Surface attachment induces *Pseudomonas aeruginosa* virulence

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*Pseudomonas aeruginosa* infects every type of host that has been examined by deploying multiple virulence factors. Previous studies of virulence regulation have largely focused on chemical cues, but *P. aeruginosa* may also respond to mechanical cues. Using a rapid imaging-based virulence assay, we demonstrate that *P. aeruginosa* activates virulence in response to attachment to a range of chemically distinct surfaces, suggesting that this bacterial species responds to mechanical properties of its substrates. Surface-activated virulence requires quorum sensing, but activating quorum sensing does not induce virulence without surface attachment. The activation of virulence by surfaces also requires the surface-exposed protein PilY1, which has a domain homologous to a eukaryotic mechanosensor. Specific mutation of the putative PilY1 mechanosensory domain is sufficient to induce virulence in non-surface-attached cells, suggesting that PilY1 mediates surface mechanotransduction. Triggering virulence only when cells are both at high density and attached to a surface—two host-nonspecific cues—explains how *P. aeruginosa* precisely regulates virulence while maintaining broad host specificity.

bacterial mechanosensation | PilY1 | von Willebrand factor | host detection | contact regulation

The bacterium *Pseudomonas aeruginosa* is a metabolically versatile pathogen that inhabits diverse environments and infects a remarkable range of hosts, including mammals, insects, worms, amoebae, fungi, and other bacteria. *P. aeruginosa* produces a large number of secreted and cell-associated virulence factors that are redundant and multifactorial (1, 2). Many of *P. aeruginosa*'s virulence factors—including pyocyanin, elastase, and hydrogen cyanide—are host-nonspecific (3–5), bolstering the ability of *P. aeruginosa* to attack a large range of hosts. Although many of the virulence factors in *P. aeruginosa* have been identified, the cues that regulate their activity are less understood. Because many of the virulence factors are host-nonspecific, we explored whether virulence in *P. aeruginosa* is regulated by host-nonspecific cues.

Host cell membranes and cell surfaces are the first line of defense against bacterial toxins and invasion. *P. aeruginosa* attaches to host cell surfaces early during the infection process. The presence of a surface could thus act as a cue for *P. aeruginosa* signaling the presence of a host. Surface attachment is also a critical initial step that enables the establishment of biofilms (6–8). Although biofilms are clearly important for pathogenesis, it remains unclear whether they directly promote host cell killing or mediate other important processes such as long-term colonization.

One host-nonspecific cue that could regulate virulence is the mechanical force that bacteria experience upon surface attachment. *P. aeruginosa* performs surface-associated behaviors (7, 8) such as swarming and twitching (9, 10), but it remains unclear whether *P. aeruginosa* senses the chemical or mechanical properties of surfaces. There is precedence for mechanotransduction in eukaryotes, in which surface substrate recognition is an important regulator of development and behavior (11). In prokaryotes, surface mechanical forces affect the binding affinity of cells to substrates (12, 13) and alter the rotation of flagella (14, 15). However, the effects of mechanical forces on cell behaviors other than motility are not understood, and the regulation of virulence by mechanical cues has not been explored.

Here, we show that attachment to surfaces induces *P. aeruginosa* to become virulent. Virulence is activated on a variety of chemically distinct abiotic and host surfaces, suggesting that mechanical cues associated with surface attachment activate virulence. We identify PilY1 as a key mediator of surface-activated virulence. PilY1 is a cell-surface–exposed protein that regulates a number of surface-associated behaviors and contains a mechanosensitive von Willebrand Factor A (VWFa) domain. Although *P. aeruginosa* lacking PilY1 cannot activate virulence upon surface contact, bacteria with a specific deletion of the VWFa domain hyperactivate virulence, even in the absence of surface contact. Together, our results suggest that cells detect mechanical cues associated with surface attachment through a mechanosensitive pathway that requires the PilY1 protein. We suggest that detecting mechanical cues associated with surface attachment enables *P. aeruginosa* to induce virulence toward a broad range of hosts without relying upon chemical recognition of any specific host factor.

**Results**

**Surface Attachment Rapidly Induces *P. aeruginosa* Virulence.** Traditional bacterial virulence assays involve prolonged exposure of bacteria to rigid surfaces such as culture plates or agar dishes and thus cannot establish the specific contribution of surface contact.

**Significance**

*Pseudomonas aeruginosa* is a pathogen that kills a remarkably wide range of hosts. The environmental cues that regulate *P. aeruginosa* virulence have remained unclear. Here, we develop a rapid imaging-based virulence assay to quantify virulence. We find that association with rigid surfaces induces virulence toward multiple hosts. Virulence induction depends on the mechanical, but not chemical, properties of the surfaces and requires the surface-exposed protein PilY1, which has homology to the mechanosensitive von Willebrand factor A domain. Specific mutation of this mechanosensitive domain is sufficient to constitutively activate virulence independent of surface attachment. Mechanosensitive virulence induction can explain how *P. aeruginosa* infects a broad range of hosts while tightly regulating virulence. Consistently, association with one host induces virulence toward other hosts.

Author contributions: A.S., G.A.O., and Z.G. designed research; A.S. performed research; A.S., S.L.K., and G.A.O. contributed new reagents/analytic tools; A.S. analyzed data; and A.S. and Z.G. wrote the paper.

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Data deposition: The raw microarray data have been deposited in the Princeton University MicroArray (PUMA) database, puma.princeton.edu (accession no. 20141009PA14).

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to virulence. We developed a virulence assay that uses single-cell fluorescence imaging to directly monitor virulence at short time-scales, enabling us to separately assay the virulence of planktonic (liquid-grown) and surface-attached bacterial subpopulations in shaking cultures. We initially focused on the amoeba Dictyostelium discoideum as a model host. D. discoideum is a natural host for P. aeruginosa and is functionally similar to mammalian macrophages, exhibiting chemotaxis toward and phagocytosis of bacteria. Genetic studies have validated that the virulence factors that act on mammalian and amoeba hosts are largely identical (16, 17). We monitored virulence by mixing amoebae with planktonic or surface-attached bacteria, confining all cells to a single plane using an agar pad, and measuring cell viability using single-cell time-lapse imaging in the presence of calcein acetoxymethyl ester (calcein-AM) (Fig. 1A and SI Appendix, Fig. S1). Calcein-AM does not permeate the D. discoideum cell membrane and fluoresces when it is cleaved by intracellular esterases. Healthy D. discoideum cells are flat, motile, and produce little or no calcein-AM fluorescence, whereas unhealthy D. discoideum cells are rounded, immotile, and produce strong calcein-AM fluorescence before lysing (Fig. 1B and SI Appendix, Fig. S2A). We compute a host killing index, a measure of host cell viability, by integrating the calcein-AM fluorescence of individual amoebae, dividing by the cell area, and averaging this value over many cells (SI Appendix, Fig. S1).

Our imaging-based virulence assay revealed a striking difference between planktonic and surface-attached P. aeruginosa. Amoebae that were mixed with planktonic P. aeruginosa flattened and exhibited robust motility and phagocytosis, but little or no calcein-AM fluorescence (Fig. 1B and C and Movie S1). These behaviors were, in all respects, similar to those observed in the presence of nonpathogenic Escherichia coli B/r (Fig. 1B). In contrast, amoebae that were exposed to surface-attached P. aeruginosa became rounded, did not exhibit motility or phagocytosis, and typically produced calcein-AM fluorescence within 15-20 min (Fig. 1B and C and Movie S1). We note that amoebae that are exposed to conditioned medium only (Fig. 1C) enter a starvation phase, which appears to make them more susceptible to secreted P. aeruginosa host killing factors (2-5). We confirmed the increased virulence of surface-attached P. aeruginosa using three other host viability reporters (SI Appendix, Fig. S2B). To establish the multiplicity of infection (MOI), we showed that P. aeruginosa cell density correlates with the number of P. aeruginosa cells in our imaging assays and then used P. aeruginosa density as a proxy for MOI in subsequent experiments (SI Appendix, Fig. S3). The significantly increased virulence of surface-attached P. aeruginosa cannot be attributed to differences in MOI, because surface-attached P. aeruginosa kill amoebae at significantly lower cell numbers than planktonic P. aeruginosa (Fig. 1D).

**Virulence Activation Is Surface- and Host-Independent.** Bacteria could sense surfaces by detecting chemical or mechanical features of the surface. To differentiate these possibilities, we assayed a range of chemically distinct rigid surfaces. P. aeruginosa virulence was similarly activated by glass (Fig. 1B), plastic (Fig. 1C), polyacrylamide (Fig. 2A), and agar (Fig. 2A) surfaces. Thus, virulence activation requires a mechanically rigid surface, but does not depend on the surface’s specific chemical composition.

Because P. aeruginosa infects a broad range of host types (18), surface contact could serve as a nonspecific cue for host infection. In such a model, P. aeruginosa mechanically detects the presence of a host and broadly activates virulence factors, predicting that surface contact with one type of host induces virulence factors that kill other hosts. We tested this prediction by culturing P. aeruginosa cells on the surface of one host, a pothos plant (Epipremnum aureum), and monitoring the virulence of P. aeruginosa toward a second host, D. discoideum (Fig. 2B-D). Attachment to the surface of a pothos leaf stimulated virulence toward D. discoideum (Fig. 2B-D), demonstrating that surface contact is a host-nonspecific cue that induces P. aeruginosa virulence.

The mechanical cues associated with surface contact are not host-specific, suggesting that surfaces could also stimulate P. aeruginosa virulence toward hosts other than amoebae. We therefore compared the viability of mouse macrophages in the presence of planktonic and surface-attached P. aeruginosa. To quantify macrophage health, we used propidium iodide, a nucleic acid dye that cannot permeate healthy cells. Uninfected macrophages and macrophages that were mixed with planktonic P. aeruginosa were largely motile and displayed no propidium iodide fluorescence (Fig. 2E). In contrast, surface-attached P. aeruginosa were more virulent and caused the majority of macrophages to lyse (Fig. 2E).

**Quorum Sensing Is Necessary but Not Sufficient for Surface-Activated Virulence.** There are at least two explanations for the increased virulence of surface-attached bacteria relative to their planktonic counterparts. Bacteria could develop virulence once they encounter a surface, or surface adhesion could enrich for a subgroup of cells that are already highly virulent. By isolating P. aeruginosa from
different growth phases, allowing them to surface-attach for 1 h, and then exposing them to amoebae to assay their virulence, we found that virulence dramatically increases between mid-exponential (OD_{600} = 0.5) and late-exponential (OD_{600} = 0.9) growth phases (Fig. 3 A and B). The number of P. aeruginosa cells attached to the surface was lower for late-exponential cultures (Fig. 3B), indicating that virulence is not due to an increase in MOI. Although some surface-activated virulence was also observed after <1 h of attachment, the host killing was heterogeneous (SI Appendix, Fig. S4A). Because virulence was most activated at the late-exponential growth phase and host killing was homogeneous after 1 h of attachment, we performed the rest of our virulence assays using cells from this growth phase and after at least 1 h of surface attachment. We found that P. aeruginosa treated with fosfomycin, an antibiotic that inhibits growth and division but does not block protein synthesis, became virulent after a similar duration (Fig. 3C), confirming that virulence development is not a result of increased bacterial growth or division. In contrast, inhibitors of protein synthesis such as gentamycin and tetracycline blocked induction of virulence by surface attachment (Fig. 3C). Once the bacteria had become virulent, treatment with gentamycin no longer inhibited virulence (SI Appendix, Fig. S4B), indicating that new protein synthesis is required for the development of virulence, but not for the subsequent process of killing host cells.

The development of virulence in late-exponential phase, when cell density is high, suggested that quorum sensing could influence surface-activated virulence. To examine the role of quorum sensing, we assayed a ΔlasR mutant. This mutation significantly disrupted surface-activated virulence (Fig. 3 D and E), indicating that quorum sensing is necessary for surface-activated virulence. However, two lines of evidence suggest that quorum sensing is not sufficient to activate virulence in the absence of a surface. First, high-density (quorum-sensing-activated) planktonic P. aeruginosa cells did not lyse amoebae (Fig. 1 B–D). Second, hyperactivating quorum sensing by administering the LasR and RhlR autoinducers N-(3-oxododecanoyl)-3-oxo-homoserine lactone (3OC12-HSL) and N-butyryl-homoserine lactone (C4-HSL), respectively (19, 20) (SI Appendix, Fig. S5), did not activate virulence in planktonic bacteria (Fig. 3F). The finding that quorum sensing is necessary but not sufficient for surface-activated virulence is consistent with the hypothesis that surface activation of virulence also requires detection of a mechanical stimulus. Furthermore, these results explain why planktonic cells were not virulent when they were placed on surfaces with agar pads for 1 h (Fig. 1C), because the agar pads were made with fresh medium and thus diluted the quorum-sensing activator.

**Surface Detection Is Mediated by PilY1, a Putative Mechanosensor.** Which genes mediate the ability to sense surfaces and activate virulence? Although flagella are regulated by mechanical forces (14, 15), surface-activated virulence does not require flagella (SI Appendix, Fig. S6), suggesting that P. aeruginosa uses a previously uncharacterized mechanosensory system. Because many signaling pathways autoregulate gene expression upon activation, we performed gene-expression profiling and operon analysis of planktonic and surface-attached P. aeruginosa to identify candidate surface signaling pathways. Consistent with previous studies suggesting that P. aeruginosa virulence is multifactorial (1) and our finding that virulence induction requires new protein synthesis (Fig. 3C), attachment to a surface for 1 h led to the induction of multiple genes previously associated with virulence (SI Appendix, Table S1). Because candidate surface sensors should be present on the bacterial cell surface, our attention was drawn to one of the most highly activated operons in surface-attached P. aeruginosa (SI Appendix, Fig. S7 A and B), which encoded the cell-surface–associated protein PilY1 (21, 22) and the minor pilin proteins FimU, PilW, PilX, and PilE. The minor pilins and PilY1 have been primarily characterized as pilus biogenesis factors (22–24), and mutations in these genes cause defects in type IV pilus production (23, 25, 26). We found that ΔpilY1, ΔpilW, and ΔpilX mutants were defective for surface-activated virulence toward amoebae, whereas ΔfimU and ΔpilE partially retained virulence (Fig. 4A). Importantly, the lack of pili cannot explain the reduced virulence of ΔpilY1, ΔpilW, and ΔpilX in our assay because other mutants that lack type IV pili, such as ΔpilB and ΔpilC mutants, remain virulent (SI Appendix, Fig. S6). Minor pilins are incorporated into type IV pili by a process that requires PilD (27), but ΔpilD mutants also retained virulence (SI Appendix, Fig. S6). Furthermore, although pilus mutants are partially defective in surface attachment (6), these mutants retained virulence in our assays, indicating that virulence is independent of pilus-mediated surface attachment. Together, our results suggest that PilY1 and the minor pilins
regulate surface-activated virulence independently of pilus assembly and function. PilY1 has a domain that shares homology with the mechanosensitive VWFa domain (28) and could consequently serve as a sensor of the mechanical cues associated with surface contact. To characterize the mechanism of PilY1 activity, we performed a structure–function analysis of the domains required for PilY1 surface sensing. The N-terminal region of PilY1 encodes a signal sequence and a VWFa domain, whereas the C-terminal region encodes a PilC domain (28). Deletion of the signal sequence, the whole N-terminal region, or the PilC domain abrogated surface-induced virulence, mimicking the loss of the full-length PilY1 protein, whereas VWFa deletion mutants retained virulence on surfaces (Fig. 4B). We hypothesized that the deletion of the putatively mechanosensitive VWFa domain places PilY1 in a constitutively active state. As predicted, the VWFa domain deletion induced virulence even in planktonic \( \text{P. aeruginosa} \) cells (Fig. 4C). Given that PilY1 is present on the bacterial cell surface, up-regulated upon surface contact, necessary for surface-activated virulence, and contains a domain homologous to a known mechanosensor whose mutation hyperactivates virulence in the absence of surface contact, we propose that the VWFa domain of PilY1 is responsible for surface detection and that the specific loss of this domain constitutively activates the surface-detection response.

Our data implicate PilY1 and LasR as two master regulators of \( \text{P. aeruginosa} \) virulence. To determine the effectors that are regulated by each pathway and whether the pathways have overlapping targets, we examined the transcriptional profiles of WT, \( \Delta \text{pilY1} \), and \( \Delta \text{lasR} \). The profiles revealed that PilY1 and LasR regulate distinct targets (SI Appendix, Fig. S7C and Tables S2 and S3). For example, expression of the pilY1 operon was not significantly changed in the \( \Delta \text{lasR} \) mutant, and expression of the major quorum-sensing controlled genes was not significantly altered in the \( \Delta \text{pilY1} \) mutant (SI Appendix, Tables S2 and S3). Furthermore, comparing the transcriptional profiles revealed that surface-attached \( \Delta \text{pilY1} \) cells were more similar to planktonic WT cells (\( R = 0.77 \)) than to surface-attached WT cells (\( R = 0.69 \)) (Fig. 4D and SI Appendix, Fig. S7D). Genes up-regulated by surface attachment in WT are also significantly less induced in \( \Delta \text{pilY1} \) (SI Appendix, Table S1). Unlike \( \Delta \text{pilY1} \),
 surface-attached ΔlasR more closely resembled attached WT (R = 0.76) than planktonic WT (R = 0.39) cells (Fig. 4D and SI Appendix, Fig. S7D). Together, transcriptional profiling suggests that PilY1 is required for the bulk of the surface-induced virulence response and that P. aeruginosa still senses surface contact in the absence of LasR.

If PilY1 is a general sensor for surface contact, its loss should disrupt a wide range of surface-regulated behaviors. Indeed, PilY1 regulates multiple surface-associated behaviors such as swarmig, twitching, cyclic diguanosine monophosphate (c-di-GMP) signaling, and biofilm formation (28–30). These surface-associated behaviors could be mediated by the PilY1-dependent transcriptional response to surface attachment identified in this work (SI Appendix, Table S1).

Multiple Redundant Virulence Factors Likely Function Downstream of Surface Detection. To understand which genes are activated downstream of surface detection, we investigated the role of previously implicated virulence factors and regulators in surface-activated virulence toward D. discoideum using P. aeruginosa mutants defective in type III secretion, type VI secretion, flagella, type IV pili, fimbiae, exopolysaccharide, quorum sensing, c-di-GMP signaling, two-component signaling, chemotaxis, sigma factor control, or secreted effectors (SI Appendix, Fig. S8). With the exception of mutants of the Las quorum-sensing system characterized above (Fig. 3D and E), none of the 42 mutants assayed significantly disrupted surface-activated virulence. The lack of a pronounced virulence defect in any one virulence factor mutant supports the hypothesis that P. aeruginosa induces multiple virulence factors that function in a redundant manner (1–2).

Although type III secretion was not required for virulence in our assay, which was performed over a short timescale (hours) at moderate MOI, type III secretion was previously shown to be important for P. aeruginosa virulence toward D. discoideum when assayed over the course of days at high MOI (16). We suggest that these results indicate that type III secretion contributes to later stages of pathogenesis but is not necessary for the early stages of host cell killing that follow host cell contact.

C-di-GMP is an established regulator of surface-associated behaviors and biofilm formation (31). We were thus surprised that rocA and sadC, which encode two of the diguanylate cyclases that synthesize c-di-GMP and are responsible for biofilm formation (32), were dispensable for surface-activated virulence (SI Appendix, Fig. S9). Surface-activated virulence also remained intact in wspA and wspR mutants (SI Appendix, Fig. S8), which have also been implicated in surface-activated c-di-GMP signaling (7, 8). The independence of surface-activated virulence from biofilm formation was further supported by the lack of a virulence defect in the algR and pelA exopolysaccharide mutants (SI Appendix, Fig. S8). Our results thus suggest that c-di-GMP signaling and biofilm formation are not necessary for surface-activated P. aeruginosa to kill host cells. Biofilm formation may contribute to host killing in a manner that is redundant with other virulence factors and facilitate pathogenesis by promoting other pathogenesis-associated behaviors such as persistence within a host.

Discussion

Bacteria Regulate Their Behaviors in Response to Their Mechanical Environment. What types of environmental signals do cells detect? Historically, studies of cell behavior have focused on chemical signals, such as nutrients and signaling molecules. Our study suggests that bacteria also detect mechanical signals associated with growth in different environments and use mechanical sensing of surface contact to induce virulence. In mammalian cells, the mechanical detection of substrates is an established driver of development and behavior (11). Because regulatory systems in bacteria generally have reduced complexity compared to their eukaryotic counterparts, the identification and characterization of a bacterial virulence “touch sensor” may represent a simplified model by which cells sense mechanical forces and transduce them into biochemical signals.

The importance of mechanosensing to the biology of P. aeruginosa is underscored by the fact that it regulates virulence, one of the most complex and highly regulated of all bacterial behaviors. Although many of the P. aeruginosa virulence factors have been identified, the signaling cues that regulate the expression of virulence factors remain less clear, in part because virulence regulation is performed by complex networks involving hundreds of components (1, 2). Surface detection appears to be a master regulator in the virulence-regulation hierarchy for P. aeruginosa, because surface-attached cells activate virulence toward both unicellular eukaryotic and mammalian hosts.

How does P. aeruginosa detect mechanical cues associated with surface attachment? The transition from planktonic growth to surface attachment involves a significant change in the mechanical properties of the growth environment in our experiments, because surface-attached cells are subjected to large shear forces generated by the movement of fluid across the attachment surface. We propose a model in which membrane-associated PilY1 (21, 22, 28) mechanically detects surfaces by mediating contact between the outer membrane and the surface substrate. Shear forces exerted on surface-attached cells shift PilY1 into an active stretched state. Supporting its role as a surface-attachment mechanosensor, PilY1 is a surface adhesin (21) and contains a putatively mechanosensitive VWFa domain that is stretched by shear force (33, 34). Because PilY1 is found on the cell surface, a periplasmic protein and/or an inner-membrane-associated protein are likely required to transduce the surface-attachment signal into the cell. In the future, it will be important to determine whether this transduction is achieved through known mechanisms, such as two-component signaling or CAMP induction, or through a novel, as-yet-uncharacterized mechanism. In addition, the existence of other surface-responsive systems, including flagella (14, 15) and the chemosensory-type Wsp system (7, 8), which do not appear to activate virulence, suggests that multiple signaling pathways distinguish different aspects of the mechanical environment.

The regulation of virulence by mechanical cues has not been explored in other bacteria. However, PilY1 is conserved across multiple gamma- and beta-proteobacteria, including several broad-host–spectrum pathogens such as Burkholderia and Acinetobacter species (SI Appendix, Fig. S10). We thus suggest that PilY1 could be part of a general mechanism by which pathogens use mechanosensation to regulate virulence in a host-nonspecific manner.

Requiring both Mechanosensation and Quorum Sensation Enables Host-Independent Virulence Regulation. Because surface attachment is one of the initial steps of host interaction, activation of

Fig. 5. Surface detection is a host-nonspecific signal that activates virulence. (A) Schematic of proposed model in which bacterial cells detect host surfaces through mechanical cues during initial attachment. The induction of virulence through this detection mechanism establishes an environment that is suitable for bacterial colonization of the host. (B) An ‘AND’ gate model for surface-activated virulence in which both surface sensing (mediated by PilY1) and quorum sensing (mediated by LasR) are required for activating virulence.
virulence at this early stage suggests that *P. aeruginosa* initially weakens the host to establish an environment that is suitable for long-term growth (Fig. 5A). In support of this model, we found that biofilm formation genes are dispensable for virulence activation, suggesting that long-term colonization acts downstream of surface-activated virulence. In a mammalian host setting, early phase virulence may be useful for killing host cells that provide the first line of defense, such as neutrophils and macrophages. Once colonization has been firmly established, virulence activation may no longer be necessary for maintaining the community. Indeed, clinical isolates from long-term infections of human lungs lose many of their virulence factors (35).

Our findings indicate that activating *P. aeruginosa* virulence requires two conditions to be met: Bacterial cell density must be high, and a rigid surface must be detected. In a simplified model, the quorum- and surface-sensing pathways can be described as a coincidence detector or an “AND” logic gate whose output results in the activation of virulence genes (Fig. 5B). *P. aeruginosa* activates virulence in response to attachment to surfaces that have a broad range of properties, including porosity, stiffness, and chemical composition (Figs. 1 B and C and 2 A–D). Together, surface sensing and quorum sensing thus form a signaling network that is host-independent. Because surface activation of virulence is a developmental process that requires the induction of a large proportion of the genome (*SI Appendix*, Table S1), the transition to a virulent state is energetically expensive. The requirement that two distinct conditions be met ensures that cells commit to activating virulence only when they can effectively kill host cells.

Surface sensing and quorum sensing are both host-independent signaling pathways that work together to tightly regulate expression of virulence factors while maintaining the ability to target a wide diversity of host cell types. Coupled with host-specific responses, these pathways may facilitate the ubiquitous and far-reaching pathogenesis of *P. aeruginosa*. Given the redundancy of *P. aeruginosa*’s multiple virulence factors, targeting PIY1-mediated mechanosensation as a global virulence regulator represents an attractive therapeutic strategy for future exploration.

**Materials and Methods**

*P. aeruginosa* PA14 cells were grown in LB or PS:DB medium at 37 °C. *D. discoideum* (amoeba) AX3 cells were grown axenically in PS medium. Planktonic or surface-attached *P. aeruginosa* cells were mixed with amoebae, imaged using fluorescence microscopy, and analyzed using our own software to compute host killing indexes. Mouse macrophage J774A.1 cells were grown at 37 °C with 5% CO2 in DMEM. Details of media, strain construction, virulence assays, cell-density quantification, microscopy, image analysis, quorum-sensing induction, biofilm density quantification, and microarray analysis are described in *SI Appendix, SI Materials and Methods*.

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Figure S1. Schematic depicting software-based automated analysis of the rapid host killing assay. Phase contrast and calcein-AM fluorescence images are acquired of amoebae mixed with *P. aeruginosa* cells that are immobilized to a single imaging plane by an agar pad. Applying an edge detection algorithm to phase contrast images identifies the boundaries of host cells, which are used to construct cell boundary masks. The masks are combined with calcein-AM images in order to isolate the fluorescence of individual host cells. The average calcein-AM intensity ($I_n$) for each host cell is computed by integrating the individual calcein-AM fluorescence pixel intensities ($I_i$) over the entire cell and dividing by the cell size. The host cell killing index is computed by averaging $I_n$ over many cells.
Figure S2

Using fluorescent markers to assess amoeba cell death. (A) Phase contrast and calcein-AM fluorescence images of untreated amoebae and amoebae treated with 1% H$_2$O$_2$. Host cells (red) are identified in phase contrast images using an edge detection algorithm. (B) Phase contrast or bright-field images and fluorescence images of amoebae that have been mixed with surface-attached *P. aeruginosa* cells and with Syto 9, which stains nucleic acids and does not significantly permeate healthy amoeba membranes, or carboxy-H$_2$DF-FDA or DC-FDA, which indicate the production of reactive oxygen species. Host cells (red) are identified in phase contrast or bright-field images using an edge detection algorithm. Scale bars represent 50 μm.
Figure S3

Figure S3. Correlation between surface cell density and cell number. (A) Phase contrast images at the resolution used for both host killing assays and measuring *P. aeruginosa* cell densities and at the resolution for counting individual *P. aeruginosa* cells. The scale bars for the cell density and cell counting images represent 50 μm and 10 μm, respectively. (B) A graph indicating the relationship between *P. aeruginosa* cell density and the number of cells in the same imaging field. The least squares fit to a line and coefficient of determination ($R^2$) are shown. Cell density and cell counting measurements are described in the Materials and Methods section.
Figure S4. Effects of attachment time and protein synthesis inhibitors on surface-induced virulence. Phase contrast, calcein-AM fluorescence, and merged images of *P. aeruginosa* that were surface-attached for 45 minutes. Only a fraction of the amoebae are calcein-AM fluorescent (green in merged image), indicating that virulence induction is heterogeneous during this attachment period. (B) Phase contrast and calcein-AM fluorescence images and host killing indexes for *P. aeruginosa* cells that were surface-attached for more than 1 hour, treated with gentamycin, and mixed with amoebae, indicating that virulence is not inhibited by protein synthesis after it has been induced. Scale bars represent 50 µm. Bars represent the average of three independent experiments and error bars indicate standard deviation.
Figure S5. Hyper-activation of quorum sensing in planktonic cells. Fluorescence intensities of *P. aeruginosa* cells that contain a transcriptional fusion of the rhlA promoter to the gene encoding yellow fluorescent protein (YFP). The expression of YFP was measured in planktonic *P. aeruginosa* cells from mid-exponential or stationary phase cultures supplemented with either DMSO or 5 μM 3OC12-HSL and 10 μM C4-HSL. Bars represent the average of three independent experiments and error bars indicate standard deviation.
Figure S6. Virulence of surface-attached flagella and pilus mutants toward amoebae. Phase contrast, host-cell mask overlays (red), and calcein-AM fluorescence images of surface-attached wildtype, ΔflgK, ΔpilB, ΔpilC, or ΔpilD P. aeruginosa cells that have been mixed with amoebae. The scale bar represents 50 μm.
Figure S7

**A** Operon Analysis

**B**
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**Figure S7. PilY1 mediates a transcriptional surface-attachment response.** (A) Transcriptional profiling and operon analysis for the identification of operons activated by surface attachment. Microarray indicates expression of genes that are decreased (blue) or increased (yellow) by surface attachment. Operon analysis was performed to identify gene expression changes in operons, as described in the Materials and Methods. (B) The scores of the nine highest gene cluster expression changes are listed. (C) Diagram indicating the number of genes in surface-attached cells regulated at least 3-fold by PilY1, LasR, or both. The genes regulated by PilY1 or LasR are shown in Tables S2 and S3, respectively. (D) Hierarchical clustering, correlation plots, and Pearson correlation coefficients for transcriptional profiles from microarrays for surface-attached (SA) or planktonic (P) sub-populations of wildtype, ΔlasR, and ΔpilY1 cells for genes that are activated by at least 4-fold by surface attachment in overnight cultures. Axes of the correlation plots indicate fold upregulation compared against a common mixed reference. The 68% confidence interval ranges for each correlation coefficient are given in parentheses.
Figure S8. Targeted screen for loss of surface-activated virulence. Phase contrast images of amoebae that have been mixed with surface-associated wild-type or mutant *P. aeruginosa* strains. Cells round up when mixed with all mutants except for the LasR and LasI mutants. The scale bar for wildtype represents 50 μm and the magnification for images of all the mutants matches that of wildtype.
**Figure S9.** Surface virulence and biofilm formation in c-di-GMP mutants. (A) Phase contrast, host cell overlay (red), and calcein-AM images and (B) host killing indexes of surface-attached \( \Delta \text{roeA}, \Delta \text{sadC}, \) or \( \Delta \text{roeA} \Delta \text{sadC} \) double deletion \( P. \text{aeruginosa} \) mutants. (C) The biofilm density of \( \Delta \text{roeA} \) and \( \Delta \text{roeA} \Delta \text{sadC} \) double deletion mutants, as measured by optical density at 550 nm of crystal violet stained surface-attached cells. Bars represent the average of three independent experiments and error bars indicate standard deviation.
Figure S10. The occurrence of PilY1 among bacteria, eukaryotes and archaea. The sequence similarity to *P. aeruginosa* PilY1 is indicated by a continuous scale indicating no homology (white), intermediate sequence conservation (red) and strong sequence conservation (black). The table was produced using the STRING database (24) version 9.1.
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Table S1. Genes that are induced by surface attachment in wildtype and ΔpilY1 cells determined by microarray analysis. The ratio of gene expression in surface attached cells to that of planktonic cells from the same culture for 1 hour of surface attachment beginning at late-exponential phase is shown for all predicted genes where the ratio is at least 1.5. Gene expression ratios are the average of three independent experiments. The average up-regulation and corresponding standard error for each cell type are given. The Pearson correlation coefficient between wildtype and ΔpilY1 cells and the range of correlation coefficients for a 68% confidence interval are shown in parentheses.
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Table S2. Genes that are regulated in surface-attached ΔphnY cells. The ratio of gene expression in ΔphnY cells to that of wildtype cells is shown for all predicted genes that are regulated at least 3-fold in surface-attached cells from cultures that were grown overnight. The inverse of the ratio is shown for down-regulated genes and is indicated by a minus sign. Gene expression ratios are the average of three independent experiments.
Table S3. Genes that are regulated in surface-attached ΔlasR cells. The ratio of gene expression in ΔlasR cells to that of wildtype is shown for all predicted genes that are regulated at least 3-fold in surface-attached cells from cultures that were grown overnight. The inverse of the ratio is shown for down-regulated genes and is indicated by a minus sign. Gene expression ratios are the average of three independent experiments.

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Table S3: Genes that are regulated in surface-attached ΔlasR cells. The ratio of gene expression in ΔlasR cells to that of wildtype is shown for all predicted genes that are regulated at least 3-fold in surface-attached cells from cultures that were grown overnight. The inverse of the ratio is shown for down-regulated genes and is indicated by a minus sign. Gene expression ratios are the average of three independent experiments.
Table S4

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<td><strong>E. coli strain</strong></td>
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<td>B/r</td>
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<td><strong>P. aeruginosa strains</strong></td>
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<td>Plasmid expressing Flp to recombine FRT sites</td>
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<td>pMQ80</td>
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Table S4. Strains and plasmids used in this study. Details of strain and plasmid construction are described in the Supplemental Materials and Methods section.
SUPPLEMENTAL MATERIALS AND METHODS

Media

PS:DB medium consists of Development Buffer (DB) (5 mM KH₂PO₄, 5 mM Na₂HPO₄, 2 mM MgCl₂, 1 mM CaCl₂, pH 6.5) containing 10% (v/v) PS medium (10 g/L Special Peptone LP0072 (Oxoid, Hampshire, United Kingdom), 7 g/L Yeast Extract LP0021 (Oxoid, Hampshire, United Kingdom), 10 mM KH₂PO₄, 0.45 mM Na₂HPO₄, 1.5% (w/v) D-glucose, 30 nM Vitamin B12, 180 nM Folic acid, pH 6.5). GYP plates consist of 1 g/L D-glucose, 2 g/L Bacto Peptone, 0.25 g/L Yeast Extract, 31 mM KH₂PO₄, 19 mM Na₂HPO₄, 2.5% agar. PS:DPBS medium is a 1:9 mixture of PS and DPBS (Gibco, Grand Island, NY) supplemented with 1 mM MgCl₂ and 1 mM CaCl₂.

To test for dependence of surface-activated virulence on protein synthesis and cell division, gentamycin at 100 μg/mL, tetracycline at 200 μg/mL, or fosfomycin at 100 μg/mL were added 5.5 hours following dilution of cultures into petri dishes and allowed 6 hours of additional incubation with drugs with shaking at 37°C (Fig. 3C). To test the effect of gentamycin on the host cell killing process (Fig. S4 in Supplementary Information), gentamycin was added at 17 hours following dilution and given 3 hours of additional incubation with shaking at 37°C.

For experiments in Supplementary Information Fig. S2B, calcein-AM was replaced in agar pads by 5 μM Syto 9 (Invitrogen), 0.1 mM carboxy-difluorodihydrofluorescein diacetate (carboxy-H₂DF-FDA) (Invitrogen) or by direct addition of 1 mM dichlorofluorescein diacetate (DC-FDA) (Sigma-Aldrich, St. Louis, MO) to the amoebae culture.

Strain construction

A strain that constitutively expresses GFP was constructed by amplifying the hybrid sequence corresponding to the -51 to 0 region of P_A1/04 (20) and the +1 to +28 region of P_A1/03 (20) (herein referred to as the P_A1/04/03 promoter) from a PA14-derived strain (gift of Kolter lab). The promoter sequence was joined with the sequence encoding mCherry using overlap extension, cloned into the KpnI and HindIII sites in the plasmid pUC18-mini-Tn7T-Gm (18), and integrated into the chromosome of PA14 using pTNS2 (18). The sequence encoding mCherry was replaced with that of GFP by flipping out the drug resistance marker of the chromosomal integration with pFLP2 and electroporating a PCR product containing the sequence that encodes GFP and a drug resistance marker using the λ Red recombination plasmid pUCP18-RedS (19), producing AFS64.

The ΔlasR::aacC7 allele was moved into the ΔpilY1 strain using λ Red recombination as described previously (19). To construct a strain that expresses a transcriptional fusion of the rhlA promoter to the gene encoding yellow fluorescent protein (YFP), the region encoding P_rhlA⁻yfp was cloned into the mini-CTX2 plasmid using overlap extension and integrated into the chromosome of a strain that constitutively expresses mCherry under the control of the tetA promoter, yielding UTD5.2.

The regions encoding wildtype PilY1-His, or the ΔSS, ΔN-term, ΔPiiC, and ΔVWFa mutant versions of PilY1-His were amplified from pPilY1-His, pSMC263, pSMC264, pSMC265, pSMC266, respectively, using the upper primer 5'-CATAAAGTTGGGAGCCAGCGCATGATTCCACCGATTCCCG-3' (or 5'-CATAAAGTTGGGAGCCAGCGCATGATTCCACCGATTCCCG-3' to amplify pSMC263) and lower primer 5'-CATAACCGGGAAGAGCTGTGGCGAGAAGAC-3', cloned into the SpeI and Xmal sites in the mini-Tn7 delivery vector pUC18-miniTn7-Lac, and integrated into the ΔpilY1 strain by co-transforming with pTNS2, resulting in AFS72-1, AFS72-2, AFS72-4, AFS72-5, and AFS72-6, respectively. Sequencing revealed that the plasmid containing the ΔVWFa deletion
also contains a point mutation in the PilC domain at G677D. The plasmid pUC18-miniTn7-Lac was co-transformed with pTNS2 into the ΔpilY1 strain, yielding the empty vector strain AFS56.

Phase contrast and fluorescence microscopy

Imaging was performed using a Nikon Ti-E microscope (Nikon, Melville, NY), a 10X Plan Fluor Ph1 Nikon objective (0.3 NA), a 20X Super Plan Fluor Ph1 Nikon objective (0.45 NA) or 100X Plan Apo VC Nikon objective (1.4 NA), a Prior Lumen 200 Pro, and an Andor Clara camera, an Andor iXon DU-897 EMCCD, a Hamamatsu Orca-R2 (Hamamatsu, Bridgewater, NJ) camera, or a QImaging Rolera (QImaging, Surrey, Canada BC) camera. DAPI, Calcein-AM, or propidium iodide/mCherry fluorescence were imaged using the 89014 filter set (Chroma, Bellows Falls, VT). The ET402/15x excitation and the ET455/50m emission filters were used to image DAPI fluorescence. The ET490/20x excitation and the ET535/50m emission filters were used to image Calcein-AM fluorescence. The ET572/35x excitation and the ET632/60m emission filters were used to image propidium iodide/mCherry fluorescence. CFP or YFP fluorescence was imaged using the 89002 filter set (Chroma) and ET430/24x excitation and the ET500/20x excitation and ET535/30m emission filter, respectively.

Amoeba cell viability assay

Dictyostelium discoideum AX3 cells were inoculated from frozen stocks into an overnight LB culture of E. coli B/r. The mixture was plated on GYP plates and incubated for 4-6 days at 22°C until D. discoideum spores formed. Individual spores were picked, inoculated into PS medium supplemented with Antibiotic-Antimycotic solution (GIBCO, Grand Island, NY) that was diluted to 1/4 of the working concentration, and grown axenically at 22°C to an optical density measured at 600 nm (OD_{600}) of 0.2 to 0.5, at which time D. discoideum cells were mixed with P. aeruginosa for host killing assays.

Agar pads were prepared by pouring molten 1% (w/v) Bacto Agar (BD Bioscience, San Jose, CA) in DB buffer and containing 1 μM Calcein-AM (Invitrogen, Grand Island, NY) on top of a glass surface and cutting the pad into individual 1.5 cm x 1.5 cm sections. P. aeruginosa cultures were picked from individual colonies on agar plates and grown overnight from at 37°C to saturation, diluted 1:100 or 1:1000 into PS:DB, and shaken in petri dishes on a benchtop rotator at 100 rpm at 37°C. Surface-attached cells were isolated by removing all the liquid medium from petri dishes and rinsing with DB buffer. Planktonic cells were isolated by transferring 10 μL of culture from petri dishes to a clean petri dish. Pre-conditioned media was isolated by centrifuging cultures at 13 k x g and filtering the supernatant with a 0.2 μm filter. 10 μL of amoebae were added to surface-attached or planktonic cells, confined to the same plane as P. aeruginosa by placing an agar pad on top, and imaged using fluorescence microscopy (details in Supplementary Information). Attachment to chemically distinct surfaces was performed by first pouring molten agar or polyacrylamide into petri dishes and soaking overnight in water, and inoculating cultures as described above. Attachment to glass or plant leaf surfaces was performed by adding a glass coverslip or live Epipremnum aureum plant leaf mounted on a glass coverslip (details in Supplementary Information), respectively, to cultures that were shaken in petri dishes.

Images were analyzed using software written in Matlab (Mathworks, Natick, MA) that was modified from (21). An edge detection algorithm was applied to phase contrast images to construct masks of amoebae and isolate the fluorescence of individual cells (Fig. S1). The average calcein-AM intensity for each amoeba was computed by integrating the calcein-AM fluorescence intensities over the entire cell and dividing by the cell size. The host cell killing
index was computed as the average of the calcein-AM intensities of at least 100 amoebae in experiments that were performed in triplicate unless otherwise noted.

**Macrophage cell death assay**

Cells from the mouse macrophage line J774A.1 (TIB-67 (authenticated by Cytochrome Oxidase I testing and verified to be mycoplasma free), ATCC, Manassas, VA) were grown at 37°C with 5% CO₂ in DMEM (with 4.5 g/L D-Glucose, and L-Glutamine) (Gibco) with 10% fetal bovine serum and Penicillin-Streptomycin solution (Invitrogen, Grand Island, NY). Cells were passed by scraping cells from the surface when confluence reached 70-90%.

*P. aeruginosa* cultures were grown in PS:DPBS (recipe in Supplementary Information) overnight on a benchtop rotator at 100 rpm at 37°C in polystyrene multi-well culture plates in which the bottom was lined with 1% agar made with PS:DPBS medium. Planktonic cells were taken from liquid portion of the culture. Surface-attached cells were isolated by removing the liquid portion, washing with DPBS, and cutting a slice of the agar pad from the bottom of the well. Propidium iodide was added at 1 μg/mL (accounting for the volume of the agar pad) and the pad was inverted and placed on top of macrophages that had been cultured in separate culture plates. Time-lapse imaging was performed at room temperature and approximately 250 cells were analyzed for experiments that were repeated in duplicate.

**Surface cell density, cell number, MOI**

The density of *P. aeruginosa* cells on surfaces was measured from amoebae cell viability assay images that were acquired using a 10X or 20X objective. The IJ_Isodata algorithm (ImageJ 1.44o) was applied to phase contrast images to construct cell boundary masks. The cell density was computed by dividing the area above the threshold in the mask by the total area. To determine the relationship between the cell density and the number of cells, planktonic *P. aeruginosa* cells were prepared as described in the amoebae viability assay except that no amoebae cells were added. Phase contrast and GFP fluorescence images of *P. aeruginosa* cells (strain AFS64) of the same imaging field were acquired using both 20X and 100X objectives. Cell density was measured as described above from phase contrast images acquired with the 20X objective. The number of cells was measured from phase contrast and GFP fluorescence images acquired using the 100X objective. Fluorescence was quantified using our own software written in Matlab modified from (21). The number of cells was computed by dividing the total cell fluorescence by the fluorescence of a single cell.

The multiplicity of infection (MOI) for planktonic cells for experiments was increased by concentrating cells from liquid cultures by centrifugation at 8 k x g. Cells in all virulence assays were kept at room temperature and imaged using phase contrast and fluorescence techniques typically 1 to 2 hours after the agar pad was added.

**Plant surface attachment virulence assay**

Saturated *P. aeruginosa* PS:DB cultures were diluted 1:1000 into petri dishes and grown to an OD₆₀₀ of 0.3. Live *Epipremnum aureum* plant leaves were cut in 1.5 x 1.5 cm squares, mounted onto slides using molten valap (vaseline/lanolin/paraffin mixture), and added to the bottom of petri dishes containing *P. aeruginosa* cultures. Cultures were shaken at 100 rpm at 37°C until they reached an OD₆₀₀ of 0.9, at which time the plant sections were removed and rinsed with DB to remove unattached cells. Axenically growing amoebae in PS medium were supplemented with 0.04 μm orange fluorescent (540/560) FluoSpheres (Invitrogen/Life Technologies) and shaken at 22°C for 1 hour before use. The virulence of plant surface attached *P. aeruginosa* was assayed by adding amoebae to the surface of the plant leaf, and immobilizing cells using a 1% agar pad containing 5 mM Calcein-AM Violet (Invitrogen/Life
The virulence of planktonic *P. aeruginosa* cells was assayed on leaves by mixing amoebae with planktonic cells, adding the mixture to leaves that were mounted on slides but not placed in cultures, and immobilizing cells on the leaf surface with the agar pad. Amoebae cell bodies were imaged using a YFP filter and Calcein-AM Violet fluorescence was imaged using a CFP filter (Fig. 2C). Amoeba host killing indexes were computed as described above for experiments repeated in biological triplicate. Amoebae and plant surface-attached *P. aeruginosa* were imaged together (Fig. 2B) by adding a mixture of amoebae that had been grown with orange FluoSpheres and *P. aeruginosa* that had been labeled with AlexaFluor 488 carboxylic acid, succinimidyl ester (Invitrogen/Life Technologies) to a mounted plant leaf. The sample was imaged using DAPI (for plant surface autofluorescence), mCherry (for amoebae containing beads), and GFP (for *P. aeruginosa*) filters. Images from different z-planes were assembled by identifying the regions in focus in each plane using the Canny detection algorithm and combining in-focus regions from different z-planes into a single image using our own software written in Matlab.

**Hyper-stimulation of quorum sensing**

*P. aeruginosa* cells were inoculated into LB, grown overnight to saturation, diluted 1:1000 into PS:DB supplemented with DMSO or 5 μM 3OC12-HSL (22) and 10 μM C4-HSL (23), and either grown to mid-exponential phase and harvested or grown in petri dishes for 16 hours, at which time planktonic and surface-attached cells were isolated for fluorescence measurements and assayed for virulence towards amoebae. Cells containing a *P. aeruginosa* transcriptional reporter (UTD5.2) were imaged using single-cell fluorescence techniques by immobilizing cells on agarose pads as described previously (21).

**Biofilm density quantification**

The density of biofilms in polystyrene petri dishes was measured by staining dishes with crystal violet, washing with water and transferring the stain to a cuvette using 95% ethanol. The absorbance at 550 nm was measured using a spectrophotometer.

**RNA isolation and microarray analysis**

*P. aeruginosa* cells were inoculated into LB, grown overnight to saturation and diluted 1:1000 into PS:DB medium. Cultures were grown overnight in petri dishes shaken at 100 rpm and harvested for RNA. Planktonic cells were isolated from the liquid medium, pelleted and snap frozen over liquid nitrogen. Surface-attached cells were isolated by washing petri dishes with DB to remove unattached cells, treating with DB supplemented with approximately 100 μg/mL of alginate lyase (Sigma-Aldrich, St. Louis, MO), and removing cells from the surface using a cell scraper (Fisher Scientific, Pittsburgh, PA). Cells were pelleted and snap frozen over liquid nitrogen. To create a mixed reference, a portion of all harvested cells were mixed together. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA), treated with RNase-Free DNaseI (Ambion/Life Technologies, Grand Island, NY), and re-purified using the RNeasy Mini Kit (Qiagen). To characterize the transcriptional response to 1 hour of surface attachment, PS:DB cultures were grown to an OD<sub>600</sub> of 0.9, transferred to a petri dish containing a glass surface, and shaken at 100 rpm for 1 hour. Planktonic and surface-attached populations were harvested for RNA instead using a lysozyme solution with 1% SDS and hot phenol extraction. For all microarray experiments, complementary DNA (cDNA) libraries containing Cy3- or Cy5- labeled dUTP (Enzo Life Sciences) were synthesized from the purified RNA using SuperScript III Reverse Transcriptase (Invitrogen/Life Technologies, Grand Island, NY). RNA was degraded by adding sodium hydroxide and the reaction was subsequently neutralized by addition of hydrochloric acid. The library was purified using the PCR Purification Kit (Qiagen).
and measured for Cy3 and Cy5 incorporation using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Experimental libraries were normalized for cDNA concentration, combined with mixed reference libraries, and hybridized to customized Agilent microarrays (designs 28678 or 43307, Agilent Technologies, Santa Clara, CA) using the Agilent Gene Expression Hybridization Kit. Microarrays were designed using the Agilent eArray tool (https://earray.chem.agilent.com/earray/) and contain on average 2 probes per gene. Samples were hybridized for 17 hours at 65°C with continuous rotation at 10 rpm. Microarrays were scanned using an Agilent G2505B scanner and analyzed using Agilent Feature Extract software versions 9.5 and 11. Resulting microarray intensity data were submitted to the PUMA Database (https://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=561) for archiving.

Operon analysis was performed by sorting microarray data by gene locus and applying the metric \( c \sum_{j=-2}^{1} l_j \) at each gene locus, where \( l = \log_{10} \left( \frac{R_{SA}}{R_{Planktonic}} \right) \), \( c \) is a fixed scaling factor, \( j \) denotes the relative position from each locus, and \( R \) is the \( \log_{10} \) of the ratio of the expression of the experimental group (surface-attached or planktonic) to that of the mixed reference. The resulting scores were averaged and sorted, producing a list of genes ranked by gene cluster score (Fig. S7B). The linear correlation between expression profiles (Fig. S7D) was determined by computing the Pearson correlation coefficient for genes that are activated by surface attachment. A threshold of 4-fold activation was imposed in order to reduce the contribution of noise on the correlation coefficient while allowing for sufficient gene sampling (N > 250). A 68% confidence interval for each correlation coefficient was computed using Fisher’s r-to-z transformation, for which the standard error was computed using \( \sigma_z = \frac{1}{\sqrt{N - 3}} \), where N is the number of genes used for the correlation. A hierarchical clustering tree was constructed in Matlab using the shortest distance linkage method and the Pearson correlation metric.
SUPPLEMENTAL REFERENCES


12. Liberati NT, et al. (2006) An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proceedings of the National Academy of Sciences of the United States of America 103(8):2833-2838.


Movie S1. Composite phase contrast (grayscale) and calcein-AM fluorescence (green) time-lapse video of amoebae mixed with planktonic or surface-associated *P. aeruginosa*. The indicator at the lower right gives elapsed time, in minutes, following mixing of amoebae and *P. aeruginosa*.

Movie S1

Other Supporting Information Files

SI Appendix (PDF)