CELL BIOLOGY

Second messenger–mediated tactile response by a bacterial rotary motor

Isabelle Hug,[1] Siddharth Deshpande,[2]* Kathrin S. Sprecher,[1] Thomas Pfohl,[2]† Urs Jenal[1]‡

When bacteria encounter surfaces, they respond with surface colonization and virulence induction. The mechanisms of bacterial mechanosensation and downstream signaling remain poorly understood. Here, we describe a tactile sensing cascade in *Caulobacter crescentus* in which the flagellar motor acts as sensor. Surface-induced motor interference stimulated the production of the second messenger cyclic diguanylate by the motor-associated diguanylate cyclase DgcB. This led to the allosteric activation of the glycosyltransferase HfsJ to promote rapid synthesis of a polysaccharide adhesin and surface anchoring. Although the membrane-embedded motor unit was essential for surface sensing, mutants that lack external flagellar structures were hypersensitive to mechanical stimuli. Thus, the bacterial flagellar motor acts as a tetherless sensor reminiscent of mechanosensitive channels.

Mechanical cues are perceived by bacteria during surface contact, leading to a rapid change in behavior through the induction of adherence and biofilm formation, surface motility, or virulence (*1*–*3*). Structures involved in bacterial surface interaction include pili and flagella. Models for bacterial mechanosensation proposed that pili transiently establish surface contact, eliminating the space required for free rotation of the flagellum and increasing the load on the motor (*1*, *4*–*6*). As a consequence, intracellular sensory proteins could detect conformational rearrangements of motor proteins or an altered proton flux through the motor (*7*, *8*). However, these models remain controversial, and recent studies have challenged the role of the flagellum as a key player in surface detection (*9*, *10*). We hypothesized that some of these inconsistencies could be attributed to analyzing surface colonization at different time scales, a problem that can be avoided with rigorous separation of the individual stages of surface colonization through careful experimental design. We chose the surface-stimulated synthesis of the adhesive holdfast of *Caulobacter crescentus* to dissect bacterial mechanosensation. Newborn *C. crescentus* swarmer (SW) cells are equipped with a single flagellum (Fig. 1A) and are motile for a defined period before shedding their flagellum and secreting the holdfast, a polysaccharide adhesin that permanently anchors cells to the surface. However, when SW cells encounter a solid substratum, they deploy a holdfast within seconds, a process that can be imaged in real time by using fluorescently labeled holdfast-specific lectins (*6*, *11*).

To experimentally assess surface-stimulated holdfast formation, we used a set of microfluidics-based techniques that impose close surface contact on *C. crescentus* (Fig. 1, B and C) (*12*). Offspring of attached mothers are exposed to surface as a consequence of medium flow over the crescentoid dividing cells, which positions the flagellated and piliated pole in close proximity to the substratum (Fig. 1B) (*9*). Under these conditions, ~50% of the daughters remained attached directly downstream of their mothers and often displayed a holdfast before separating (Fig. 1, B, D, and E; fig. S1, A to D; and movie S1). Thus, future SW cells acquired the ability to respond to surface exposure before separation. At this stage, the flagellum is fully assembled and actively rotating (movie S2). High-speed imaging of cells dividing under flow revealed that SW offspring rotating around their long axis before separation were generally washed away, whereas SW cells that managed to adhere remained static or stopped their rotation before budding off (fig. S1E and movies S2 to S4). These observations are in agreement with pili-mediated immobilization of the cell body and flagellar obstruction being vital for SW cells to sense surface. Consistent with this, polar pili were strictly required for rapid attachment (Fig. 1E). When grown under flow, a single mother generated small microcolonies through cycles of division and attachment of SW offspring (Fig. 1F and movie S5). The area covered by such colonies was proportional to the attachment efficiency of SW progeny (Fig. 1, E and G). Surprisingly, SW cells of mutants that lack outer parts of the flagellum—including proximal and distal rods (Δ*flgFG*), hook, and filament (Δ*flgDE*) (Fig. 1A)—showed higher propensity to attach than did the wild type (Fig. 1, E and G), arguing against the flagellum acting as a simple tactile tether. This was not due to an altered developmental timing of holdfast formation but rather to a hypersensitive tactile response (fig. S2). By contrast, mutants that failed to assemble the inner parts of the motor (Δ*fliFG*) as well as mutants with a fully assembled but paralyzed flagellum (Δ*motA*, Δ*fliL*, Δ*motB*, and $motB_{D33N}$) failed to attach effectively (Fig. 1, E and G). Alleles that compromise internal motor components or the pilus were dominant over alleles that affect external flagellar parts (Fig. 1G), demonstrating that cells that lack hook and filament but have an intact internal motor remained fully responsive to surface. Thus, the integrity and activity of the motor but not the external parts of the rotary flagellum are required for surface sensing.

To corroborate these findings and to address the role of the pili in surface signaling, we designed thin, diffusion-controlled quasi–two-dimensional chambers (*12*) to follow SW progeny microscopically from the moment of birth at division to the onset of holdfast production (Fig. 1C). With a height of only 0.75 μm, such microchambers offer cells constant surface interaction opportunities without medium flow. Accordingly, we observed the formation of holdfast within a few seconds after newborn SW cells were released from their mothers (Fig. 1H). The tight geometry of these chambers alleviated the strict requirement of adhesive pili (Δ*pilA*) and putative pili motor components (Δ*cpaE* and Δ*cpaF*) for surface recognition (Fig. 1H). By contrast, a paralyzed mutant with a MotB stator subunit unable to conduct protons ($MotB_{D33N}$) (*13*, *14*) but harboring unaltered pili developed holdfast only ~10 min after division, corresponding roughly to the time required for the completion of the developmental program leading to holdfast formation (Fig. 1H). Thus, rather than playing a direct role as surface sensors, the function of pili is to bring the cell pole into close proximity with the underlying surface to facilitate effective sensing by the motor.

The machinery for holdfast biosynthesis is on standby in dividing and newborn SW cells, waiting for an activating trigger (*15*, *16*). This, and the observed speed of holdfast production, argued that the tactile response is regulated posttranslationally. A prime candidate to fulfill this function is the second messenger cyclic diguanylate (c-di-GMP), which promotes surface adaptation in many bacteria (*17*) and controls holdfast production during *C. crescentus* development (*18*, *19*). To investigate the role of c-di-GMP in the *C. crescentus* surface response, we used a strain in which the c-di-GMP concentration can be experimentally tuned. In this strain, all genes encoding endogenous diguanylate cyclases and phosphodiesterases were deleted and substituted with the P_{lac}-driven *dgcZ* diguanylate cyclase gene form *Escherichia coli* (rcdG^0 P_{lac}::*dgcZ*). Although the rcdG^0 strain itself is unable to assemble pili and holdfast and thus fails to adhere to surfaces, moderate induction of

[1]Biozentrum, University of Basel, Klingelbergstrasse 50/70, CH-4056, Switzerland. [2]Department of Chemistry, University of Basel, Klingelbergstrasse 80, CH-4056, Switzerland.
*Present address: Department of Bionanoscience, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, Netherlands.
†Present address: Institute of Physics, University of Freiburg, Hermann-Herder-Strasse 3, 79104 Freiburg, Germany.
‡Corresponding author. Email: urs.jenal@unibas.ch

dgcZ restored organelle assembly (*18*) and enabled the formation of microcolonies under flow conditions (Fig. 2). Constitutive synthesis of c-di-GMP bypassed the requirement of an active motor for rapid attachment (Fig. 2), whereas pili remained important for attachment (Fig. 2) but were also not required for holdfast biogenesis (fig. S3). Thus, c-di-GMP acts downstream of the motor to induce a rapid surface response. When analyzing *C. crescentus* mutants that lack individual diguanylate cyclases, only deletion of *dgcB* strongly reduced attachment of newborn SW cells, a defect that could be complemented by adding *dgcB* in trans (Fig. 2 and fig. S4). Likewise, a point mutation abolishing DgcB catalytic activity (E261Q) eliminated rapid surface attachment (Fig. 2). (Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S,

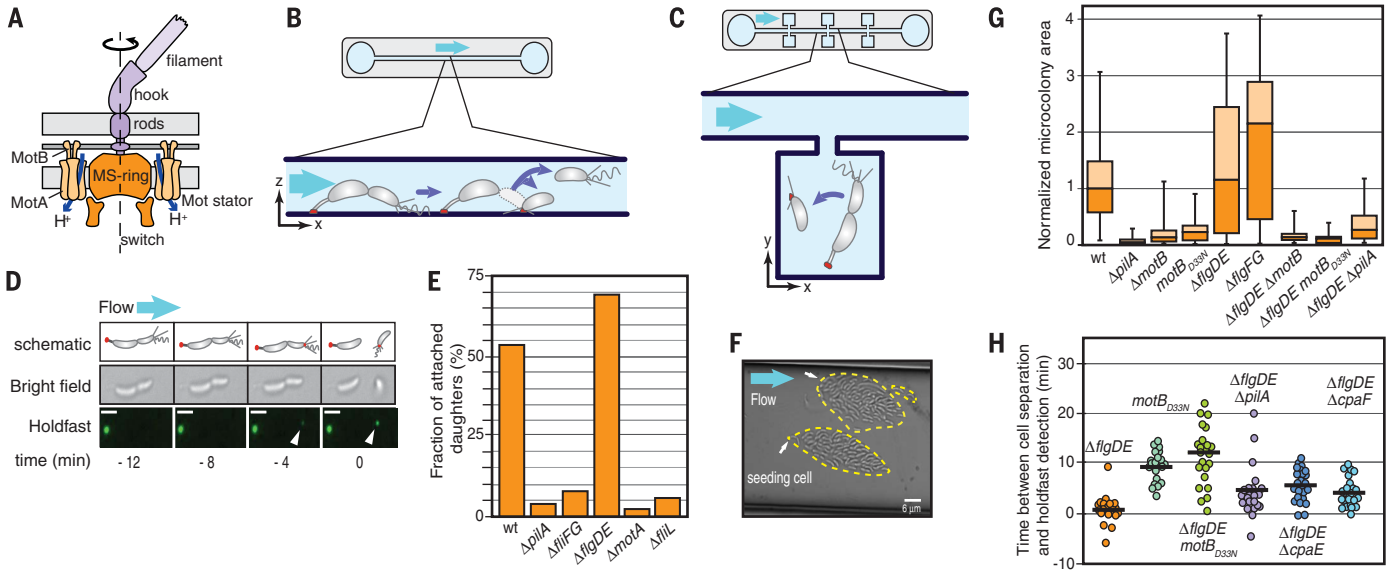
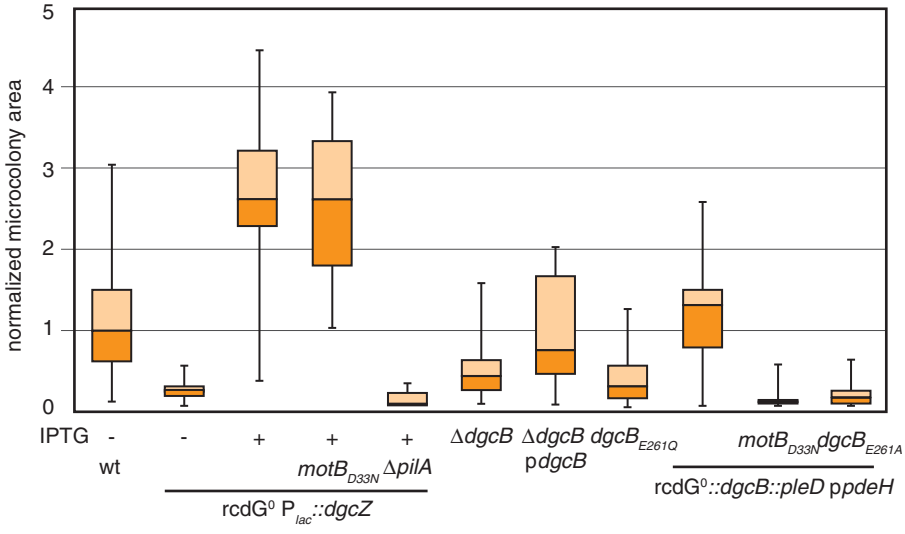


Fig. 1. The flagellar motor is a tactile mechanosensor. (**A**) Schematic representation of the bacterial flagellum. (**B**) Progeny of mother cells attached to the glass surface in a microfluidic channel were either carried away after release or expressed a holdfast (red) before separating to remain attached downstream of the mother cell. Medium flow and chamber dimensions are indicated by light blue and black arrows, respectively. (**C**) Newborn cells were observed in microchambers of 0.75 µm height with fluorescent wheat germ agglutinin (WGA) lectin added to the medium in order to determine the time span between daughter cell release and the appearance of the holdfast (red). (**D**) *C. crescentus* cell dividing in a flow channel. Newly formed holdfast at the flagellated pole of a dividing cell (arrowheads) and time relative to cell division are indicated. Scale bars, 2 µm. (**E**) Efficiency of SW cell attachment under constant flow. Data are based on 232 [wild type (wt)], 160 (Δ*pilA*), 88 (Δ*fliFG*), 139 (Δ*flgDE*), 88 (Δ*motA*), and 107 (Δ*fliL*) individual separation events. (**F**) Microcolonies formed from growth and attachment of single ancestors (arrows) in the flow channel. The area covered by the colony (dashed yellow line) served as a measure for the efficiency of rapid holdfast expression. (**G**) Areas of microcolonies after 15 hours of growth in a flow channel illustrate the efficiency of daughter cell attachment. Box plots show median (horizontal black lines), lower and upper quartiles (dark and light orange boxes, respectively), and extreme values (whiskers). Values were normalized to the median of the wild-type colony area. (**H**) Time between release of SW progeny from their mothers to detection of holdfast. The strains were grown as illustrated in (C). Because motile daughters are difficult to reliably track in this assay because of their swimming speed, only nonmotile mutants were used for this analysis. Averages are shown as black lines. Measurements for Δ*flgDE* Δ*pilA* are significantly different from measurements for Δ*flgDE motB*$_{D33N}$ (Students *t* test, $P < 0.001$).

Fig. 2. The diguanylate cyclase DgcB is essential for surface sensing and rapid attachment. Attachment of the strains indicated was scored as outlined in Fig. 1G. The *C. crescentus* rcdG^{0} P$_{lac}$::*dgcZ* strain lacks all diguanylate cyclases and phosphodiesterases but carries a plasmid-born copy of the P$_{lac}$-driven *dgcZ* gene from *E. coli*. In the rcdG^{0}::*dgcB*::*pleD ppdeH* strain, two diguanylate cyclase genes, *dgcB* and *pleD*, are retained. In addition, the strain carries a plasmid-born copy of the *E. coli pdeH* gene, encoding a phosphodiesterase. The *dgcBE261A* allele carries a point mutation in the active site, rendering its product catalytically inactive. Box plots show the median (horizontal black lines), lower and upper quartiles (dark and light orange boxes, respectively), and the extreme values (whiskers). Values were normalized to the median of the wild-type colony area.



Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. In the mutants, other amino acids were substituted at certain locations; for example, E261Q indicates that glutamic acid at position 261 was replaced by glutamine.) Together with the observation that DgcB does not contribute to the c-di-GMP pool in liquid cultures (*18*), these data inferred a specific role for DgcB in *C. crescentus* surface sensing.

DgcB localized to the flagellated pole (*20*) in 16.1% of dividing and in 30.6% of SW cells (n = 242 and 292 cells, respectively) (fig. S5A and movie S6). Although polar localization of DgcB did not depend on the presence of components of the flagellar motor (fig. S5A), pulldown experiments revealed MotA in the elution fraction of tagged DgcB. Inversely, DgcB was pulled down when tagged MotA served as bait (fig. S5B). This argued that DgcB is located in close proximity of the stator units and that spatial coupling may facilitate signal transduction. DgcB has a C-terminal catalytic GGDEF domain and an N-terminal domain of unknown function. DgcB homologs with an orthologous N-terminal domain are widespread in bacteria, including *Vibrio* or *Pseudomonas* species (fig. S6, A and B). A motif search with amino acids conserved in the N-terminal domain of DgcB (fig. S6C) identified diguanylate cyclases, oxidoreductases, and inner-membrane transporters (table S1), suggesting that these domains might tap into the redox/energy status at the inner membrane. Mutating highly conserved residues of this motif (N35A, F36A, and W39A) strongly reduced *C. crescentus* surface-induced attachment (fig. S6D) without affecting DgcB protein stability (fig. S6E). This is in line with the idea that the N terminus serves as an input domain to regulate DgcB activity. Thus, *Caulobacter* senses mechanical cues via its polar flagellar motor, which in turn emits a physical or chemical signal that is converted by DgcB into a pulse of c-di-GMP.

To identify the molecular target of c-di-GMP that is responsible for rapid holdfast production, we used capture compound mass spectrometry (CCMS) (*21*). One of the proteins that was specifically pulled down was HfsJ, a glycosyltransferase essential for holdfast biogenesis (*22*). Biochemical analyses confirmed that HfsJ bound c-di-GMP directly and specifically (Fig. 3A). HfsJ harbors an arginine-rich N-terminal region, which is only conserved in homologs of close relatives of *C. crescentus* (Fig. 3B). This region features a triple repeat of the RXXXR motif also found in other c-di-GMP effectors (*23*). Replacing R23 or R27 abolished c-di-GMP binding without affecting protein stability (Fig. 3C and fig. S7A) and strongly reduced holdfast production, akin to an HfsJ catalytic mutant (R247Q) (Fig. 3D). Overexpression of *hfsJ* augmented holdfast formation in *C. crescentus* wild type and restored holdfast formation in the cdG^0 strain (fig. S7B), arguing that HfsJ activity constitutes the rate-limiting step in holdfast biosynthesis. Likewise, HfsJ variants unable to bind c-di-GMP, but not an HfsJ catalytic mutant, restored holdfast biogenesis in the cdG^0 strain when overexpressed (fig. S7C). Thus, these mutations affected the regulation of HfsJ but not its catalytic activity per se. HfsJ is also targeted by a small protein inhibitor, HfiA, which controls holdfast formation in response to nutritional cues (*22*). Rapid attachment of a Δ*hfiA* mutant retained its strict dependency on DgcB and on a functional motor, excluding a role for HfiA in mechanosensation (fig. S7D). Thus, HfsJ is a critical bottleneck in holdfast biogenesis, and its activity is stimulated by c-di-GMP to rapidly boost holdfast expression when DgcB is activated by tactile sensing or when c-di-GMP levels rise during SW cell development.

Our results have uncovered an important role for the *C. crescentus* flagellar motor as a tactile sensor. A simple model for sensing by means of surface-mediated obstruction of flagellar rotation (*1*, *4*–*6*) is not supported by our data. Rather, the strict need for an intact motor argues that flagellar Mot proteins may

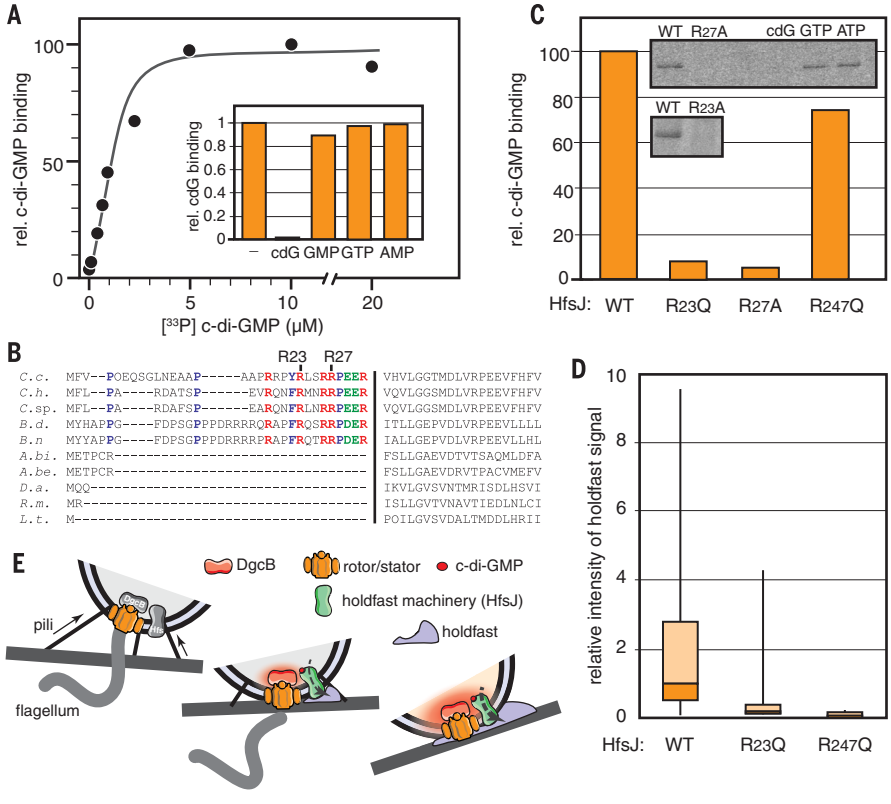


Fig. 3. C-di-GMP binding to HfsJ initiates holdfast biosynthesis. (**A**) Binding of c-di-GMP to HfsJ (solid circles) was measured with increasing concentrations of radiolabeled ligand as indicated, revealing a dissociation constant (K_d) of 2.7 μM. (Inset) Binding is specific for c-di-GMP. Radiolabeled c-di-GMP (5 μM) was competed with a 100× excess of the nucleotides indicated, and overall binding was determined. (**B**) Alignment of the N-termini of HfsJ orthologs from *C. crescentus* (*C.c.*) and the following closely related species: *Caulobacter henricii* (*C.h.*), *Caulobacter* sp. K31 (*C.sp.*), *Brevundimonas diminuta* (*B.d.*), *Brevundimonas naejangsanensis* (*B.n.*), *Asticcacaulis biprosthecium* (*A.b.*), *Asticcacaulis benevestitus* (*A.b.*), *Deinococcus apachensis* (*D.a.*), *Rhodothermus marinus* (*R.m.*), and *Lihuaxuella thermophile* (*L.t.*). Conserved residues are highlighted, with arginines in red, negative charges in green, and proline or aromatic amino acids in blue. (**C**) Binding studies with HfsJ identified R23 and R27 as critical residues for c-di-GMP binding. The highly conserved catalytic residue R247 is not required for c-di-GMP binding. (**D**) In vivo activity of HfsJ. Cells of *C. crescentus* wild type (n = 764 cells) and *hfsJ* mutants (R23Q, n = 1077 cells; R247Q, n = 6 cells) were grown in the presence of Oregon Green 488–labeled WGA lectin, and holdfast biogenesis was measured as relative fluorescence intensities of individual cells. (**E**) Model of *C. crescentus* surface sensing. Initial surface adherence is mediated by pili and pili retraction positioning the flagellar pole in close contact with the surface. The physical pressure applied on the cell envelope by the surface affects the function of the flagellar rotor-stator components, generating an unknown signal that is sensed by DgcB and converted into a burst of c-di-GMP (red). The second messenger initiates rapid holdfast biogenesis and permanent attachment by activating the key glycosyltransferase HfsJ.

act as mechanosensitive channels. Similar to bacterial osmoregulators MscL/S (*24*) or Piezo2 channels in mammalian epidermal Merkel cells (*25*, *26*), surface exposure of *C. crescentus* may be communicated to the cytosol via a change in ion flux through the stators, which is inflicted by conformational changes when mechanical forces affect the cell envelope. This could result in a transient pH change inside the cells, which is then picked up and amplified by enzymes such as DgcB (Fig. 3E). Such a model conforms well with the strong pH-dependent stimulation of DgcB activity in vitro (*20*) and with the observation that the $MotB_{D33N}$ mutant, which is unable to conduct protons, failed to respond to surfaces similar to strains lacking components of the rotor.

REFERENCES AND NOTES

1. G. A. O'Toole, G. C. L. Wong, *Curr. Opin. Microbiol.* **30**, 139–146 (2016).
2. A. Siryaporn, S. L. Kuchma, G. A. O'Toole, Z. Gitai, *Proc. Natl. Acad. Sci. U.S.A.* **111**, 16860–16865 (2014).
3. A. Persat *et al.*, *Cell* **161**, 988–997 (2015).
4. L. McCarter, M. Hilmen, M. Silverman, *Cell* **54**, 345–351 (1988).
5. E. Karatan, P. Watnick, *Microbiol. Mol. Biol. Rev.* **73**, 310–347 (2009).
6. G. Li *et al.*, *Mol. Microbiol.* **83**, 41–51 (2012).
7. P. P. Lele, B. G. Hosu, H. C. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 11839–11844 (2013).
8. K. L. Van Dellen, L. Houot, P. I. Watnick, *J. Bacteriol.* **190**, 8185–8196 (2008).
9. A. Persat, H. A. Stone, Z. Gitai, *Nat. Commun.* **5**, 3824 (2014).
10. A. Persat, Y. F. Inclán, J. N. Engel, H. A. Stone, Z. Gitai, *Proc. Natl. Acad. Sci. U.S.A.* **112**, 7563–7568 (2015).
11. M. D. Hoffman *et al.*, *Anal. Chem.* **87**, 12032–12039 (2015).
12. S. Deshpande, T. Pfohl, *Biomicrofluidics* **6**, 34120 (2012).
13. J. Zhou *et al.*, *J. Bacteriol.* **180**, 2729–2735 (1998).
14. S. Kojima, D. F. Blair, *Biochemistry* **40**, 13041–13050 (2001).
15. A. Levi, U. Jenal, *J. Bacteriol.* **188**, 5315–5318 (2006).
16. G. G. Hardy *et al.*, *Mol. Microbiol.* **76**, 409–427 (2010).
17. U. Jenal, A. Reinders, C. Lori, *Nat. Rev. Microbiol.* **15**, 271–284 (2017).
18. S. Abel *et al.*, *PLOS Genet.* **9**, e1003744 (2013).
19. K. S. Sprecher *et al.*, *MBio* **8**, e00294–e17 (2017).
20. S. Abel *et al.*, *Mol. Cell* **43**, 550–560 (2011).
21. J. Nesper, A. Reinders, T. Glatter, A. Schmidt, U. Jenal, *J. Proteomics* **75**, 4874–4878 (2012).
22. A. Fiebig *et al.*, *PLOS Genet.* **10**, e1004101 (2014).
23. S.-H. Chou, M. Y. Galperin, *J. Bacteriol.* **198**, 32–46 (2016).
24. I. R. Booth, S. Miller, A. Müller, L. Lehtovirta-Morley, *Cell Calcium* **57**, 140–150 (2015).
25. R. Ikeda *et al.*, *Cell* **157**, 664–675 (2014).
26. S.-H. Woo *et al.*, *Nature* **509**, 622–626 (2014).

ACKNOWLEDGMENTS

We thank S. Abel, A. Dürig, A. Levi, and D. Meyer for providing plasmids; P. Jenö and S. Moes for technical assistance; and S. Ozaki, M. Sangermani, and C. von Arx for manuscript comments. This work was supported by the Swiss National Science Foundation (grant 310030B_147090 to U.J.) and by the European Research Council (ERC) Advanced Research Grant (322809 to U.J.). S.D. and T.P. gratefully acknowledge the support by the Swiss Nanoscience Institute (SNI). The data reported in this paper are tabulated in the supplementary materials. I.H., S.D., T.P., and U.J. designed the study; I.H., S.D., and K.S.S. collected and processed data; I.H., S.D., and T.P. performed the analyses; and I.H. and U.J. wrote the paper.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/358/6362/531/suppl/DC1
Materials and Methods
Figs. S1 to S7
References (*27*–*31*)
Tables S1 to S4
Movies S1 to S6

27 April 2017; accepted 15 September 2017
10.1126/science.aan5353

Science

Second messenger–mediated tactile response by a bacterial rotary motor

Isabelle Hug, Siddharth Deshpande, Kathrin S. Sprecher, Thomas Pfohl and Urs Jenal

Science **358** (6362), 531-534.
DOI: 10.1126/science.aan5353

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.

Science, this issue p. 531, p. 535; see also p. 446