Second messenger-mediated tactile response by a bacterial rotary motor

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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 bacterial flagellar motor acts as a 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.

Mecchancial 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 substrate, 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 microfluidic-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 holdfast 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 F). With a height of only 0.75 μm, such microcolonies 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 (ΔpilA) and putative pili motor components (ΔcpaE and ΔcpaF) for surface recognition (Fig. 1H). By contrast, a paralyzed mutant with a MotB stator subunit unable to conduct protons (MotBD33N) (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 Pbil-driven digZ1 diguanylate cyclase gene form Escherichia coli (redG D Pbil digZ). Although the redG strain itself is unable to assemble pili and holdfast and thus fails to adhere to surfaces, moderate induction of...
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 pilA 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,

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**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 (ΔpilA), 88 (ΔflgDE), 139 (ΔmotA), and 107 (ΔfligL) 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 ΔflgDE ΔpilA are significantly different from measurements for ΔflgDE motBD33N (Students t test, P < 0.001).

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**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 rcdGΔ Pbc::dgcZ strain lacks all diguanylate cyclases and phosphodiesterases but carries a plasmid-born copy of the Pbc-driven dgcZ gene from E. coli. In the rcdGΔ::dgcB::pleD::pdeH 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.
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 transduction. DgcB bound to c-di-GMP directly and specifically (fig. 3A). Replacing R23 or R27 abolished c-di-GMP binding without affecting protein stability (fig. 3C and fig. S7A). Thus, 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 biosynthesis (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 biosynthesis 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 MfiA 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 biosynthesis, 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 serve as cues to the flagellar motor, which in turn emits a physical or chemical signal that is converted by DgcB into a pulse of c-di-GMP.

**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. Radio-labeled 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 Caulobacter (C.h.), Caulobacter sp. K31 (C.s.p.), Brevundimonas diminuta (B.d.), Brevundimonas naegeansenensis (B.n.), Asticacaulis biprosthecium (A.b.), Asticacaulis benedixitus (A.b.), Deinococcus apachensis (D.a.), Rhodothermus marinus (R.m.), and Lihuaxuella thermophile (L.t.)保守 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 MotBD33N mutant, which is unable to conduct protons, failed to respond to surfaces similar to strains lacking components of the rotor.

REFERENCES AND NOTES

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