Type IV pili mecanochemically regulate virulence factors in \textit{Pseudomonas aeruginosa} \\

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Bacteria have evolved a wide range of sensing systems to appropriately respond to environmental signals. Here we demonstrate that the opportunistic pathogen \textit{Pseudomonas aeruginosa} detects contact with surfaces on short timescales using the mechanical activity of its type IV pili, a major surface adhesin. This signal transduction mechanism requires attachment of type IV pili to a solid surface, followed by pilus retraction and signal transduction through the Chp chemosensory system, a chemotaxis-like sensory system that regulates cAMP production and transcription of hundreds of genes, including key virulence factors. Like other chemotaxis pathways, pili-mediated surface sensing results in a transient response amplified by a positive feedback that increases type IV pilus activity, thereby promoting long-term surface attachment that can stimulate additional virulence and biofilm-inducing pathways. The methyl-accepting chemotaxis protein-like chemosensor PilJ directly interacts with the major pilin subunit PilA. Our results thus support a mecanochemo model where a chemosensory system measures the mechanically induced conformational changes in stretched type IV pili. These findings demonstrate that \textit{P. aeruginosa} not only uses type IV pili for surface-specific twitching motility, but also as a sensor regulating surface-induced gene expression and pathogenicity.

Numerous bacterial species exhibit a range of behaviors that are specific to life on surfaces. For many pathogens, surface-specific phenotypes are often associated with virulent activity, because many infection strategies require contact with a host (1). For example, in \textit{Pseudomonas aeruginosa}, the type III secretion system, which injects toxins, requires attachment of individual cells to host-cell membranes (2). The genetic pathways that regulate \textit{P. aeruginosa} secretion systems have been well characterized (3), but the environmental signals that activate these pathways remain poorly defined (4). Given the requirement for host-cell contact for efficient infection, \textit{P. aeruginosa} could leverage surface contact as a signal to properly activate and coordinate pathogenicity. Indeed, our laboratory recently demonstrated that the Chp system, a chemotaxis-like sensory system and TFP are regulators of this mechanically induced response. Here we demonstrate that the Chp system is activated by mechanical cues transduced by TFP upon surface contact. It has been previously shown that the cAMP-dependent operon \textit{PaQa} is up-regulated in colonies growing on solid medium (13). We show that the \textit{PaQa} operon is directly regulated by contact between the cell and the substratum, independently of surface chemistry. We also demonstrate that the Chp chemosensory system and TFP are regulators of this mechanically induced response. We show that \textit{P. aeruginosa} up-regulates \textit{PaQa} in response to TFP extension, attachment, and retraction, thus identifying TFP as a mecanotransduction system. Finally, we demonstrate that the Chp system, a complex two-component signal transduction system that resembles the flagellar chemotaxis system, has previously been implicated as a regulator of TFP; in the absence of Chp proteins, \textit{P. aeruginosa} exhibits defective TFP assembly and twitching motility, although pilin subunits are still produced (8). In addition, the Chp system has been shown to regulate the level of the signaling molecule cAMP (9, 10), which in turn binds to the transcription factor Vfr (virulence factor regulator) to activate the transcription of more than 100 genes (11). Vfr regulates multiple virulence factors in \textit{P. aeruginosa}, including the type II and III secretion systems and quorum sensing (11). Because these virulence pathways have a high metabolic cost, such complex regulatory networks are typically optimized to sense and respond to specific environments, thereby inducing virulence in the presence of a host. The homology between the Chp system and other well-characterized chemotaxis systems (e.g., the Che system) suggested that Chp stimulates twitching upon sensing specific solutes present in the environment. However, no compound has been shown to clearly activate the Chp system (12).

Here we investigate the hypothesis that the Chp system is activated by mechanical cues transduced by TFP upon surface contact. It has been previously shown that the cAMP-dependent operon \textit{PaQa} is up-regulated in colonies growing on solid medium (13). We show that the \textit{PaQa} operon is directly regulated by contact between the cell and the substratum, independently of surface chemistry. We also demonstrate that the Chp chemosensory system and TFP are regulators of this mechanically induced response. We show that \textit{P. aeruginosa} up-regulates \textit{PaQa} in response to TFP extension, attachment, and retraction, thus identifying TFP as a mecanotransduction system. Finally, we demonstrate that the Chp system, a complex two-component signal transduction system that resembles the flagellar chemotaxis system, has previously been implicated as a regulator of TFP; in the absence of Chp proteins, \textit{P. aeruginosa} exhibits defective TFP assembly and twitching motility, although pilin subunits are still produced (8). In addition, the Chp system has been shown to regulate the level of the signaling molecule cAMP (9, 10), which in turn binds to the transcription factor Vfr (virulence factor regulator) to activate the transcription of more than 100 genes (11). Vfr regulates multiple virulence factors in \textit{P. aeruginosa}, including the type II and III secretion systems and quorum sensing (11). Because these virulence pathways have a high metabolic cost, such complex regulatory networks are typically optimized to sense and respond to specific environments, thereby inducing virulence in the presence of a host. The homology between the Chp system and other well-characterized chemotaxis systems (e.g., the Che system) suggested that Chp stimulates twitching upon sensing specific solutes present in the environment. However, no compound has been shown to clearly activate the Chp system (12).

In their natural environments, bacteria frequently transition from a free-swimming state to a surface-associated state, attached to a substratum. As they encounter a surface, they may initiate developmental programs to optimally colonize this new environment and induce pathways such as virulence. Here we demonstrate that the pathogen \textit{Pseudomonas aeruginosa} uses fiber-like motorized appendages called type IV pili to sense initial contact with surfaces. This leads to a signaling cascade that results in the expression of hundreds of genes associated with pathogenicity and surface-specific twitching motility. Thus, bacteria use pili not only to attach and move, but also to sense mechanical features of their environment and regulate cellular processes of surface-associated lifestyles.

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provide evidence that the major pilin subunit PilA interacts with PilJ, the sensory protein component of the Chp chemosensory system, suggesting that Chp senses TFP structural changes upon tensile load. Altogether, these findings highlight a previously unknown function for TFP in sensing the mechanical properties of surfaces upon the initial stages of surface association. TFP in turn signal through the Chp system to induce cAMP-dependent processes such as stronger adhesion, prolonged surface association, biofilm formation, and pathogenicity.

Results

*PaQa* Is Up-Regulated upon Contact with Surfaces. To explore the role of surface contact in regulating *PaQa*, we generated a *yfp* transcriptional reporter for *PaQa* and measured its activation at the single-cell level as a ratio to a constitutively expressed *cfp* reporter. We found that single cells induced significant *PaQa* expression within 3 h of contact with a solid agarose surface (Fig. 1A). We observed a fivefold increase in the mean fluorescence intensity of a population of cells (Fig. 1B). We verified that the increase in the YFP-to-CFP ratio was mainly a result of the increased relative to liquid growth. All fluorescence values are normalized to the mean CFP fluorescence is about 10% higher in liquid than increased activity of the *PaQa* promoter in response to mechanical stimulation by surface contact. Because the *PaQa* reporter depends on cAMP signaling. We repeated the surface-sensing assay on agarose using iso- genic mutants defective in cAMP production or in the Chp chemosensory regulon. In *P. aeruginosa*, the *cyaB* gene encodes the major adenylate cyclase (11). In the *cyaB* mutant, the *PaQa* reporter showed no increase in fluorescence upon surface contact (Fig. S3A). Conversely, disruption of the gene encoding the cAMP phosphodiesterase *cpdA*, which results in elevated cAMP levels, showed constitutive induction of the *PaQa* reporter in liquid medium, without the need for surface contact (Fig. S3B). Finally, we verified that the surface-dependent induction of *PaQa* requires *vfr*, a transcription factor that regulates multiple virulence factors upon binding cAMP (Fig. S3A). Together, these results demonstrate that surface-induced *PaQa* expression requires an intact cAMP signaling pathway, indicating that surface contact is a signal for cAMP production and Vfr transcription.

The Chp Chemosensory System Regulates *PaQa* upon Surface Contact. Given the importance of cAMP, we investigated the role of the Chp chemosensory pathway, a known regulator of cAMP production and surface-associated behaviors such as twitching motility and TFP assembly (10, 12). The induction of *PaQa* upon surface contact was inhibited in mutants in the Chp chemosensory system, including mutants in *pilU*, a methyl-accepting chemotaxis protein (MCP)-like chemosensory receptor, and *chpA*, the CheA two-component signaling histidine kinase homolog (Fig. 2A). Classic chemotaxis systems use CheY as the response regulator downstream of CheA (18). The Chp system has two apparent CheY homologs, PilG and PilH, which have opposite effects on cAMP levels (9). The *pilH* mutant exhibits elevated cAMP levels and, as predicted, demonstrated constitutive induction of *PaQa* expression in liquid (Fig. S3C). Together these results indicate that the Chp and cAMP pathways mediate the response to mechanical stimulation by surface contact. Because the Chp system is homologous to a chemotaxis system, this raises the possibility that Chp senses a signal specifically induced upon surface contact to regulate cAMP production.

Surface Sensing Requires TFP and Its Motors. How might the Chp chemosensory system sense physical contact with the surface? TFP mechanically couple the cell with the substrate. Mutants in
Disrupting the major pilin subunit, PilA, completely abolished sensitivity to contact with the hydrogel (Fig. 2C). This loss of sensitivity also demonstrates that flagella do not mediate this response, because pilA mutations do not affect the swimming motility system (19). Disrupting the TFP extension motor, PilB, or simultaneously disrupting the two TFP retraction motors, PilT and PilU, reduced the magnitude of the surface sensitivity compared with WT. Mutants lacking the PilTU retraction motors exhibited an increase in PaQa transcription compared with mutants lacking PilA or the PilB extension motor. Feedback regulation maintains PilA levels relatively constant in motor mutants such that these mutants’ perturbed surface sensing can be attributed to altered motor activity. Thus, surface sensing requires PilA and is amplified by TFP extension and retraction. We note that in a simultaneous study Luo et al. showed that cAMP production increases in a PilA- and PilJ-dependent manner when P. aeruginosa are grown on an agar surface (10).

In a recent study we demonstrated that the TFP-associated protein PilY1 works with quorum sensing to stimulate virulence upon prolonged surface contact (5). Whereas PilB is required for the short timescale PaQa response investigated here, PilB was not required for the long timescale virulence response to surface association (5), suggesting that the Chp and PilY1 systems are distinct. To further distinguish the PilY1- and Chp-dependent surface responses, we characterized the effect of deleting pilY1 on the surface sensitivity of the PaQa reporter. The PaQa reporter could still be induced by contact with agarose hydrogels in the pilY1 mutant (Fig. S4). However, similar to the effect of PilY1 on its own expression (10), the magnitude of the PaQa induction was decreased in pilY1 compared with WT. Therefore, PilY1 affects short timescale mechanosensation but is not absolutely required. This effect could be explained by the fact that pilY1 mutants are partially defective in TFP assembly and retraction (20).

We further tested the possible dependence of PaQa induction upon surface contact on quorum sensing. On a solid substrate, a single cell grows into a dense colony (Fig. 2B), raising the possibility that high cell density activates quorum sensing-dependent genes via the LasR and RhlR regulons. To determine whether these regulators play a role in PaQa induction, we tested the effect of mutations in their corresponding genes on PaQa induction. Both lasR and rhlR mutants showed significant PaQa reporter induction upon surface growth (Fig. S5). The magnitude of the response was similar to that of WT cells, demonstrating that surface sensing is independent of quorum sensing despite the rapid increase in cell density. These results support the hypothesis that the short timescale initial surface association response is mediated by TFP and is independent from the long timescale response mediated by PilY1 and quorum sensing.

**TFP Attachment to Surfaces Stimulates PaQa Expression.** Given that TFP are necessary for mechanosensation and that their extension and retraction motors amplify this signal, we sought to determine how TFP are mechanically coupled to the surface. In particular, do TFP need to attach to a surface or is PilA required through some indirect mechanism? To determine whether more frequent TFP attachment stimulated surface sensing, we varied surface substrate density by exposing cells to engineered hydrogels with decreasing pore sizes (21). Here, smaller pore sizes lead to higher substrate density and thus a higher probability of TFP attachment upon extension. Time-lapse imaging of colonies enabled quantitative measurement of PaQa induction upon contact with the hydrogels of various pore size (Fig. 3). Fluorescence measurements showed that the increase in PaQa reporter fluorescence was slowest at the largest pore size (0.75% agarose), intermediate at the intermediate pore size (1%), and fastest at the smallest pore size (1.5%). We furthermore observed that the rates of change in fluorescence induction reach their maximum
The activity of PaQa upon surface contact depends on hydrogel mechanical properties. The relative fluorescence intensity of the PaQa YFP reporter (normalized to RpoD-mKate) per cell is averaged for each colony and reported as a function of time. Dashed lines represent the mean over multiple colonies (n = 12) and the shaded regions represent 95% confidence intervals. (Inset) The rate of change of fluorescence F. Higher agarose concentration yields hydrogels with smaller pore size, thus increasing interaction between TFP and the substrate.

values at ~150 min on all three hydrogels, before returning to near-zero levels thereafter. This indicates that the differences of signal between the hydrogels are not due to a kinetic effect (i.e., where it takes longer to reach identical cAMP levels on larger pores), but rather on instantaneous sensing. The distinct steady-state value of PaQa induction on liquid and solid show that cAMP levels likely adapt to the distinct substrates (i.e., cAMP level is a memory of the attachment state of a cell).

When assembled, TFP undergo thermal fluctuations in the vicinity of the substrate (22). Attachment of a TFP tip depends upon encountering the agarose surface. We estimate that the timescale of TFP attachment scales with the diffusion time of its tip across the area of a pore. In our experiments, mean pore size varies between about 100 nm (1.5% agarose) and 300 nm (0.75%) (21). Therefore, in the range of agarose concentration tested here, the mean area of a pore decreases as the square of this difference, or roughly 10-fold between 0.75% and 1.5% gels. Balancing this area with the mean square displacement of a TFP tip (which scales approximately with time) shows that TFP attachment is roughly 10 times more frequent in the 1.5% gel compared with 0.75%, leading to stronger PaQa induction. We note that higher gel concentration results in harder gels, so that TFP possibly attach to and pull on stiffer gel fibers, which may also in part contribute to stronger signal (23). We thus conclude that P. aeruginosa directly senses TFP attachment as a means of sensing the extent of surface association, indicating that TFP coupled with Chp constitute a mechanosensitive machinery.

PilA Interacts with the Chemosensory Protein PilJ. How do TFP signal to the Chp system? We used the bacterial two-hybrid system (BACTH) to test the interaction between the two most likely candidates: PilJ, the MCP homolog, and PilA, the pilus subunit (Fig. S6d). PilA is associated with the inner membrane and has a very short cytoplasmic extension before being processed by a prepilin peptidase (7). PilJ has two transmembrane domains, and both its N- and C-termini are cytoplasmic (24). The BACTH system functions by using the interactions between two test proteins to reconstitute cAMP activity by bringing together the two subdomains (T18 and T25) of adenylate cyclase (AC) to reconstitute a functional enzyme. In the N terminus PilA fusion, the AC fragment will be localized in the cytoplasm (Fig. S6b). Both N- and C-terminal fusions of PilJ to either AC subunit should localize AC to the cytoplasm. Coexpression of the N-terminal PilA fusion with the N- or C-terminal T25 or T18 PilJ fusion, respectively, resulted in a large increase in AC activity (Fig. S6a). We verified that this signal was not a result of nonspecific interactions between the N-terminal fusion to PilA with another integral membrane protein by measuring the interaction with fusions to FimV (8), which were both negative (Fig. S6a). Also, fusions to PilJ lacking the transmembrane domains resulted in loss of signal (Fig. S6a). Together these results indicate that PilJ and PilA can interact. Because PilA does not have a cytoplasmic domain, PilA and PilJ likely interact through their periplasmic domains. The periplasmic domain of PilJ includes its chemosensory domain, indicating that the Chp system may respond to the PilA ligand as a signaling input.

Mechanotransduction Requires Both TFP Tension and Retraction. How could a PilA–PilJ interaction sense TFP attachment and induce surface mechanosensing? Attached TFP are under tension during retraction and this tension could modify TFP, for example by inducing a modification in PilA. PilJ could sense such tension-induced changes at the attachment point between the cell body and the TFP, where PilJ colocalizes (25). Alternatively, PilJ could sense TFP modification during retraction, as PilA subunits depolymerize into monomers and are incorporated back into the inner membrane. These two models differ in their dependence on retraction; if PilJ senses tension at the base of TFP, then in the absence of retraction, placing TFP under tension would restore signaling. In contrast, if stretched TFP activate PilJ as they retract, then tension in the absence of retraction would not further stimulate signaling. To differentiate these two models, we generated tension in TFP of a pilTU mutant, defective in TFP retraction (Fig. S7). We initially attached pilTU cells onto the glass surface of a microfluidic channel. Here, unretracted TFP allow for efficient attachment to glass. To generate tension in TFP of attached cells, we applied flow in the microchannel. Shear stress generates a force on the cell body that is parallel to the surface and is balanced by tension in TFP. We set the flow rate to generate roughly 10 pN of force, which is on the order of forces generated by TFP retraction (26).

We measured the response of the PaQa reporter in the pilTU background in the presence and absence of flow. Similarly to the case of growth on agarose pads, we measured an increase in the fluorescence intensity of the reporter upon attachment in both flow and no-flow conditions (Fig. S7). However, fluorescence intensities measured in flow and no-flow conditions had similar amplitudes, indicating that further TFP tension generated by flow does not increase the response. In the no-flow case, the response upon surface attachment may originate from mechanical perturbations, for example tension induced by Brownian motion of the cell body. Importantly, flow-induced TFP tension failed to restore PaQa promoter activity to the levels measured on agarose pads (Figs. 2C and 3). These results indicate that efficient PilJ signaling via TFP activity requires not only extension and attachment but also the internalization of stretched TFP, and refutes a mechanism where PilJ senses TFP tension without retraction. The residual induction observed in the absence of PilTU could be explained by interactions between PilJ and the small number of PaQa subunits found at the base of the pili. Weak interactions would also explain the low level of PaQa induction observed in pilB mutants (Fig. 2C).

Discussion

We demonstrated that TFP not only function as a structure enabling surface motility, but also as a force transducer promoting the induction of virulence factors when cells encounter surfaces such as host tissues. Based on our results, we propose a mechanomechanical model for surface sensing where TFP function both as mechanical actuators and sensors whose output is
Inhibiting TFP extension in the initially sensitive to surface attachment, but with reduced sensitivity. When cells encounter a surface, their TFP adhere to the substrate. Retraction generates tension in the TFP between its anchor and the cell. This tension induces an unknown modification in TFP to interpret its mechanical environment. In P. aeruginosa, TFP exhibit conformational changes upon stretching (28) and stretching reduces TFP width. Our findings suggest that P. aeruginosa measures mechanically induced modification in TFP to interpret its mechanical environment. In Neisseria gonorrhoea, TFP exhibit conformational changes upon stretching (28) and stretching reduces TFP width. Interestingly, these changes expose hidden PilA epitopes, indicating that PilA itself may change conformation upon tension. Our results suggest that the Chp system measures tension in nonretracted fibers. Thus, PilJ could interact with PilA at the stretched TFP (27). The difference of signals measured in these two mutants is therefore an effect of TFP extension and retraction, prompting a positive feedback loop to interpret tension in nonretracted fibers. This interaction could be a ruler for TFP width, allowing the cell to read out tension in the TFP.

Our findings also suggest that P. aeruginosa has evolved a sensory system that is specific to surface contact by coupling TFP to an MCP. TFP already mechanically couple the bacteria to the surface, and are repurposed here to sense attachment. In this scenario, PilJ might act as a ruler for TFP width.

P. aeruginosa seems to have evolved a sensory system that is specific to surface contact by coupling TFP to an MCP, two systems that are widespread in bacteria. TFP already mechanically couple the bacteria to the surface, and are repurposed here to sense attachment. In this scenario, PilJ might act as a ruler for TFP width. The Chp system is unique because it repurposes a chemotaxis-system to sense surface contact by coupling TFP to an MCP, two systems that are widespread in bacteria. TFP already mechanically couple the bacteria to the surface, and are repurposed here to sense attachment. In this scenario, PilJ might act as a ruler for TFP width.
help decipher the role of mechanics in their activation. Additionally, other bacterial species have the ability to mechanically probe their environment. For example, a flagellum-dependent mechanism modulates transcription, surface motility, and biofilm formation in *Escherichia coli* (31). Inhibiting flagellum rotation potentially disrupts the flow of ions through the flagellar motor, thereby transducing a mechanical signal into a chemical signal. This system differs from our model in that it uses flagellar function as a mechanosensor and may be activated by swimming in fluids more viscous than water. In contrast, the system we describe here requires TFP to mechanically attach to a surface, a feature that is independent of fluid properties. The role of TFP as the mechanical component prevents nonspecific activation of the pathway by the chemical environment.

*P. aeruginosa* possess multiple surface sensing systems to coordinate various virulence pathways (Fig. 4B and Fig. S8). The mechanosensitive TFP system enables surface sensing on short timescales on abiotic surfaces. Swimming *P. aeruginosa* cells harbor low but significant numbers of TFP (27), enabling the ability to sense a surface upon contact with the substrate. Transient contact leads to activation of the Chp system, simultaneously stimulating cAMP production and TFP extension–retraction. Increased cAMP leads to Vfr-dependent transcription, activating the type II and III secretion systems (12). Vfr also regulates the LasR quorum sensing system and the minor pilin PilY1 (10, 32). Consequently, surface sensing by TFP increases sensitivity to quorum sensing, possibly activating other surface-specific virulence factors, for example, via the synthesis of cyclic-di-GMP (5, 10). Thus, *P. aeruginosa* harnesses the TFP surface sensing system to rapidly initiate infections upon surface contact.

On a longer timescale (multiple hours or days), cells grow into biofilms and induce other virulence factors (Fig. S8). Activating virulence pathways in response to mechanical contact, in lieu of sensing specific chemical cues, could promote the ability of *P. aeruginosa* to infect a wide range of hosts. Inducing virulence upon association with abiotic surfaces may also help combat invasion by competing microbes, thereby promoting robust surface colonization. Most studies on bacterial development have focused on the role of chemical environments in shaping physiological adaptations. Mechanics are a ubiquitous feature of bacterial habitats; our results highlight the possibility that they actively sense mechanical forces to adapt to their environments.

**Materials and Methods**

To measure reporter activity, overnight cultures of PA01 containing the PaQa reporter plasmid (SI Materials and Methods and Table S1) were diluted into fresh LB and grown at 37 °C to midexponential phase. One hundred microliters of culture was then spread and dried onto each hydrogels and incubated for 3 h at 37 °C. Cells were then harvested by washing the hydrogels and mechanically scraping their surface with 400 μL of PBS. We delivered a 1-μL drop of the harvested population onto an agarose pad then covered it with a no. 1.5 coverslip. Finally, we acquired phase contrast and fluorescence images of the cells (SI Materials and Methods). These images were then segmented using phase contrast and the cell contours were used as masks to measure the relative fluorescence of the PaQa transcriptional reporter to the reference (YFP/CFP). We measured this ratio for more than 100 cells per experiment and reported the average of four replicates with their 95% confidence intervals based on a t distribution.

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Supporting Information

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**SI Materials and Methods**

**Growth and Media.** We used the *Pseudomonas aeruginosa* PAO1 as the parental strain (1). Cells were grown at 37 °C in LB. Cloning was performed in *Escherichia coli* S17.1. We used the following antibiotic concentrations in media: 200 μg/mL carbenicillin (300 μg/mL on plates) or 60 μg/mL tetracycline (100 μg/mL on plates) for PAO1, and 50 μg/mL carbenicillin (100 μg/mL on plates) or 10 μg/mL tetracycline (20 μg/mL on plates) for *E. coli*.

**Strains and Plasmids.** To generate the transcriptional reporter for the PaQa operon, we incorporated the fluorescent reporter system pZS2-123 (2) into the plasmid pUCP18 (3). We first amplified the fluorescent reporter section of the pZS2-123 scaffold containing yfp and cfp using primers P1 and P2. We then ligated the fragment at the SfiI and PciI sites of pUCP18 to generate pAP02. We amplified the promoter sequence of PaQa and the housekeeping gene rpoD using primer pairs (P3, P4) and (P5, P6), respectively. We PCR-amplified pAP02 using P7 and P8 and used Gibson assembly to insert the rpoD promoter fragment upstream of cfp. The PaQa promoter was then inserted into the XhoI and BamHI sites. For constructs used in the timecourse experiments, we replaced the cfp by mKate by amplifying the reporter plasmid using primers P9 and P10, separately amplified the mKate2 gene coding for a red fluorescent protein using P11 and P12, and assembled both fragments using Gibson assembly. These plasmid constructs were then electroporated into PAO1. In-frame deletions in PAO1 were described previously (4). Quorum sensing mutants lasR::Tn5 and rhlR::Tn5 were obtained from the PAO1 transposon mutant library (5).

**BACTH Plasmid Construction.** The *P. aeruginosa* genes pilA, pilI, and FimV were cloned into pKT25, pKNT25, pUT18, and pUT18C (Euromedex). The pilA, pilI, and FimV genes were PCR-amplified from purified PAO1 genomic DNA, pMBAD-pilI-GFP, and pMBAD-FimV-GFP, respectively, using primer pairs (P13, P14), (P15, P16), and (P17, P18) using phusion polymerase (NEB). The pilA and FimV PCR fragment was inserted into the linearized vectors using the BD In-fusion cloning kit (Clontech) and the pilI PCR fragment was digested with BamHI-HF and KpnI-HF (NEB) and ligated into the above vectors previously digested with the same restriction enzymes using Fast-Link DNA ligase (Epicentre). The PilIΔmt constructs were generated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and primer pairs (P19, P20).

**BACTH Assay.** BTH101 chemically competent cells were generated using the TSS protocol (6). Competent cells were cotransformed with 20 ng of each pK and pUT constructs and plated on MacConkey agar (Difco) supplemented with 1% maltose, 50 μg/mL kanamycin, 100 μg/mL ampicillin, and 0.5 mM isopropyl β-D-1-thiogalactopyranoside. Plates were incubated at 30 °C for 48 h and scored by comparing the color of the colonies to purple positive control (pKT25-zip + pUT18C-zip) and white negative control (pKT25 + pUT18C) colonies.

**Hydrogel Preparation.** One percent (wt/vol) agarose hydrogels were obtained by dissolving powdered agarose in LB and autoclaved for 15 min. The molten gels were subsequently cooled down to 60 °C and poured in dishes. The 1% (wt/vol) gellan hydrogel was prepared by dissolving Geltite (Sigma) in LB, autoclaved for 15 min, and cooled to 60 °C before adding MgCl₂ hexahydrate to 3 mM while stirring. The 1% alginate gel was obtained by autoclaving a 1% solution of sodium alginate (wt/vol) in deionized water, cooling, and adding calcium carbonate to 30 mM before pouring. After hours of cooling, the alginate gels were washed in vast amounts of LB for 24 h at 4 °C. The gels were dried in a fume hood for 1 h before seeding cells. PVA/PVP hydrogels were made using a freeze–thaw method described in ref. 7. We prepared a solution of 10% (wt/vol) PVA and 1% (wt/vol) PVP, which we autoclaved for 15 min. After cooling while stirring, we poured the molten gel in Petri dishes, which were frozen at –20 °C for 24 h and brought back to room temperature for another 24 h. We repeated this freeze–thaw cycle three times. The hydrogels were then incubated in a bath of LB for 24 h at 4 °C, transferred into a new dish, and briefly dried in a fume hood before inoculation.

**Fluorescence Microscopy.** Images of cell populations were obtained on an upright Nikon 90i microscope controlled with NIS Elements software equipped with a Hamamatsu Orca R2 CCD camera. Time-lapse and flow experiments were obtained on an inverted Nikon TiE microscope controlled with NIS Elements, equipped with a 40× 0.95 N.A. Plan Apo objective. Images were acquired using an Andor Clara CCD camera. Snapshots images were generated with ImageJ and all data were analyzed with MATLAB.

**Time-Lapse Imaging of *P. aeruginosa* Colonies.** Cells containing the PaQa reporter plasmid with mKate as reference reporter were grown overnight in LB containing carbenicillin. We pelleted 1 mL of this culture and washed twice with M9 salts. Cells were then diluted and grown at room temperature in M9 salts containing mucins. M9 mucin was prepared by dissolving 0.25% (wt/vol) mucins (type III from porcine stomach; Sigma) in 1x M9 salts containing 2 mM MgSO₄ and filtered through a Nalgene 0.22-μm filter. We transferred 1 μL of the midexponential phase culture onto agarose pads (161-3100; BioRad) prepared with the same M9 mucin growth medium. The pad was then transferred to a glass bottom dish (no. 1.5 coverslip; Mattek) and immediately visualized on the inverted microscope. To measure colony growth, we initially focused on single isolated cells and imaged YFP and mKate2 fluorescence every 15 min for 5 h. The movies were then segmented into isolated cells from the mKate2 images. We then computed the relative intensity YFP/mKate for each cell in the colony at each time and averaged this intensity across the whole colony to yield the mean intensity per cell. PaQa activity curves dF/dt correspond to a five-point moving average of the derivative of fluorescence with time.

**Time-Lapse Imaging in Flow Chambers.** One milliliter of overnight culture of PAO1 containing the fluorescent reporter was pelleted and washed twice with M9 salts. Cells were then diluted and grown at room temperature in M9 salts containing 0.25% (wt/vol) filtered mucin for WT and M9 salts supplemented with 0.2% tryptone for pilTU. We grew all cells to early exponential phase and injected 10 μL of the culture in a polydimethylsiloxane microchannel fabricated using standard soft lithography. We then connected tubing to the inlet and outlet of the channel. The inlet was connected to a sterile syringe filled with media (MW with mucin or tryptone) and the outlet to a waste container. The microfluidic chip was then mounted on the inverted microscope stage. We initiated flow of medium using a syringe pump and immediately monitored fluorescence of single isolated cells growing into colonies for pilTU (with 100× magnification) and the fluorescence of the surface-associated population of WT (40× magnification).

**Fig. S1.** Fluorescence levels of the reference reporter fusion to the rpoD promoter are similar between liquid- and solid-grown cells. (A) The distribution of fluorescence intensity per cell per unit area only slightly differs between liquid-grown WT cells (blue bars) and cells associated to 1% agarose for 3 h (red bars). The fluorescence only slightly shifts toward lower intensity in solid grown cells, suggesting a slight change in cell metabolism. The histograms are each generated from measuring CFP fluorescence of 700 cells. (B) The mean CFP fluorescence intensity per cell only slightly decreases between liquid and solid growth. Error bars represent 95% confidence intervals (n = 6, each measurement is the average fluorescence about 100 cells).

**Fig. S2.** *P. aeruginosa* respond to a surface environment with mucins as they develop into surface-associated colonies. (A) The relative fluorescence intensity of the PaQa YFP reporter (normalized to rpoD-mKate) was measured in response to binding to mucins under fluid flow shear stress (0.1 Pa at the channel centerline), in an environment that recapitulates infections. The solid line represents the mean over multiple fields of view (n = 10) and the shaded regions correspond to the 95% confidence interval. (Inset) YFP fluorescence of a colony at 15 h. (B) Using mucin-based medium does not modify the magnitude of the reporter response on agarose compared with the tryptone-based LB medium. Error bars represent 95% confidence intervals (n = 4, each measurement is the average fluorescence about 100 cells).
cAMP and Vfr regulate PaQa activity upon contact with surfaces. The relative fluorescence intensity of the PaQa YFP reporter (normalized to rpoD-CFP) upon binding to a 1% agarose hydrogel was compared between (A) WT, in a mutant defective in the major adenylate cyclase (cyaB), and in a mutant defective in Vfr, which encodes a cAMP-dependent transcription factor that regulates the transcription of >200 genes; (B) WT and a mutant defective in the phosphodiesterase (cpdA), which degrades cAMP, and (C) WT and pilH, a predicted response regulator for ChpA and associated with increased cAMP production. Error bars represent 95% confidence interval (n = 4, each measurement is the average fluorescence from more than 100 cells).

Mutants lacking the minor pilin gene pilY1 remain sensitive to surface contact. The relative fluorescence intensity of the PaQa YFP reporter (normalized to rpoD-mKate) in a pilY1 background increases upon binding to a 1% agarose hydrogel. We attribute the reduction of sensitivity compared with WT to the defect in TFP assembly and function observed in pilY1 mutants. Error bars represent 95% confidence intervals (n = 7, each measurement is the average fluorescence from more than 100 cells).
Fig. S5. Mutants with disrupted quorum sensing regulation remain sensitive to surface contact. The relative fluorescence intensity of the PaQa YFP reporter (normalized to rpoD-CFP) in a WT, lasR::Tn5, and rhlR::Tn5 backgrounds all increase upon binding to 1% agarose hydrogel with similar amplitudes. We note the slight increase in surface sensitivity in the rhlR mutant, suggesting that the associated quorum sensing pathway may repress surface sensing. Error bars represent 95% confidence intervals (n = 6).

Fig. S6. BACTH reveals an interaction between PilA and the MCP-like chemosensory protein PilJ via their periplasmic domains. (A) We fused the T18 and T25 fragment to either the N or C terminus of PilA (an inner membrane-associated protein with a short N-terminal cytoplasmic tail) and fused the T25 and T18 fragment to the N or C terminus of PilJ, which possess two inner membrane domains [green in schematic (B)] and a periplasmic domain. Both N- and C-terminal constructs of PilJ localize T18 and T25 to the cytoplasm. The N-terminal PilA fusion (T25-PilA) showed a strong BACTH signal with either N- or C-terminal fusions to PilJ. We also verified that the transmembrane domain of PilJ is required for interaction with PilA, because the PilJΔtm failed to induce BACTH signal. These results suggest that PilA interacts with the periplasmic domain of PilJ, predicted to include its sensory domain. Thus, PilJ may use PilA as a ligand for an input signal, transducing a mechanical cue into a chemical response. Fusions to the integral membrane protein FimV failed to induce a BACTH signal with both PilA fusions, showing that our positive BACTH signals are not a result of nonspecific interactions between membrane proteins. T18-zip and T25-zip refers to T18 and T25 fused to a leucine zipper and vector refers to T18 and T25 vectors alone. These are standard controls for nonspecific interactions in the BACTH assay.
Fig. S7. The activity of PaQa upon surface contact depends on TFP tension and retraction. The relative fluorescence intensity of the PaQa YFP reporter in the retraction deficient pilTU strain was measured as a function of time in the presence (∼2 Pa applied flow to generate a force of ∼10 pN per cell) or absence of flow. Note that the amplitude of fluorescence is similar to that measured on agarose pads (Fig. 2C). We measured the fluorescence of single cells that were initially attached to the glass surface. The shaded regions correspond to 95% confidence intervals (n = 19 for no flow, n = 34 with flow).

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Fig. S8. A proposed model for the regulation of multiple surface-activated virulence programs in P. aeruginosa. (i) Upon encounter with a surface, TFP activity generates a signal that leads to the production of cAMP and the transcription of hundreds of genes via Vfr, including the up-regulation of LasR and PilY1. (ii) Cells grow into dense microcolonies stimulating contact between cells. (iii) The combined effects of Wsp and PilY1 surface sensing and LasR quorum sensing systems induce the activation of behaviors associated with high levels of cyclic-di-GMP, including the formation of biofilms. (iv) Biofilms persist on abiotic and biological surfaces on longer timescales and are associated with increased antibiotic resistance.
### Table S1. Primers used in this study

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**Movie S1.** Time lapse of PaQa-yfp reporter activity during colonization of a glass surface with flow of mucin. *P. aeruginosa* containing the PaQa reporter system are initially seeded in the microchannel. Flow of filtered minimal medium containing 0.25% of mucins is initiated at $t = 0$. In the first few hours of the movie, single cells are attached to the surface and appear motile. They initiate the formation of surface-associated microcolonies before growing into larger multicellular structures exhibiting high PaQa activity. The pixel intensity in this movie is shown on a logarithmic scale to enhance visualization.

**Movie S1**