PilU with the model giving the best score shown. The *Pa* PA14 amino acid sequences for each protein were used to build the model. The pLDDT scores have been mapped onto the structure in Fig. S1. (F) AlphaFold-Multimer was used to predict the structure of the PilT hexamer bound to the hexamer PilU as well as the PilC trimer with the model giving the best score shown. The PA14 amino acid sequences for each protein were used to build the model. The pLDDT scores have been mapped onto the structure in Fig. S1.

cells that are unable to perform twitching motility and are phage resistant, indicating a complete lack of any T4P retraction. Despite the lack of function, AIRNLIRE mutants are still able to oligomerize and retain ATPase activity, indicating an unexplained role of this motif in motor function (122).

T4P motors function as hexamers. Oligomerization occurs by the NTD of one monomer binding to the CTD of adjacent monomers (Fig. 3C). The ATP binding occurs between monomers in the core of the hexamer (17, 19, 123, 124). The ability to bind and hydrolyze ATP leads to different stable conformations. Three stable conformations have been observed for the PilT hexamer of *Pa*. A fully ATP bound C6 conformation is thought to allow binding between the PilC platform protein and the PilT hexamer, although this state could have been an artifact of the purification conditions. The C2 conformation of PilT, where only two ATP molecules are bound, is thought to be the active conformation of PilT while powering disassembly. The observed C3 conformation has no known physiological role (17).

As part of developing a model for PilT-mediated surface sensing, it is relevant to understand where PilT is localized. While all three T4P motors are cytoplasmic, their localization can vary based on other proteins as well as growth conditions (15, 121). Localization of PilT to the poles has been shown to be dependent on the actin homolog MreB (125) but does not require the platform protein PilC, indicating that polar localization could be independent of pilus machinery (121). However, whether PilT or PilU motors are unipolar or bipolar in their localization appears to vary with strain,

expression condition, and the presence of other proteins like FimX (15, 114, 125, 126). FimX has not been shown to directly bind PilT or PilU, but its effect on polar localization is possibly due to PilB, which it does bind. A mutation in the Walker-A motif of PilT (G135S) has been shown to affect polar localization, while mutations in Walker-B motif do not, potentially due to differences in ATP binding (15). While many factors appear to affect T4P motor localization, one consistent observation is that all three motors are not always present at the poles of *Pa* at the same time.

Recently, our group published a study with data that support a model whereby the retraction motor PilT senses and relays the surface sensing signal. A B2H screen identified a novel interaction between PilT and PilJ of the Pil-Chp system, leading us to hypothesize that the surface signal may be transmitted through this interaction instead of the previously proposed PilA-PilJ interaction (see above). Since the B2H screen was performed in the BTH101 *E. coli* strain in the absence of other *Pseudomonas* T4P or Pil-Chp proteins, we assume a direct interaction between PilT and PilJ. It is important to note that when functioning in the endogenous host, it is possible that the interaction between these two proteins is affected by other members of the T4P or Pil-Chp machinery.

By generating PilT mutants with differences in ATPase activity and measuring the cAMP produced when grown on a surface, as well as strength of interaction between PilT and PilJ, a linear model was developed that indicated a strong positive correlation between strength of interaction between PilJ and PilT-ATPase mutants and the amount of cAMP produced when cells were grown on a surface. This finding indicated that PilT may be "sensing" the surface through its ATPase activity and that the ability to interact with PilJ was important for cAMP production. Furthermore, by manipulating levels of the accessory motor PilU, we were able to demonstrate that the surface-dependent cAMP response could be modulated by the amount of PilU available to interact with PilT and that this effect of PilU on cAMP was dependent on the presence of PilT (118).

Taken together, these data regarding PilT and PilJ led us to propose the following model of motor-dependent surface sensing by *Pa*. In this model, PilT binds to PilC to drive retraction of extended T4P. If T4P are unbound to a surface, then retraction does not require maximal force and PilT is able to retract pili in the absence of PilU. Support for PilT functioning in the absence of PilU comes from the *pilU* mutant remaining sensitive to a phage that requires a retractile T4P for infection (14, 76, 117). However, if T4P are bound to the surface, then PilT may be unable to power disassembly in the absence of PilU, as this accessory retraction motor provides the high T4P retractive forces that are not achieved in a Δ*pilU* mutant (14, 69). If there are not enough PilU motors to aid in retraction or if the PilU motor is at the opposite pole, PilT-mediated retraction will stall, potentially leading to novel hexameric conformations due to improper ATP binding or ADP diffusion out of the hexamer (17). We believe this stalled conformation then allows PilT to interact with PilJ to relay the surface signal to Pil-Chp. At this stage, the nature of the PilJ-PilT interaction is still an open question.

Interestingly, this model accounts for why a Δ*pilU* mutant strain would have elevated levels of cAMP; that is, in the absence of PilU, every attempt to retract surface-bound T4P would result in the "stalled conformation" of the motor, which we propose is the signaling conformation of PilT. An increase in the likelihood of this conformation of PilT would then lead to an increase in PilT-PilJ interaction, PilJ activation, and thus surface-dependent cAMP production, which is what we have observed (24, 117, 118, 127).

However, one piece of data that did not fit this model was the ability of ATPase-deficient PilU protein, PilU-WA, to lower cAMP in a Δ*pilU* background when PilU-WA was expressed from a plasmid. As previously mentioned, T4P in a Δ*pilU* mutant background are functionally equivalent to T4P in a Δ*pilU* background expressing *pilU*-K136A. Despite both strains being deficient in twitching motility while remaining phage susceptible, the strain expressing PilU-WA has significantly less cAMP than WT, whereas the Δ*pilU* strain has elevated levels of cAMP. One possible explanation for this discrepancy is that PilU

and PilJ share a binding site on PilT such that expression of a defective PilU lowers cAMP by blocking signaling to PilJ by PilT (118). Unfortunately, at the time of the publication of the PilT-PilU interaction model (118), we had no evidence to support this hypothesis because the residues on PilT that mediate interaction with its binding partners, PilU and PilC, were unknown. Below, we use structural models as well as amino acid conservation information to show that PilJ and PilU potentially use the same binding site on PilT. This is a hypothesis worth testing and could help explain a surface sensing model whereby PilT could be sensing a surface and relaying this information to the Pil-Chp system.

USING COMPARATIVE GENOMICS TO DEVELOP A MODEL OF SURFACE SENSING ACCOUNTING FOR THE PilT MOTOR, THE PilJ MCP, AND THE PilU ACCESSORY MOTOR

To predict residues that might mediate binding of PilT to its two partners (PilJ and PilU), we used a combination of analyzing sequence conservation with structural modeling. We reasoned those conserved residues on the surface of PilT likely mediated the interactions between its binding partners. To predict which surface residues of PilT might use to mediate binding to PilJ versus PilU, we used the tool AlphaFold-Multimer (128–133). We used this approach to generate hypotheses that could serve to guide future experiments.

To determine candidate residues mediating PilU binding to PilT, we first identified the residues on the surface of PilT that likely mediate interaction with PilC. We performed this analysis first for several reasons. First, we knew that PilT is able to interact with PilC in the absence of PilU, given that *Pa* remains phage susceptible in a *pilU* mutant background (76). Second, we reasoned that because PilC, PilT, and PilU must all form a complex to power twitching motility (7, 19, 134), that the residues mediating PilT-PilC interaction could *not* be the residues mediating PilT-PilU interaction. Third, consistent with this supposition, cryoET structures have shown that the PilT hexamer likely interacts with the PilC platform protein through either its N- or C-terminal face (119). Therefore, whichever face of the PilT hexamer was mediating interaction with PilC would be unavailable for PilU binding; thus, we could likely discern which face of PilT was using to interact with PilC versus PilU.

We speculate that mutations that disrupt interaction between PilT and PilC would phenocopy a Δ*pilUT* mutant strain in terms of T4P retraction but would likely not affect ATPase activity or oligomerization as the residues that facilitate these functions are near the NTPase core of PilT and not on the surface of the protein. Such phenotypes occur when mutating the AIRNLIRE motif of PilT, which is located on the C-terminus of the protein (122). Another piece of data supporting this hypothesis is that of the 4 PilT motors in *Geobacter sulfurreducens*; only PilT motors that contain the AIRNLIRE motif are able to complement a Δ*pilT* PAK *Pa* strain to support twitching motility (135); this observation led us to predict that the C-terminal face of PilT mediates interaction with PilC. In agreement with this prediction, the highest scored AlphaFold-Multimer prediction for PilT and PilC showed interaction of PilC via PilT hexamer's C-terminal face, which includes the AIRNLIRE motif (Fig. 3D). All structures generated by AlphaFold-Multimer has PilT binding PilC via its PilT's C-terminal face.

To assess the extent of conservation of residues on the C-terminal surface of the PilT hexamer (Fig. 4A), we collected the homologs of the PilT protein for all pseudomonads and then binned sequences by whether the genome also contained a *pilU* gene. Conservation scores were then calculated for each amino acid in PilT using the Consurf webserver (133, 136, 137). Here, a score of 7 or greater was considered "conserved," and a score of 3 or less was considered "variable." We observed that the C-terminal surface of the PilT hexamers is highly conserved for all pseudomonads, whether or not they contain a PilU (Fig. 4B). This observation is in agreement with the structural predictions, as all PilTs of T4aP must bind a PilC so this PilT-PilC interaction face should be well conserved regardless of the presence or absence of PilU.

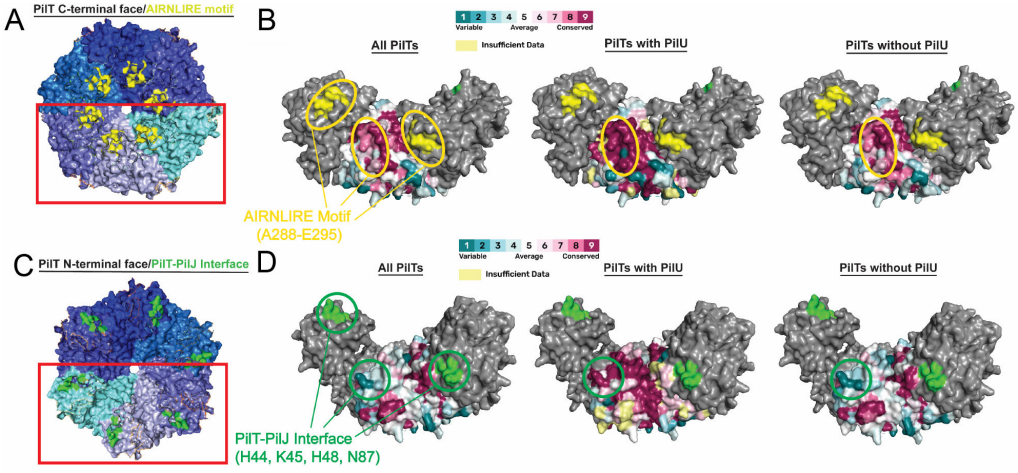

FIG 4 Predicting the interface between the N-terminal face of the PilT hexamer and the PilU or PilC hexamers. (A) The C-terminal face of the PilT hexamer with PilC removed. Each PilT monomer is in a different shade of blue, and the conserved AIRNLIRE motif is yellow. A red box encircles the monomers with conservation information mapped on the surface as shown in panel B. (B) A BLAST search was performed on taxid 286 for the genus *Pseudomonas* using the PA14 PilT sequence as a search query. Homologs were identified, and genomes were pooled by whether a homolog of PilU could also be identified (center) or not (right). An MSA was then generated using Clustal Omega, and the resulting MSA (see Fig. S2) was used with CONSURF to calculate and map conservation scores for each residue on the center monomer of the trimer to visualize the conserved areas of the protein, as previously described (138–142). PDB: 3JVV chain B was used to map the conservation information onto each residue. The legend shows the extent of sequence identity for each residue for the central monomer. The AIRNLIRE motif (A288-E295) in the left and right monomers is highlighted in yellow. A yellow ellipse encompasses the AIRNLIRE motif on each monomer of PilT. (C) N-terminal face of the PilT hexamer with PilU removed. Each PilT monomer is in a different shade of blue, and the potential PilT-PilJ binding interface (H44, K45, H48, and N87) is in green. A red box includes the monomers with conservation information mapped on the surface of the central monomer as shown in panel D. (D) A BLAST search was performed on taxid 286 for the genus *Pseudomonas* using PA14 *pilT* as a search query. Homologs were identified, and genomes were pooled by whether a homolog of PilU could also be identified (center) or not (right). An MSA was then generated using Clustal Omega (Fig. S3 and S4) with CONSURF to calculate and map conservation scores for each residue on the center monomer of the trimer to visualize the conserved areas of the protein as previously described (138–142). PDB: 3JVV chain B was then used to map the conservation information onto each residue. The legend shows the extent of sequence identity for each residue. In green are the residues that are predicted to mediate binding with PilJ on the left and right monomers. A green ellipse encompasses the predicted PilT-PilJ interface on each PilT monomer. MSA, multiple sequence alignment.

Given that data from the literature (143), structural predictions (Fig. 3), and our observation that the conservation of amino acids across PilT homologs all indicated that PilT binds PilC via the C-terminal face of the PilT hexamer, we reasoned that the N-terminal face of the PilT hexamer would be available for PilU binding. The highest scoring AlphaFold-Multimer prediction also agreed with this hypothesis and showed the N-terminal face of the PilT hexamer binding PilU (Fig. 3E).

We note that Fig. 3 and Fig. 4 show predictive structures that have not been verified. All structures generated by AlphaFold-Multimer have the N-terminal face of PilT interacting with PilU. When analyzing the three proteins together, we obtain a composite of the two predictions which shows the PilT hexamer's C-terminal face binding PilC and the N-terminal face of the PilT hexamer binding PilU (Fig. 3F).

Upon revisiting the conserved surface residues of PilT, we noticed that the PilT hexamer's N-terminal face (as opposed to the C-terminal face as outlined above) showed few conserved amino acids (Fig. 4C through D). However, when we performed this analysis with the PilT sequences from organisms which also encoded a PilU in the same genome, we find that the N-terminal face of the PilT hexamer has patches of conserved

amino acids (Fig. 4D, center versus right), which would make sense if these residues were under selection to maintain binding with another protein for function (i.e., PilU; Fig. 3E).

The N-terminal face of the PilT hexamer is also the location of residues that mediate PilT-PilJ binding as determined in our previous study (Fig. 4C) (118). In an attempt to isolate mutants of PilT that retain twitching motility but no longer bind to PilJ, a genetic screen was performed that isolated alleles of *pilT* that produced motors capable of powering twitching motility in *Pa* but are unable to bind to PilJ in the B2H system. When mapping these mutations onto the PilT structure, 28% of the mutations mapped to a four-residue patch on the N-terminal face of the PilT hexamer, indicating that this was the face of PilT that interacted with PilJ. Of the four mutants making up this patch, only one of the alleles produced a stable protein when expressed in *Pa*, but surprisingly, there was no difference in the amount of cAMP observed for this H44L mutant when grown on a surface (118). However, when examining the conservation score of this residue for PilT motors that also have a PilU in the genome, this residue was the *least* conserved of the four residues identified in our screen (118), indicating that this residue was the least important for interacting with PilJ and providing a possible explanation as to why a change in cAMP was not observed.

Finally, based on the lack of PilJ-PilU binding, we would expect that the conserved four-residue patch required for PilT to interact with PilJ should be not conserved in PilU. When we perform the same analysis as above, this time examining PilU residues, we see that the four residues that we predict would facilitate PilJ interaction with PilT (residue numbers 44, 45, 48, and 87) are not conserved for PilU. These findings are consistent with the model presented above. As expected, the most conserved regions of PilU are the domains responsible for ATP binding and hydrolysis, as well as residues that facilitate oligomerization (not shown).

Together these data support the hypothesis that PilU can modulate PilT signaling to PilJ not only by providing force during retraction and preventing the conformational change in PilT that allows for binding to PilJ, but by also blocking the binding site for PilJ even when PilU is not actively aiding in retraction.

Consistent with our model, signaling by T4P motors has been previously reported for *Myxococcus xanthus*. In this organism, EPS production only occurs when T4P can interact with a surface. Mutations in the extension motor PilB were able to restore EPS production in a T4P-deficient background of *Myxococcus*. A Walker-A mutation in PilB was also able to stimulate EPS production without restoring T4P motility. The PilB-WA hexamer resembles the WT PilB in the apo form, leading to the conclusion that the unbound form of PilB was the signaling conformation. Further work has demonstrated that the binding state of PilB to c-di-GMP, ATP, and ADP influences the conformation of PilB and controls whether this motor is actively polymerizing pili or performing EPS signaling (144–146). Thus, a role for T4P motors in transmitting surface signals may be a conserved mechanism. Furthermore, conformational changes in the retraction motor PilT have been implicated in *Neisseria gonorheae* when tension is applied to pilus fibers. It is thought that these conformational changes may occur in the motor when the load is near or over the motor stalling limit and may serve as a signal within cells (19, 147). These data indicate that the T4P motors not only sense tension on pili but also can serve as signaling proteins in other organisms as well.

While we have identified residues on PilT that facilitate interaction on PilJ, we have not identified the residues on PilJ that facilitate binding with PilT. One difficulty is the lack of structural information for PilJ. The domain architecture of PilJ, as well as sequence homology, indicates that the protein is similar to methyl-accepting chemotaxis proteins, but neither the crystal structure of PilJ nor whether PilJ forms hexameric arrays typical of chemoreceptors has been determined (148). Furthermore, it is unclear whether PilT must remain associated with the rest of the T4P machinery to signal to PilJ. If PilT undergoes a particular conformational change due to tension on the pilus, it stands to reason that by disassociating from the pilus fiber, PilT would exit this conformational change, meaning that PilT must interact with PilJ while PilT still bound to the rest of the pilus

machinery. Importantly, a previous work has shown that PilT can exist in several different stable conformations due to differences in ATP binding (17). Thus, it is possible that pili tension prevents the conformational changes of PilT that are necessary for active ATP hydrolysis and pilus disassembly. This stalling could then lead to altered ATP binding by the monomers, which could result in the hexameric signaling conformation that could remain stable even when dissociated from the pilus fiber. Testing these ideas is the goal of future studies.

OPEN QUESTIONS

The opportunistic pathogen *P. aeruginosa* PA14 serves as an excellent model organism to study the challenging problem of relating T4P function to surface sensing. A foundational understanding of this relation impinges on an important aspect of this bacterium's lifestyle and its ability to be a pathogen. Current data point to a model where *Pa* uses T4P to sense a surface and transmit that signal to the cell likely through its primary retraction motor PilT. We propose that this mechanism of surface sensing leads to the production of cAMP due to motor stalling.

Just as T4P and the flagellum regulate levels of c-di-GMP by controlling the binding partner and subsequent activity of SadC, these two appendages may be regulating levels of cAMP by controlling CyaB activity. As previously mentioned, these two second messengers oscillate at the single-cell level over multiple generations (23, 92). The generation of fluorescent reporters capable of showing levels of both cAMP and c-di-GMP in the same cell will enable researchers to investigate how these oscillations relate to each other during surface sensing by *Pa*. Furthermore, these second messengers have been shown to affect different surface behaviors including twitching motility and cellular spinning when attached to a surface (23, 59). How these surface behaviors are coordinated to efficiently colonize a surface while also forming a biofilm is also an area of current research (84). As demonstrated above, the interaction of motility appendages with a surface is critical to surface sensing. In an attempt to prevent biofilm formation, engineers have begun designing surfaces that can prevent these mechanisms of surface sensing in order to prevent the attachment and growth of microbial cells in industrial settings (148–150).

Pseudomonas phages have also been shown to interfere with T4P function. Viral proteins can prevent T4P function, which can inhibit viral superinfections, but can also likely affect surface sensing and biofilm formation (151–153). Furthermore, investigation of how biofilm formation and surface sensing by *Pa* are altered when in a polymicrobial community is also being conducted. Other pathogens like *S. aureus* are known to produce molecules that can affect T4P surface behavior. How that change in surface behavior affects biofilm phenotypes is currently being investigated (108, 109). Finally, the environments in which *Pa* colonizes during infection are distinct from ideal laboratory conditions with differences in oxygen concentrations, surface stiffness, viscosity, and carbon and nitrogen sources that can affect *Pa* physiology, as well as biofilm formation (154). As mentioned above, *Pa* is known to directionally twitch toward many different biologically-relevant molecules such as mucin, BSA, and oligopeptides, which also lead to an increase in intracellular cAMP (111). How all of these environmentally relevant factors affect surface sensing during infection and biofilm formation through the mechanisms outlined above will be a major area of research in the coming years.

It remains to be seen how the principles learned in *Pa* surface sensing can be applied to other microbes, including those that lack motility appendages altogether.

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AUTHOR CONTRIBUTIONS

C. J. Geiger, Conceptualization, Visualization, Writing – original draft, Writing – review and editing | G. C. L. Wong, Conceptualization, Writing – review and editing.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Data File S1 (JB00442-23-S0001.pdf). Full MSA of all PilT homologs.
Data File S2 (JB00442-23-S0002.pdf). Full MSA of PilT homologs from organisms that also have a PilU homolog.
Data File S3 (JB00442-23-S0003.pdf). Full MSA of PilT homologs from organisms that do not have a PilU homolog.
Supplemental material (JB00442-23-S0004.pdf). Fig. S1-S4; supplemental methods.

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