CELL BIOLOGY

Obstruction of pilus retraction stimulates bacterial surface sensing

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It is critical for bacteria to recognize surface contact and initiate physiological changes required for surface-associated lifestyles. Ubiquitous microbial appendages called pili are involved in sensing surfaces and facilitating downstream behaviors, but the mechanism by which pili mediate surface sensing has been unclear. We visualized *Caulobacter crescentus* pili undergoing dynamic cycles of extension and retraction. Within seconds of surface contact, these cycles ceased, which coincided with synthesis of the adhesive holdfast required for attachment. Physically blocking pili imposed resistance to pilus retraction, which was sufficient to stimulate holdfast synthesis without surface contact. Thus, to sense surfaces, bacteria use the resistance on retracting, surface-bound pili that occurs upon surface contact.

The role of pili in surface sensing by bacteria has been difficult to assess, in part because of the difficulty in visualizing their dynamic activity and the time required for surface contact–induced responses. We used the rapid surface contact–stimulated synthesis of the adhesive holdfast of *Caulobacter crescentus* to study surface sensing. Newborn, nonreproductive swarmer cells harbor multiple tight adherence (Tad) pili and a flagellum at the same pole (figs. S1 and S2). When swarmer cells encounter a surface, holdfast synthesis at the flagellar pole is stimulated within seconds in a Tad pili–dependent process and is concurrent with an arrest of flagellum rotation (*1*, *2*). To determine whether the flagellar motor is required for surface stimulation of holdfast synthesis, we tracked the time of holdfast synthesis in single cells after surface contact (fig. S3A). A mutant lacking the MotB flagellar stator showed wild-type levels of surface stimulation of holdfast synthesis, whereas a mutant lacking the pilus filament subunit PilA did not respond to surface contact (Fig. 1).

For systems in which pili are required for surface-stimulated phenotypes, it has been proposed that the tension exerted on retracting pili when they attach to a surface is used for surface sensing (*1*, *3*–*5*). Because Tad pili were not known to retract, and they lack a homolog of known retraction adenosine triphosphatases (ATPases), we labeled them fluorescently to study their behavior. Inspired by a technique for labeling flagellar filaments (*6*), we replaced a native residue within the major pilin subunit with a cysteine for subsequent labeling with thiol-reactive maleimide dyes (AF488- or AF594-mal) (figs. S3B and S4, C and E). The *C. crescentus* PilAT36C strain showed wild-type levels of attachment to surfaces and sensitivity to the pilus-dependent bacteriophage ΦCbK (fig. S5, A and B). Cryo–electron tomography (cryo-ET) showed that neither the introduction of the cysteine residue nor the addition of maleimide dye affected the structure of the pilus fiber or the cell envelope (Fig. 2B and movie S1). Fluorescently labeled Tad pili were highly dynamic and capable of both extension and retraction (Fig. 2A and movie S2).

Cells synthesized an average of two pili per minute at an average length of 1.08 μm (fig. S6, A and B). To measure the strength of pilus retraction, we used an elastic micropillars assay (*7*). In this assay, pili from the same cell bind adjacent micropillars, and their subsequent retraction causes micropillar bending, which allows calculation of a retraction rate and force (fig. S7A and movies S3 to S7). *C. crescentus* pilus retraction generated an average force of 14 pN, and labeling did not affect retraction force (Fig. 2C). Extension and retraction rates of labeled pili under agar pads averaged 0.14 and 0.16 μm/s, respectively, and the latter were consistent with retraction rates measured by the micropillars assay (fig. S7, B and C). Thus, Tad pili retract, and

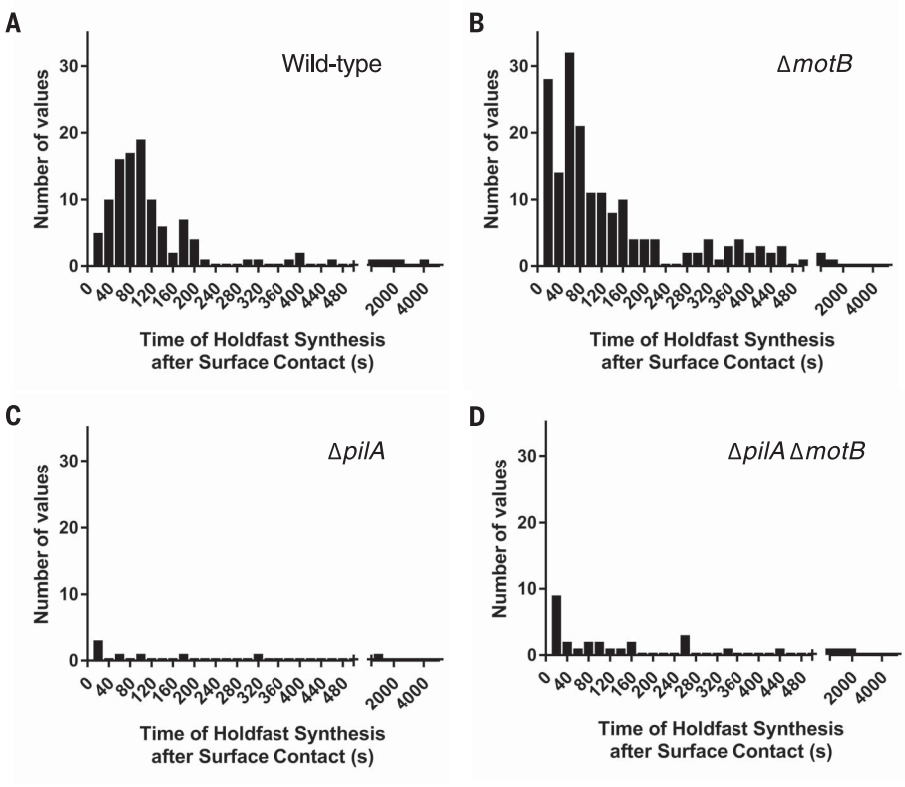

Fig. 1. In *C. crescentus*, Tad pili are required for surface stimulation of holdfast synthesis. (**A** to **D**) Histogram plots showing the time of holdfast synthesis in single cells after surface contact, for two independent replicates of wild type (n = 115) (A), Δ*motB* (n = 182) (B), Δ*pilA* (n = 8) (C), and Δ*pilA* Δ*motB* (n = 33) (D). The total number of cells tracked, including cells arriving with holdfast already synthesized, was as follows: wild type, n = 241; Δ*motB*, n = 566; Δ*pilA*, n = 93; and Δ*pilA* Δ*motB*, n = 84.

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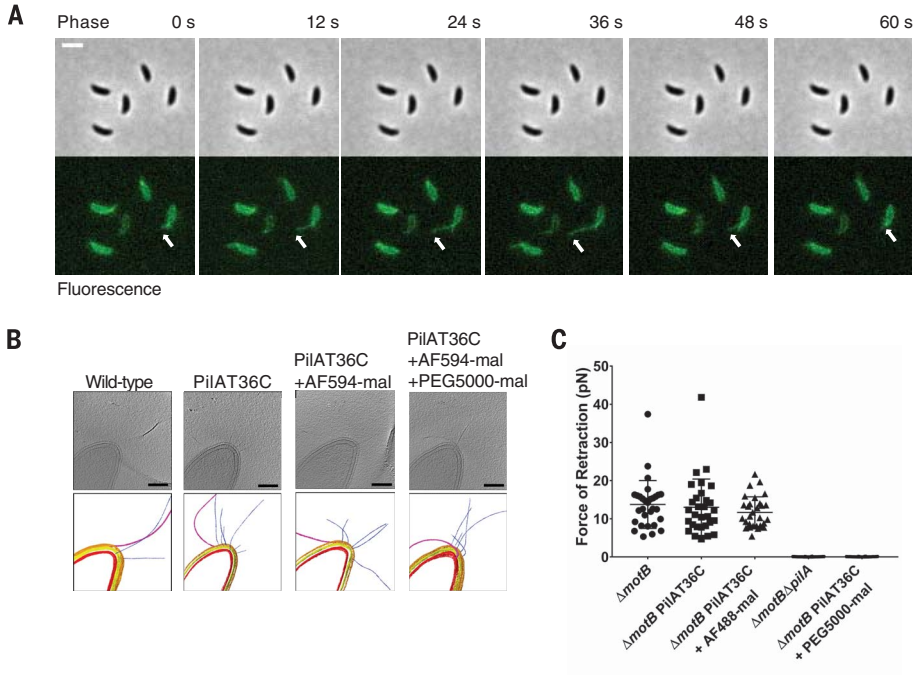

Fig. 2. Tad pili undergo dynamic cycles of extension and retraction. (**A**) Time-lapse of synchronized PilAT36C swarmer cells extending and retracting pili after labeling with AF488-mal dye. The white arrow indicates the most prominent extension and retraction event for a single cell, although all cells shown extended and retracted pili. Scale bar, 2 μm. (**B**) Slices from tomograms and corresponding three-dimensional (3D) segmentations of wild type, PilAT36C, PilAT36 labeled with AF594-mal, and PilAT36C blocked with PEG5000-mal and labeled with AF594-mal. In 3D segmentation volumes, flagella are pink, pili are blue, the S-layer is gold, the outer membrane is yellow, and the inner membrane is red. Scale bars, 200 nm. (**C**) Measurements of the force of retraction of Tad pili in flagellar motor mutant (Δ*motB*) strains, assessed by micropillars assay. Flagellar motor mutants exhibiting paralyzed flagella were used to ensure that all measurements were dependent solely on pilus activity. The mean (widest bar) ± SD (error bars) from 30 cells is indicated for each data set.

labeled Tad pili extend and retract normally. Tad pili, which we refer to as type IVc (fig. S1, A and B), are more similar to the type IV secretion system and the archaellum than to other type IV pili. Additionally, we labeled the *Vibrio cholerae* type IVa MSHA (mannose-sensitive hemagglutinin) pili and the *V. cholerae* type IVb TCP (toxin co-regulated pilus) pili (figs. S4, A, B, and D, and S8, A and B), demonstrating that this labeling method is broadly applicable to diverse pilus systems (*8*).

Mutants lacking pilus retraction machinery exhibit changes in pilin distribution (*9*). However, direct demonstration of pilin subunit recycling has proven difficult owing to limitations in techniques for studying their real-time dynamics. Upon labeling with AF488-mal, we observed that piliated swarmer cells exhibited fluorescent cell bodies, suggesting that externally labeled pilins might be internalized and recycled during pilus retraction. Using the principle of size-based exclusion by the outer membrane (OM), we labeled the wild-type and PilAT36C strains using either the large, OM-excluded AF488-mal dye (720.66 Da) or a small, OM-permeable boron-dipyrromethene (BODIPY)–mal dye (414.22 Da) to quantify fluorescent cell bodies in each population (fig. S3C and Fig. 3, A and B). The OM-permeable BODIPY-mal labeled all cell types, including stalked and predivisional cells lacking pili, from both the wild-type and PilAT36C strains. In contrast, the OM-excluded AF488-mal did not label wild-type cells or cells lacking pili within the PilAT36C population, unless their OM was compromised (Fig. 3, A and C). Thus, we hypothesized that cell body staining with AF488-mal was due to pilus retraction into the periplasm.

To test this hypothesis, we added a large polyethylene glycol maleimide conjugate (PEG5000-mal; ~5000 Da). Upon colabeling pili with PEG5000-mal

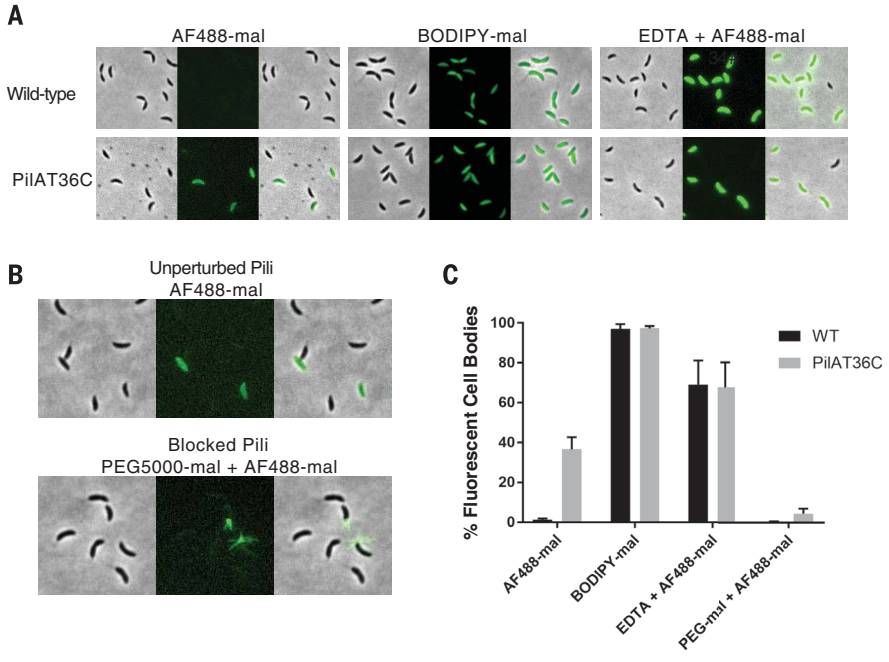

Fig. 3. Tad pilus retraction internalizes labeled pilins into a recyclable pool of subunits. (**A**) Representative images of wild-type or PilAT36C cells labeled with AF488-mal, BODIPY-mal, or AF488-mal after OM permeabilization with 20 mM EDTA (ethylenediaminetetraacetic acid). (**B**) Representative images of PilAT36C cells labeled with AF488-mal with or without PEG5000-mal. (**C**) Quantification of fluorescent cell bodies in populations of cells from images shown in (A) and (B). A minimum of 398 cells from each of three independent biological replicates were quantified. Means and SD (error bars) are shown.

and AF488-mal, labeled pili no longer exhibited dynamic activity and could not cause micropillar bending (Figs. 3B and 2C). We also observed a large reduction in the number of swarmer cells with fluorescent cell bodies and a concomitant increase in the number of cells with multiple fluorescent pili (Fig. 3, B and C). Cryo-ET of cells labeled with PEG5000-mal showed no effect on pilus structure or the cell envelope (Fig. 2B). Thus, AF488-mal is excluded by the OM, externally

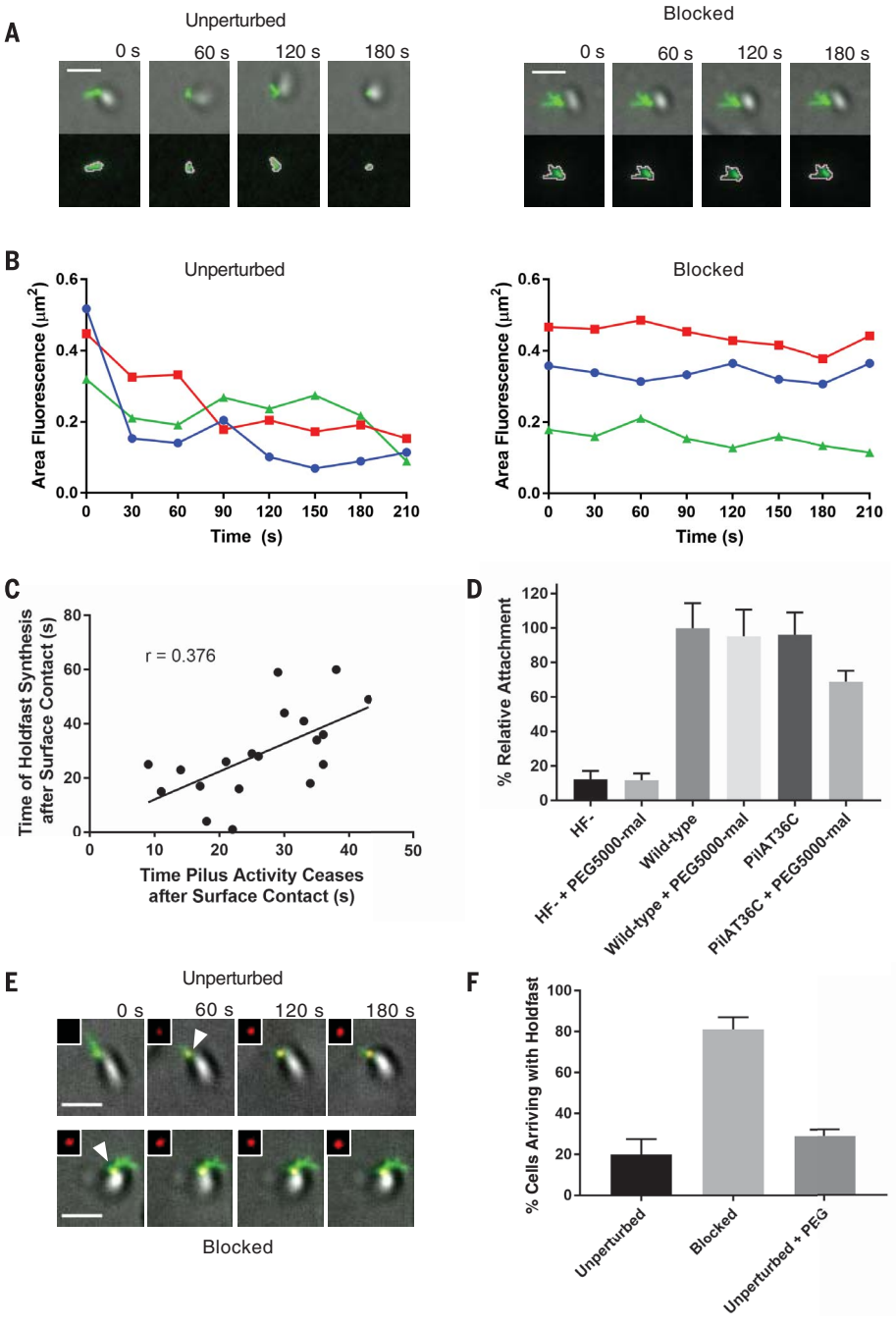

Fig. 4. Resistance to Tad pilus retraction triggers surface stimulation of holdfast synthesis. (**A**) Representative TIRF images of (left) a cell with unperturbed pili (labeled with AF488-mal), exhibiting dynamic pilus activity, and (right) a cell for which pilus retraction is blocked (labeled with both AF488-mal and PEG5000-mal), exhibiting no dynamic pilus activity. In the top images, cell bodies are shown in gray with green fluorescent pili. The bottom images show green fluorescent pili surrounded by a pink MicrobeJ overlay used to measure changes in the fluorescence area of pili (square micrometers) over the time shown in (B). Scale bars, 2 μm. (**B**) Changes in the fluorescence area occupied by pili over time for cells shown in (A) (red, blue, and green are fluorescent area traces for individual cells). (**C**) Plot showing the correlation between the time of holdfast synthesis after surface contact and the time of cessation of dynamic pilus activity after surface contact for 19 cells. *r*, Pearson's correlation coefficient. (**D**) Relative attachment assay showing the binding efficiency of strains lacking holdfast (HF−) and PilAT36C strains compared with that of the wild-type strain after 30 min binding, with or without PEG5000-mal. Data are representative of binding from three independent cultures, normalized to wild-type binding levels. Means and SD (error bars) are shown. PilAT36C plus PEG5000-mal is significantly different from all other treatments ($P < 0.03$; unpaired, two-tailed *t* test). (**E**) Representative TIRF microscopy images of cells with unperturbed (top) and blocked (bottom) pili upon surface contact (time = 0 s) in the presence of AF594-WGA. The cell body is gray, labeled pili are green, and holdfast is red (shown in the upper right inset in each image). White arrowheads indicate the first appearance of holdfast for the cell depicted. Scale bars, 2 μm. (**F**) Quantification of cells after labeling with AF488-mal (unperturbed); AF488-mal plus PEG5000-mal (blocked); or AF488-mal plus PEG5000 (unperturbed + PEG). A minimum of 30 cells from each of three independent biological replicates were quantified. Means and SD (error bars) are shown.

labeled pilins are retracted into an intracellular pool of subunits, and pilus retraction can be physically obstructed. Because newly extended pili are still labeled after removal of excess dye, the results also indicate that pilins are recycled and reused, as previously suggested (*10*).

The inability to observe pilus behavior directly when a cell makes contact with a surface has complicated analysis of the role of pilus dynamics in surface sensing. This has required the use of pilus synthesis and/or retraction mutants to infer their function by observing the behavior of a nonfunctional or genetically modified system. To bypass these problems and determine the role of pilus retraction in surface sensing, we combined our abilities to observe and physically perturb fully functional pili. We first examined the dynamics of pilus extension and retraction upon surface contact. Cells were added to microfluidic well devices, and those that were detected at the glass-liquid interface by differential interference contrast microscopy were analyzed for pilus activity. We defined the cessation of pilus activity as the first frame after which fluorescence area neither increased nor declined. Cells with unperturbed pili exhibited dynamic pilus activity, whereas cells for which pilus retraction was blocked exhibited no dynamic activity, as indicated by tracking the fluorescence area of pili associated with single cells over time (Fig. 4, A and B, and movie S8). In unperturbed cells, upon surface contact, the cessation of dynamic pilus extension and retraction was positively correlated with the stimulation of holdfast synthesis (Fig. 4C). Although holdfast synthesis did not always occur immediately upon cessation of pilus activity, the correlation between these two events implicates perturbation of pilus dynamics, and not necessarily cessation of activity, in the surface-sensing process.

The above observations are consistent with the hypothesis that resistance exerted on retracting

surface-bound pili provides a surface-sensing signal to trigger holdfast synthesis. To test this hypothesis, we blocked pilus retraction by treatment with PEG5000-mal and measured surface attachment (Fig. 4D). Cultures in which pilus retraction was blocked experienced a 27% reduction in adhesion compared with cultures of unperturbed cells, suggesting that dynamic pilus activity is important for mediating attachment. Next, we used TIRF (total internal reflection fluorescence) microscopy to track holdfast synthesis upon surface contact in piliated swarmer cells with either unperturbed or blocked pili (Fig. 4E; fig. S9, A and B; and movies S9 and S10). Whereas only 20% of unperturbed cells arrived on the surface with a holdfast synthesized before surface contact, 81% of cells for which pilus retraction was blocked had synthesized a holdfast before reaching the surface; thus, blocking pilus retraction in planktonic cells was sufficient to stimulate holdfast synthesis in the absence of surface contact (Fig. 4F). The addition of PEG5000-mal had no effect on cell swimming, indicating that blocking pilus retraction does not impede flagellum rotation (fig. S10A). Although a Δ*motB* mutant was deficient in surface colonization because it was unable to reach the surface efficiently (fig. S10B), it was stimulated for holdfast synthesis before surface contact after pilus retraction was blocked (fig. S10, C and D). Thus, blocking pilus retraction of Δ*motB* cells phenocopied blocking pilus retraction in wild-type cells, demonstrating that rotating flagella were not required for this process. The Δ*motB* mutation increased the number of piliated cells with a holdfast in the medium (fig. S10D), suggesting a regulatory interaction between the flagellum and holdfast synthesis that may reflect a role for flagellum rotation in a parallel surface-sensing pathway. These data support a model in which resistance exerted on retracting, surface-bound pili generates a surface-sensing signal, expanding on previous work in which mutants deficient in pilus retraction and extension could not sense surface contact (*4*, *5*).

Although a rotating flagellum was not required for surface stimulation of holdfast synthesis in the absence of flow, obstruction of flagellum rotation may be involved in surface sensing through an unknown mechanism (*11*, *12*). It remains unclear whether these two mechanosensors use the same signaling pathways and whether they are used to sense surfaces under different conditions.

How is the surface-sensing signal transduced? Holdfast synthesis was stimulated normally in the absence of RNA or protein synthesis (fig. S11), suggesting that it could be mediated by a second messenger molecule such as cyclic diguanylate monophosphate (c-di-GMP). Chemical signaling would be compatible with the rapidity of the response. Deletion of the diguanylate cyclase PleD reduces c-di-GMP concentration by about a third (*13*), but a Δ*pleD* mutant still synthesizes pili and holdfasts (*13*–*16*). However, the Δ*pleD* mutant was deficient in synthesizing holdfast in response to surface contact (fig. S12A). The *pleD* mutant was stimulated for holdfast synthesis upon blocking pilus retraction, albeit not to the same extent as wild-type cells (fig. S12B). These data implicate c-di-GMP levels in the surface stimulation of holdfast synthesis.

We have developed a broadly applicable pilus-labeling method that enables real-time observation of pilus dynamics and targeted physical obstruction. Tad pili can retract despite the absence of an orthologous retraction ATPase; retracted pilins are internalized into a pilin pool that is recycled and reused; resistance on retracting, surface-bound pili upon surface contact mediates surface sensing; and c-di-GMP signaling may be involved in surface sensing.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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Elucidating a bacterial sense of touch

Bacteria can adhere to surfaces within the host. This leads to tissue colonization, induction of virulence, and eventually the formation of biofilms—multicellular bacterial communities that resist antibiotics and clearance by the immune system (see the Perspective by Hughes and Berg). Hug *et al.* show that bacteria have a sense of touch that allows them to change their behavior rapidly when encountering surfaces. This tactile sensing makes use of the inner components of the flagellum, a rotary motor powered by proton motif force that facilitates swimming toward surfaces. Thus, the multifunctional flagellar motor is a mechanosensitive device that promotes surface adaptation. In complementary work, Ellison *et al.* elucidate the role of bacterial pili in a similar surface-sensing role.

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