Review

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Biofilms, flagella, and mechanosensing of surfaces by bacteria

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Formation of a bacterial biofilm is a developmental process that begins when a cell attaches to a surface, but how does a bacterial cell know it is on or near a surface in the first place? The phase of this 'swim-or-stick' switch is determined by a sensory transduction mechanism referred to as surface sensing, which involves the rotating bacterial flagellum. This review explores six bacterial species as models of flagellar mechanosensing of surfaces to understand the current state of our knowledge and the challenges that lie ahead. A common link between these bacteria is a requirement for the proper function of the flagellar motor stators that channel ions into the cell to drive flagellar rotation. Conditions that affect ion flow act as a signal that, ultimately, controls the master transcriptional regulatory circuits controlling the flagellar hierarchy and biofilm formation.

Flagellar mechanosensors and the initiation of biofilm formation

Bacteria are able to live either as independent planktonic cells or as members of organized surface-attached microbial communities called biofilms, which are composed of microorganisms and the extracellular matrix-forming polymers they produce [1]. Formation of a bacterial biofilm is a developmental process that begins when a cell attaches to a surface. Biofilms have major clinical relevance because they provide protective environments against stresses, immune responses, antibacterial agents, and antibiotics [2]. Biofilm formation on man-made surfaces (for example, heat exchangers or the hulls of ships) is the precursor to colonization by larger macro-organisms that often leads to decreased efficiency and performance, and increased removal cost [3]. Numerous studies have made it abundantly clear that biofilm formation is regulated in response to environmental conditions and cues that vary among different species. Yet, in contrast to our wealth of knowledge of the events that occur after recognition of a 'surface signal', we know relatively little about the mechanisms used to sense surfaces, and how the surface signal flips the 'swim-or-stick' switch from a motile to a sessile lifestyle. How does a bacterial cell 'know' it is on or near a surface?

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It is generally agreed that motility and biofilm development are mutually exclusive events, and a transition from motility to sessility occurs during the earliest steps in biofilm development [1,4]. The phase of the switch is determined by a sensory transduction mechanism termed surface sensing that often involves the bacterial flagellum [5]. Thus, flagella are not only required for propulsion but also have a critical mechanosensory role in surface sensing and the initial stages of surface adhesion that leads to the formation of a biofilm [6]. Thus, a long-range goal of research on biofilm formation is a thorough understanding of the surface-sensing mechanism, which will allow the discovery of drugs that inhibit the ensuing response and thereby prevent biofilm formation.

The goal of this article is to review the state of our knowledge on the role that flagella have as mechanosensors (see Glossary) of surfaces, and to highlight what we

Glossary

Bis-(3′–5′)-cyclic dimeric guanosine monophosphate (C-di-GMP): a secondary messenger used by many bacteria to regulate biofilm formation. High levels of c-di-GMP promote biofilms, whereas low levels of c-di-GMP promote motility.
Flagellin: protein subunit that comprises the flagellar filament.
Lateral flagella: flagella that are used for swarming, and that are distributed around the surface of the cell in some bacteria that also possess polar flagella. Lateral and polar flagella are encoded by different sets of genes.
Mechanosensor: an organelle, a biological complex, or individual protein that detects and responds to physical forces exerted by the local environment and transduces this signal to control the transcriptional machinery.
Membrane potential (ΔΨ): the potential (electrical charge) across a bacterial membrane relative to the fluid outside of the concentration of potassium, sodium, chloride, and other diffusible ions. ΔΨ is one component of proton motive force. (The second is ΔpH.)
Polar flagella: flagella used for swimming, and localized to one or both ends of a rod-shaped bacterium.
Proton motive force (PMF): energy that is generated by the transfer of protons across a membrane that can be used for a variety of purposes, including synthesizing ATP. It is composed of the difference in proton concentration (ΔpH) and the electrical charge (ΔΨ) across a membrane.
Swarming: is a bacterial flagella-dependent motile behavior that allows cells to move over surfaces in a coordinated manner and expand the population to new locations. The process of swarming is distinct from swimming in that swarming is a multicellular process that occurs on solid surfaces or in viscous liquids, and requires differentiation of a vegetative swimmer cell into a specialized cell type called a swarmer cell.
Swarmer cell differentiation: morphological change of some bacteria from a planktonic or vegetative form that moves by swimming in liquid medium to a form in which the cells move across solid surfaces. Swarmer cell differentiation results in an increased number of flagella per cell and, in some bacteria, an alteration in how the flagella are distributed around the cell surface, as well as a significant elongation of the swarmer cells due to an inhibition of septation.
Two-component regulatory system: in its simplest form, composed of a membrane-bound sensor histidine kinase protein that senses specific environmental stimuli and its cognate response regulator protein that mediates the response, frequently through direct binding to DNA and subsequent differential expression of target gene transcription.

know and do not know about the molecular mechanism (or mechanisms) underlying this sensory transduction process. Further information and details of biofilm formation can be found in several excellent reviews [7–9].

Structure, function, and regulation of bacterial flagella

Early studies using *Escherichia coli* found that half of the transposon insertion mutants deficient in biofilm formation had defects in flagellar functions [10]. Thus, motility itself, and not chemotaxis, is required to form a biofilm [11]. In the past several years, it has become recognized that control of the swim-or-stick switch leading to biofilm formation involves the inhibition of flagellar synthesis and rotation coupled with increased synthesis of the polymers and structures that are required for long-term attachment to a surface and biofilm formation: that is, pili, fimbriae, holdfasts, capsules, and so on. Accordingly, the following general review of flagellar structure and the regulation of flagellar assembly is provided. Further detail may be found in a recent review [8].

Bacteria swim by rotating of one or more rigid helical flagella. Flagella are extremely effective organelles of locomotion that permit bacteria to achieve speeds exceeding many cell body lengths per second [12]. Cells are propelled forward when flagella turn counterclockwise, creating thrust to propel the cell to 'run', while changes in course are affected when one or more flagella turn clockwise to produce a 'tumble'. Swimming cells can perform chemotaxis by moving up or down chemical gradients, using an elaborate signaling system that modulates the counterclockwise/clockwise bias of the motors [13].

The flagellum can be subdivided into three substructures that are assembled in a temporal sequence [14] (Figure 1). The first component to be assembled is the basal body, which anchors the flagellum to the cell membrane, provides the power for rotation, and secretes the more distal components. The next component is the hook, which is connected to the basal body and serves as a flexible universal joint changing the angle of flagellar rotation. The third structure is the helical filament, which is composed primarily of the protein flagellin, one of the most abundant proteins made by the cell. Hook and filament proteins are secreted through the basal body, and a substrate specificity switch ensures that hook proteins are secreted first and flagellin proteins are secreted thereafter [14]. Synthesis of the flagellum is an ordered process that is controlled by a set of hierarchical regulatory checkpoints that ensure proper synthesis and assembly of the flagellar components (Box 1).

The rotor in the hook-basal body (HBB) consists of an axial rod, the FliF (MS) ring (Figure 1), which is embedded in the cytoplasmic membrane, and the C ring, which is composed of FliG, FliM, and FliN [14]. Rotation of the HBB structure is achieved via the motor force generators, MotA

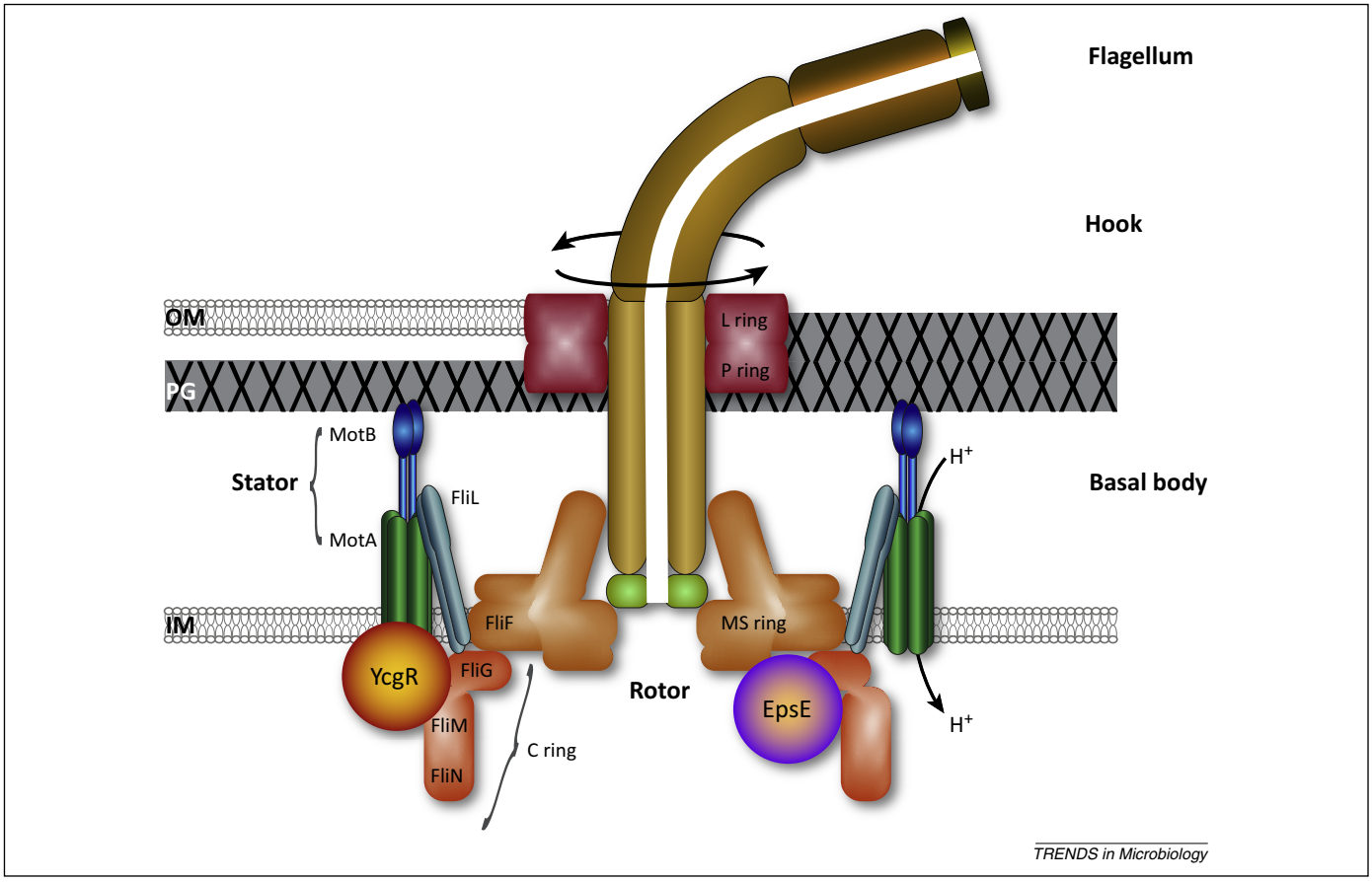

Figure 1. Flagellar structure. Simplified diagram showing the main components of the flagellum: the basal body, hook, and flagellar filament. The motor is composed of the stator (MotA and MotB proteins) and the rotor (C ring, composed of FliG, FliM, and FliN). Ion (H^+ or Na^+) flow through the MotAB channel provides the power to rotate the flagellum. On the left side of the diagram is a schematic of the Gram-negative structure, together with YcgR brake, whereas the right side depicts a Gram-positive envelope and the EpsE clutch protein. Both YcgR and EpsE are functional inhibitors of motor rotation. Abbreviations: IM, inner (or cytoplasmic) membrane; OM, outer membrane; PG, peptidoglycan.

Box 1. Regulation of flagellar genes in enteric bacteria

Enteric bacteria, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, serve as useful examples of the hierarchical control system regulating bacterial flagella. The flagellar regulon of *E. coli* and *Salmonella* is organized into a transcriptional hierarchy that is based on three promoter classes temporally regulated in response to assembly [86]. The flagellar master operon *flhDC* is at the top of this hierarchy and controls the fundamental decision of whether to produce flagella. The *flhDC* operon is expressed from the sole class 1 promoter. The FlhDC proteins form a heteromultimeric complex ($FlhD_4C_2$) that functions as a transcriptional activator to promote σ^{70}-dependent transcription from the class 2 flagellar promoters [87]. The class 2 promoters direct transcription of the genes that encode components of the flagellar C-ring (also known as the motor 'switch'), export apparatus, basal body, and hook, and are needed for the structure and assembly of the hook-basal body (HBB). They also include the gene for the flagellum-specific sigma factor FliA (σ^{28}). On HBB completion, class 3 promoters are transcribed by σ^{28} RNA polymerase [86], which is specific for flagellar class 3 promoters [88] encoding later-assembled components, including flagellin. A σ^{28}-specific anti-sigma factor, FlgM, provides feedback to σ^{28} regarding the state of flagellar assembly [14]. On HBB completion, FlgM is secreted from the cell, presumably through the completed HBB structure, and σ^{28}-dependent transcription ensues. In this way, genes such as the flagellin filament genes, the products of which are needed after HBB formation, are only transcribed when there is a functional motor onto which they can be assembled [14].

and MotB, which are anchored around the basal body and act as stators against the C-ring part of the rotor. MotB is a membrane protein with a peptidoglycan binding domain, whereas MotA interacts with FliG. MotA, MotB, and FliG are specifically involved in torque generation. The MotAB (or PomAB homolog) complex creates an ion channel (H^+ or Na^+ ions), such that ion flow induces a conformational change in MotA that interacts with the C-terminal domain of FliG, resulting in torque generation [15]. Thus, rotation of the flagellar filament is powered through the proton/sodium motive force (PMF/SMF), and not ATP [12]. The filament-generated torque powers the cell to swim through liquid or flagellum-dependent swarming over solid surfaces [8]. Although slight increases in viscosity (by the introduction of crowding polymers, e.g., Ficoll and dextran) enhance swimming speed, high viscosity generally impedes flagellar rotation and performance [16].

As one considers how flagella function to transduce surface signals into a bacterial cell, FlhDC and other master regulators of flagellar gene expression [8,14] stand out as putative checkpoints in the swim-or-stick switch, as do the major genetic regulators of biofilm formation, such as RpoS, CsgD, and CpxR [17]. For example, *rpoS* plays a key role during biofilm formation because it encodes the stationary phase sigma factor (σ^S), which regulates a number of stress-related genes, including CpxR and CsgD, that exert strong negative regulation on flagellar class 3 genes [18], including *ycgR* of *E. coli*, which encodes a protein that acts as a 'monkey wrench' that interacts with the rotor protein FliG to impair rotation [19]. Other proteins that may impair flagellum function in response to the surface-sensing signal include the *Bacillus subtilis* homolog of YcgR, YpfA, and *B. subtilis* EpsE, which acts as a clutch to decouple the motor stator [20], and CheY of *Rhodobacter sphaeroides*, which binds to the motor, acting as a brake [21]. Adding to the complexity, several of these functional regulators of the swim-or-stick switch, for example, YcgR, are controlled or influenced by the secondary messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) [22]. High intracellular cyclic di-GMP levels favor settlement, surface attachment, and biofilm formation, whereas low levels correlate with motility and planktonic behavior [23].

Making sense of the staggering array of controls on the swim-or-stick switch requires a reductionist approach. As such, I have chosen to focus on six models as examples of flagellar mechanosensing: (in order) *Pseudomonas aeruginosa, Vibrio cholerae, B. subtilis, Caulobacter crescentus, Vibrio parahaemolyticus*, and *Proteus mirabilis*. It should be emphasized that bacteria have evolved other mechanisms to sense surfaces that do not directly involve flagella: for example, the Wsp control circuit of *P. aeruginosa* [24,25]. These nonflagellar surface-sensing systems are only briefly discussed here.

Model systems of flagellar mechanosensing: *P. aeruginosa*

P. aeruginosa, a Gram-negative opportunistic pathogen, is a biofilm-forming bacterium that uses a single polar flagellum to swim in liquids and swarm over surfaces [26]. Flagellar motility is required to form a biofilm, is controlled at multiple levels, is regulated by c-di-GMP, and is recognized as a major step leading to lung infections in patients with cystic fibrosis [27]. *P. aeruginosa* responds to growth on agar surfaces by producing c-di-GMP, which stimulates biofilm formation. C-di-GMP affects the activity of the master regulator of flagellar gene expression, FleQ, which inhibits the expression of the *pel* genes required for biofilm exopolysaccharide synthesis. FleQ binds to c-di-GMP, and elevated levels of c-di-GMP *in vivo* relieve the inhibition of *pel* gene expression by FleQ [28]. Transcriptome and proteome measurements of cells in biofilms suggest that expression of virulence genes is upregulated and swarming cells are more pathogenic [29].

Viscosity-dependent regulation of flagellar reversal frequency plays a critical part in the swim-or-stick switch of *P. aeruginosa* [30,31]. Evidence for this role comes from the discovery of surface attachment defective (Sad) mutants that are defective in biofilm formation and have increased swarming motility [32]. In this category are mutants defective in SadC, a diguanylate cyclase that elevates c-di-GMP levels [32]. Strains with defects in SadC show increased flagellar reversal rates in high-viscosity media, similar to those encountered during either biofilm formation or swarming, but not when swimming in low viscosity liquids: for example, nutrient broths [30,32]. SadC receives an unknown environmental signal, perhaps contact with a surface, and transmits this information to the cytoplasm by modulating production of c-di-GMP, which affects flagellar reversals via chemotaxis cluster IV (CheIV cluster) in a viscosity-dependent fashion, which in turn influences the production of the Pel biofilm exopolysaccharide [30,32]. BifA, a c-di-GMP phosphodiesterase, counters SadC activity by decreasing c-di-GMP levels, suppressing swarming motility, and decreasing flagellar reversals [33]. It is worth noting that flagellar reversals are also important in bacterial swimming through semisolid agar: greater reversal

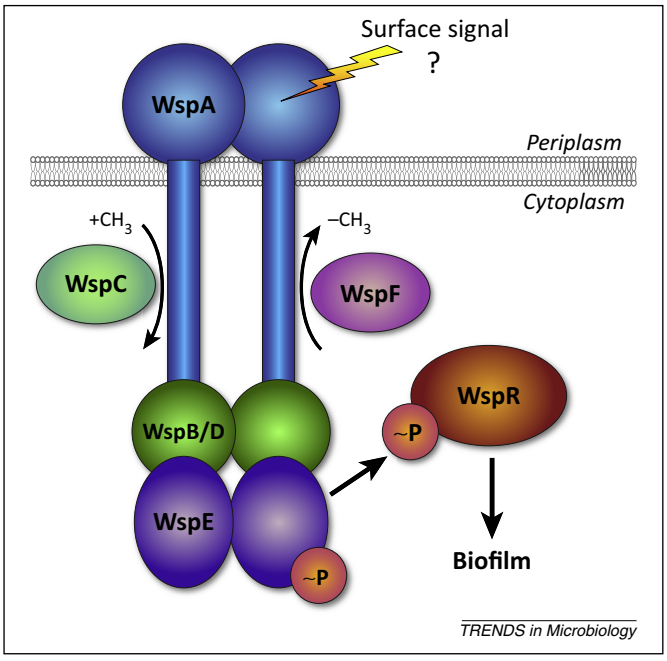

Figure 2. *Pseudomonas aeruginosa* Wsp surface-sensing system. WspA is predicted to be a membrane-bound methyl-accepting chemotaxis protein that detects an unknown signal when grown on a surface. WspB and WspD are CheW-like proteins, WspE is a CheA-like histidine kinase, WspC is a CheR-like methyltransferase, and WspF is a CheB-like methylesterase. Detection of surface growth by WspA results in phosphorylation of WspR, increased synthesis of c-di-GMP, and triggers biofilm formation. Adapted from Huangyutitham, Guvener, and Harwood [25].

frequency results in a higher rate of movement due to changes in direction that prevent the bacteria from becoming trapped in the agar matrix [34]. Adding to the evidence in favor of a flagellar mechanosensing circuit, cells harboring mutations in one of the flagellar stators, MotAB, retain wild-type swimming motility yet have defects in biofilm formation [35].

P. aeruginosa has at least one other surface-sensing system, the Wsp regulatory circuit (Figure 2), which consists of proteins that are homologs of the chemotaxis system, including a membrane-bound chemoreceptor (methyl-accepting chemotaxis protein) homolog, WspA, and a response regulator protein, WspR, which catalyzes c-di-GMP synthesis when phosphorylated [36]. WspA senses surfaces, perhaps by recognizing mechanical stress associated with a surface or cell–cell contact, although the exact nature of the signal is unknown [24]. Unlike chemotaxis chemoreceptors that localize to the pole of a cell, WspA forms patches in the cell, whereas WspR localizes as cytoplasmic clusters, and this clustering is markedly enhanced when cells are grown on a surface [37]. This surface-induced increase in WspR clusters is not affected by changes in agar concentration, viscosity, or defects that affect flagella [24], arguing against involvement of a flagellar mechanosensor in Wsp signaling.

Model systems of flagellar mechanosensing: *V. cholerae*

Flagella and motility are required for biofilm formation by *V. cholerae*, the causative agent of cholera [38,39]. Proper motor rotation is critical in the initial step of biofilm development [40]. It is thought that the attachment of the cell body and flagellum to a surface stops the flagellar motor, indicating that the bacterium senses the increased drag on the motor caused by its interaction with the surface [40]. This suggests that conditions that lead to inhibition of flagellar motor function are critical to the bacterium's response to a surface and its initiation of biofilm formation [41]. A possible mechanism to explain how *V. cholerae* senses the inhibition of flagellar rotation is that when the flagellum's rotation is stopped, the decreased ion flow through the membrane-embedded motor is interrupted. This results in an increase in membrane potential ($\Delta\Psi$) and a hyperpolarized membrane that would be sensed by putative ion flux sensors in the flagellar basal body or in the membrane [42]. This hypothesis is supported by the fact that experimental dissipation of $\Delta\Psi$ blocks transition from transient to permanent attachment [42,43].

Furthermore, the swim-or-stick switch of *V. cholerae* decreases the expression of motility genes while increasing the expression of genes involved in biofilm formation and virulence [44]. Reducing motility by inhibiting flagella rotation through disrupting the SMF using inhibitory drugs, high media viscosity, or specific mutations results in an increase in the expression of *toxT*, a gene encoding a transcription regulator of the primary virulence factors [45], suggesting a connection between flagellar motor function and the biofilm phenotype that may involve impairment of Na^+ ion flow through the stators and a disruption of SMF [46].

Model systems of flagellar mechanosensing: *B. subtilis*

B. subtilis, a Gram-positive, non-pathogenic, spore-forming bacterium, uses flagella to swim in liquid environments and to swarm over surfaces. When cells come into contact with a surface, transcription begins of genes that are required for synthesis of a biofilm matrix. This matrix is composed of multiple proteins and exopolysaccharides (EPS), such as those synthesized by the products of the *eps* operon [47], and γ-poly-DL-glutamic acid (γ-PGA), synthesized by the *pgs* operon enzymes [48]. Matrix synthesis is controlled by a complex regulatory network that includes three major transcriptional proteins: Spo0A, ComA, and DegU, the details of which may be found in Box 2 and in a recent review [7].

The *B. subtilis* flagellum serves a mechanosensory role in biofilm formation [49]. First, deletion of *motB* results in nonmotile cells that produce mucoid colonies due to increases in *pgsB* expression and γ-PGA synthesis, which coincide with increases in transcription of *aprE* and protease activity, all of which are DegU∼P-dependent processes [49,50]. Second, disrupting proton flow through the MotAB stator, and therefore flagellar rotation, increases DegU∼P activity [49]. Third, inhibition of flagellar rotation, either by overexpressing EpsE or by binding flagella with anti-Hag (flagellin) antibody results in upregulation of *degU* and an increase in exoprotease production. Fourth, a *degU32* hy mutation, known to increase DegU∼P levels [51], results in highly mucoid colonies with elevated γ-PGA biosynthesis, whereas disruption of DegS in a Δ*motB* background reverted colonies to a wild-type phenotype. Therefore, the DegS kinase is required for enhancement of the DegU∼P-dependent processes in Δ*motB* cells. The signal that is detected by the cytoplasmic DegS kinase to

Box 2. *B. subtilis* biofilm regulation

Control of *Bacillus subtilis* biofilm matrix gene expression is under the control of a complex regulatory network that includes three major transcriptional proteins: Spo0A, ComA, and DegU [7]. Signals relayed through this network ultimately trigger expression of the extracellular biofilm matrix amyloid protein, TasA, and proteins required for the synthesis of the biofilm matrix exopolysaccharides, respectively [7]. A third matrix protein, BslA (formerly YuaB), is also required for biofilm formation [89]. The expression of *bslA* gene is activated by phosphorylated DegU (DegU∼P) [90,91].

The DegS–DegU two-component regulatory circuit also acts to regulate biofilm formation. DegS is a cytoplasmic bifunctional protein that exhibits kinase and phosphatase activities. As a kinase, DegS phosphorylates DegU, which acts to control a myriad of processes involved in biofilm formation, including motility and assembly of the flagellar basal body [92], as well as synthesis of γ-PGA [48]. DegU activity is controlled at the level of *degU* transcription, through DegU phosphorylation, and by DegU∼P activity, and the protein has regulatory activity in both its unphosphorylated and phosphorylated states [92] (Figure 3).

In the absence of appropriate signals, DegS is at a low level and unphosphorylated, and the level of phosphorylated DegU is also low. Low levels of DegU∼P induce flagella biosynthesis and swarming motility by binding to the promoter region of the *fla/che* operon and thereby inducing flagellar gene transcription, which also requires the activity of two other proteins, SwrA and SwrB, as well as completion of the flagellar basal body [52].

Elevated levels of DegU∼P induce synthesis of BslA, leading to biofilm development, as well as an extracellular protease (AprE; subtilisin) and γ-PGA, all of which are regulated in part by a positive feedback loop [93]. DegU∼P inhibits the *fla-che* promoter for class 2 genes [94], and also indirectly inhibits σ^D (encoding the flagellar gene specific sigma factor) by activating the expression of the anti-sigma factor FlgM [52]. DegU∼P-dependent activation of FlgM is essential to inhibit flagellin expression when assembly of the basal body is disturbed, which results in a non-motile, cell chaining phenotype [52]. This suggests that the DegS–DegU two-component system may either directly or indirectly senses cell motility, flagellar assembly, or the status of flagellar motor's operation.

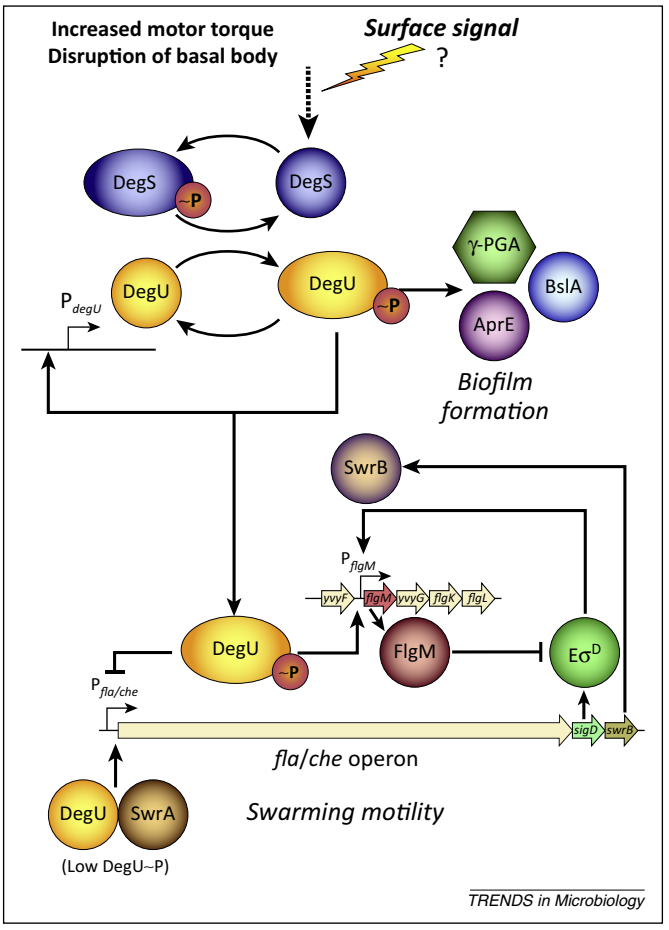

Figure 3. Inhibition of flagellum rotation increases biofilm formation and decreases swarming motility in *Bacillus subtilis*. Loss of motility or disruption of assembly of the flagellum results in an unknown signal (hashed arrow) that upregulates the DegS–DegU two-component system, leading to increase in DegU∼P, BslA, γ-PGA, and subtilisin (AprE), and a decrease in *fla/che* operon expression. Conversely, unphosphorylated DegU, along with SwrA and SwrB, increases expression of the *fla/che* operon leading to flagellar synthesis and swarming motility. Lines with arrowheads indicate positive effects, whereas T-end lines indicate inhibitory effects. Promoters are shown as small square-angled arrows. Adapted from Belas [6].

trigger phosphorylation of DegU is unknown, but regulation of *flgM* by DegU-P suggests that the DegS–DegU two-component system may either directly or indirectly sense the completion state of the flagellum [52].

Further evidence comes from a recent report by the Kearns laboratory [50]. In a screen for mutations that reduced mucoidy in a *motA* mutant, they found a class of transposon insertions that disrupted proteins involved in the assembly of the flagellar filament (FliD, FliT, and FlgL), suggesting that disruption of filament cap assembly (and hence flagellum function) reduces γ-PGA synthesis. Although the precise roles of FliD, FlgL, and FliT remain unclear, the underlying message is that the *B. subtilis* flagellar stator regulates γ-PGA synthesis and biofilm formation [50].

How does DegS sense the lack of flagellar rotation or incomplete flagellum assembly? Several scenarios are conceivable (Figure 3). First, similar to *E. coli* YcgR [19], DegS could directly interact with cytoplasmic components of the flagellar motor to act as a brake. Second, DegS may interact indirectly through a protein component of the basal body, such as the stator-associated transmembrane protein FliL, which is part of the *P. mirabilis* surface-sensing mechanism [53]. Third, inhibited flagellar rotation is likely to cause changes in $\Delta\Psi$ or cellular energy status due to restricted proton (ion) flux through the MotAB stators [42], and this change may be sensed by DegS. Restriction of rotation and the resulting torque on the flagellar motor could also induce stress in the cell wall, as described for the *E. coli* Rcs and Cpx surface-sensing circuits [54,55].

Model systems of flagellar mechanosensing: *C. crescentus*

The freshwater, Gram-negative alphaproteobacterium *C. crescentus* has a dimorphic life cycle resulting from an asymmetric cell division, during which a motile swarmer cell is born to a stalked, non-motile cell [56]. The smaller swarmer cell, which possesses a polar flagellum that is colocalized with one to seven pili, is motile and capable of chemotaxis. Flagellum-driven motility and adherence mediate the initial reversible adhesion by overcoming surface electrostatic repulsion [57]. The larger stalked cell possesses a polar stalk that attaches it to a substrate through a polysaccharide-based holdfast, an *N*-acetylglucosamine-containing polysaccharide. This permits formation of a stable biofilm [57,58].

The transition from motile to sessile phase of *C. crescentus* swarmer cells is the result of interplay between pili

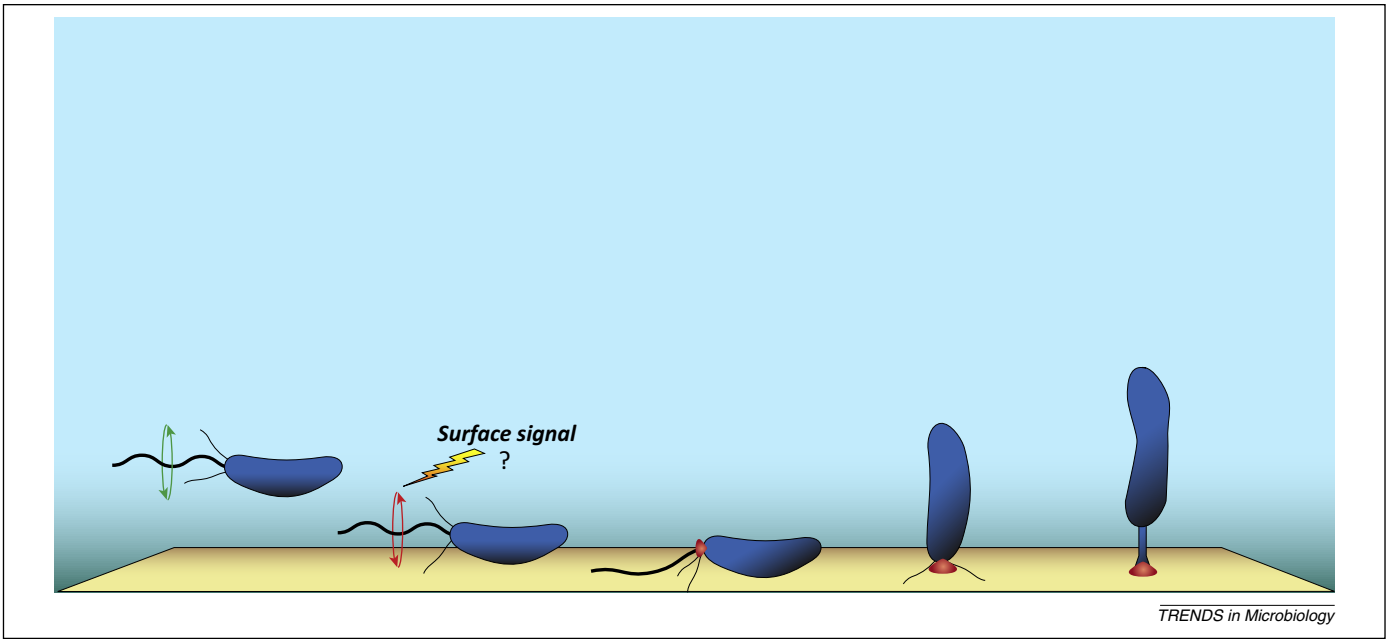

Figure 4. Flagellar mechanosensing in *Caulobacter crescentus* involves inhibition of flagellar rotation, resulting in just-in-time production of the holdfast polysaccharide adhesin. From left to right: swimming swarmer cells possess a single flagellum and polar pili. As a cell nears a surface, surface contact results in the rapid pili-dependent arrest of flagellum rotation and concurrent stimulation of polar holdfast adhesive polysaccharide (depicted here as a red cone) and ultimately the formation of a stalk cell. Green circular arrows indicate rotating flagellum; red circular arrows indicate rotation is inhibited. Adapted from Kirkpatrick and Viollier [62].

and flagellum rotation: for example, on encountering a surface with an ensuing inhibition of flagellar rotation, *Caulobacter* immediately produces holdfasts and attaches [59]. Evidence suggests that initial cell tethering occurs via the flagellum, with holdfast synthesis beginning 1 to 2 minutes after the swarmer cell encounters a surface (Figure 4). Pili do not have a major role in initial tethering to surfaces, but instead mediate the transition from reversible to irreversible adhesion by stopping flagellum rotation and the resulting activation of the holdfast synthesis machinery [59].

Inhibition of the flagellar motor is involved in *C. crescentus* biofilm development. Li *et al.* [59] used Ficoll and dextran, which reduce the solvent volume available to cells, thus obstructing flagellar rotation. Addition of these polymers resulted in rapid inhibition of flagellar rotation and holdfast synthesis. The same effect was observed with Δ*pilA* cells, indicating that alternative methods of blocking flagellar rotation are sufficient for stimulation of holdfast production, presumably by bypassing the need for pili-mediated initial attachment and jamming of the flagellum [59].

Polar polysaccharide and holdfast synthesis is a conserved phenomenon among alphaproteobacterial species [60]. Therefore it may not come as a surprise that surface-contact-mediated flagellar mechanosensing has also been observed in other alphaproteobacteria, such as *Asticcacaulis biprosthecum* and *Agrobacterium tumefaciens* [59]. In each of these species, a single cell responds to initial contact with a surface by triggering 'just-in-time' adhesion production that leads to biofilm formation [59].

We currently do not know the mechanism by which inhibition of flagellar rotation triggers holdfast production, but several possibilities exist. One possible pathway may involve FliL, an inner membrane component of the flagellar basal body that has been proposed to act as a sensor, because flagella formed by a *Caulobacter* Δ*fliL* mutant cannot rotate [61]. Alternatively, a transient change in $\Delta\Psi$ might signal holdfast production in *Caulobacter* through an indirect mechanism [62].

Model systems of flagellar mechanosensing: the swarming bacteria *V. parahaemolyticus* and *P. mirabilis*

Flagella are not only used for swimming through liquids but are also required for swarming motility over solid surfaces. Although many species swarm (defined in this review as a motile biofilm), including *Aeromonas*, *Azospirillum, B. subtilis, E. coli, Rhodospirillum, Rhizobium, Salmonella, Serratia*, and *Yersinia*, only a few (two notable examples are *V. parahaemolyticus* and *P. mirabilis*) do so after a surface-induced physiological differentiation that results in an elongated, highly flagellated swarmer cell [63]. Although there are numerous differences between the two species, the common feature uniting *V. parahaemolyticus* and *P. mirabilis* is the requirement for a flagellar mechanosensor that detects the presence of a surface and relays that information to induce swarmer-cell-dependent gene expression. The following summarizes the data leading to this conclusion.

Physical conditions that inhibit flagellar rotation induce swarmer cell differentiation. *V. parahaemolyticus* produces two types of flagella: a constitutively synthesized, single, polar, sheathed flagellum driven by SMF and surface-induced, swarmer-cell-dependent, lateral flagella that number in the hundreds to thousands per cell and whose motors rotate by PMF [64]. It is the polar flagellum that acts as a flagellar mechanosensor, with evidence coming from experiments showing that: (i) induction of lateral flagella synthesis is signaled by physiological conditions that inhibit polar flagellar rotation [65,66]; (ii) anti-flagellin antiserum is capable of tethering flagella together, and

thereby preventing their rotation, triggers differentiation of *V. parahaemolyticus* swarmer cells; (iii) the sodium-channel-blocking drug phenamil, which poisons the energy source that drives polar (but not lateral) flagellar rotation, induces swarmer cell differentiation [66]; (iv) mutations that cause defects in swimming motility by the polar flagellum induce transcription of lateral flagellar genes in liquid; and (v) mutants with defects in the motor stator proteins induce swarmer cell differentiation [67]. Together, these results suggest that when the rotation of the polar flagellum of *V. parahaemolyticus* is reduced (for example, as it comes into contact with a surface), the cell senses and responds to this signal by inducing synthesis of swarmer cells. The identity of the signal is currently not known. The cue may be torque or external force on the motor, which is then sensed by an unknown mechanism, but the results from perturbing ion flow using phenamil or through construction of stator mutants argue that what is being sensed is a reduction or change in sodium ion flux. The *V. parahaemolyticus* surface-signaling pathway is also not known, but is likely to be mediated through the σ^{54}-dependent master regulator, LafK, which controls transcription of the lateral flagella (*laf*) genes [67].

P. mirabilis, a Gram-negative *Enterobacteriaceae* that is often associated with urinary tract infections [68], synthesizes a single type of flagella, such that vegetative swimmer cells possess four to eight peritrichous flagella, whereas differentiated swarmer cells are elongated and hyperflagellated (in a similar way to *V. parahaemolyticus*). Similar to *V. parahaemolyticus, P. mirabilis* swarmer cell differentiation is triggered by physical conditions that inhibit the rotation of the peritrichous flagella of the swimmer cell [69]. Agar surfaces, viscous liquids, and antibodies specific to flagellar proteins, such as flagellin, all induce differentiation and are thought to increase torque on the motor [69]. Correct flagellar assembly is also required for differentiation, and certain flagellar mutations result in constitutive swarmer cell elongation [69,70]. In general, mutations in *P. mirabilis* flagellar genes result in cells that do not differentiate and do not swarm, but there are exceptions: mutations in *fliL*, *fliG*, and to a lesser extent *fliF*, result in the inappropriate production of swarmer-like cells (referred to as 'pseudoswarmer cells') in noninducing conditions: for example, broth [69]. The production of a pseudoswarmer cell suggests that these mutants are defective in their response to a surface and behave as though they are always on a surface [53]. Thus, the *P. mirabilis* flagellum functions as a mechanosensor of the surface signal.

The *P. mirabilis* surface signal ultimately affects the flagellar master regulator, $FlhD_4C_2$, encoded by *flhDC*, the transcription of which is upregulated during swimmer-to-swarmer cell differentiation and the onset of swarming motility [71]. Regulation of *flhDC* is complex and involves a myriad of controls; however, only a few of these regulatory circuits have been shown to interact with the *P. mirabilis* surface-sensing pathway and are discussed next.

Both lipopolysaccharide (LPS) and O-antigen play a part in *P. mirabilis* surface sensing [70,72]. Evidence includes the observation that, when placed on solid surfaces, cells with mutations in *waaL* (*rfaL*), encoding O-antigen ligase, and wzz (*cld*), encoding a chain-length determinant for O antigen, do not activate *flhDC* and the flagellar gene cascade [72]. It is believed that loss of O-antigen or perturbation of LPS composition or structure creates cell envelope stress, which is sensed by the Rcs phosphorelay regulatory circuit [73].

The enteric bacterial Rcs phosphorelay is more complicated than the canonical two-component system; it is a phosphorelay that consists of the outer membrane activator protein RcsF, the hybrid sensor kinase RcsC, the histidine phosphotransferase RcsD, the response regulator RcsB, and the transcription factor RcsA. Via an unknown mechanism, RcsF senses signals external to the cell and relays that information through the outer membrane to RcsC, which initiates the phospho-cascade. The result is phosphorylated RcsB [54]. In complex with RcsA, phosphorylated RcsB binds a DNA site downstream of the *flhDC* promoter, inhibiting transcription [74]. Mutations in *P. mirabilis* RcsD result in precocious swarming (that is, swarming motility initiates earlier than in the wild type) and a pseudoswarmer phenotype, implicating the Rcs pathway in surface sensing [75].

Work by the Rather laboratory has implicated two other proteins, UmoB and UmoD, in the pathway that leads from the external signals to the sensor kinase RcsC [72,73]. The four Umo (upregulator of the master operon) proteins (UmoA–D) are associated with the cell envelope [76]. They were discovered in a search for suppressors of the swarming defect that results from mutation in the *flgN* flagellar chaperone, and increase transcription of *flhDC* [76]. The UmoB homolog, *yrfF* (encoding IgaA), is involved in the Rcs signal transduction pathway of *Salmonella* and *Serratia marcescens* [63,77]. The homolog of UmoD is *E. coli ycfJ*, a gene of unknown function that is upregulated in *E. coli* biofilms [78].

Figure 5 depicts a potential model of surface contact and sensing that integrates the flagellar mechanosensor and accounts for LPS and O-antigen involvement in Rcs-dependent regulation of *flhDC* transcription [73]. In this model, FliL, which is part of the flagellar mechanosensor pathway [53], interacts with the Umo proteins, probably UmoA. Surface contact interactions with LPS and/or O-antigen trigger conformational changes in the outer membrane that result in decreased activity of RcsF and/or increased activity of UmoD. This results in activation of UmoB by two mechanisms, direct activation by UmoD and reduced activity of RcsF, an inhibitor of UmoB [73]. The activated form of UmoB then inhibits the Rcs phosphorelay, resulting in reduced levels of phosphorylated RcsB and derepression of the *flhDC* operon. Thus, *P. mirabilis* may have two mechanisms to sense a surface: a flagellar mechanosensor and a surface contact sensor working through the Rcs stress response to activate *flhDC*.

FliL and its role in flagellar mechanosensing

Homologs of FliL are found in nearly all flagellated bacterial species, frequently as the first gene in a class 2 operon, *fliLMNOPQR*, which includes genes for the motor/switch (*fliMN*) and the export apparatus (*fliOPQR*). FliL is a small inner membrane protein (*P. mirabilis* FliL is a 160-amino-acid, 18.2 kDa protein), with a single transmembrane helix located in the N-terminal domain

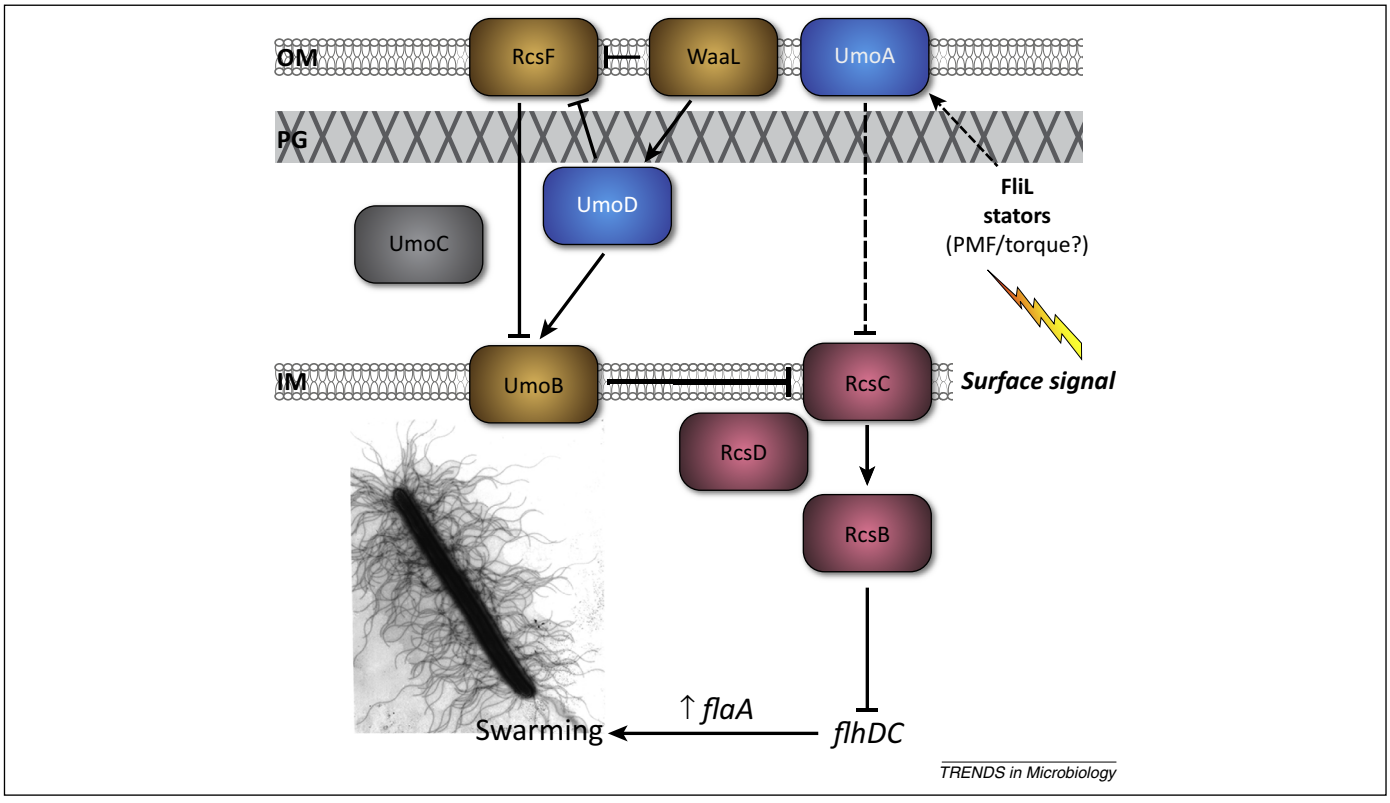

Figure 5. Surface-sensing in *Proteus mirabilis*. Conditions that inhibit flagellar rotation induce surface-dependent swarmer cell differentiation mediated by FliL, a flagellar protein thought to be associated with the MotAB stator. The role played by FliL is unknown, but it may empower the motor, perhaps through modulating ion flow. The signal mediated through FliL involves UmoA, which upregulates *flhDC*, encoding the flagellar master regulator, $FlhD_4C_2$, the activity of which is required for swarming and swarmer cell differentiation. A second nonflagellar mechanosensing circuit has been proposed that senses cell wall stress or perturbations through lipopolysaccharide (LPS) and O-antigen changes, mediated by WaaL, UmoD, and UmoB. Both mechanosensors are likely to control the activity of the Rcs regulatory circuit, which in turn inhibits *flhDC* expression. Abbreviations: IM, inner (or cytoplasmic) membrane; OM, outer membrane; PG, peptidoglycan. Unbroken lines indicate interactions determined by empirical tests, broken lines are hypothetical interactions, arrowheads indicate positive effects, whereas T-end lines indicate inhibitory effects.

(NTD), such that the NTD is in the cytoplasm, whereas the C-terminal domain (CTD) is in the periplasm [69,79,80]. Despite its ubiquity, FliL is poorly conserved among species, with only 51% identity between the FliL proteins of *P. mirabilis* and *Salmonella*.

In *C. crescentus* and *Silicibacter* sp. TM1040, mutations in *fliL* result in nonswimming cells [61,81], whereas *fliL* defects in enteric bacteria have only minor effects on swimming but prevent swarming motility over agar, in part caused by a failure of the flagellar rod to withstand higher torque encountered on the agar surface [53,79]. This implies that FliL must reside close to the flagellar HBB, and this is borne out from the results of cryo-electron tomography showing that FliL is located close to the HBB and in association with the MotAB stators in *Borrelia burgdorferi* [82]. Evidence supporting an interaction between FliL and the stators comes from *fliL* mutations in *R. sphaeroides*, where loss of FliL inhibits swimming. This defect can be overcome by suppressor mutations that map to a domain of MotB that is known to increase PMF [80]. Furthermore, Partridge and Harshey recently demonstrated that overexpressing MotAB together with FliL allows *Salmonella* to swarm over agar surfaces that prevent wild-type swarming [83].

These observations suggest that FliL enhances the output of the stators or controls the flow of ions through the motor during times of increased flagellar torque when cells contact and interact with a surface. Indeed, several of the flagellar mechanosensor model systems discussed earlier are affected by ion flow through the motor. Perhaps FliL acts as a governor or an ion flow regulator during times of increased motor torque to limit and control the flow of ions through the motor, thereby preventing hyperpolarization of the membrane. However, the function of FliL is likely to be more complicated than this; recent reports [84,85] indicate that the stators of *E. coli* change their structure in response to changes in external load imposed on the motor, irrespective of the presence or absence of FliL [85], thus putting the true function of FliL into question. Further research is needed to resolve the interaction of FliL and the stator.

FliL may have additional, species-specific functions. For example, *P. mirabilis* FliL uniquely displays viscosity-dependent functions, indicating that the protein has a direct role in the surface-sensing pathway [53]. FliL defects affect the induction of swarming-dependent proteins [69] and prevent viscosity-dependent sensing of surfaces and viscosity-dependent transcription [53]. Notable among the genes affected by FliL defects is *umoA*. Increased expression of *umoA* is apparent in *P. mirabilis fliL*-knockout mutations, including strains in which only the CTD of FliL is mutated [53]. This implicates the periplasmic domain of FliL in surface sensing. The pathway leading from FliL to UmoA is unknown, as are the downstream components of

the *P. mirabilis* surface-sensing pathway that lead ultimately to $FlhD_4C_2$.

Concluding remarks and future directions

How does a bacterium know it is in contact with a surface? Hopefully, the examples provided in this review offer one answer, if not the answer: they use a flagellar mechanosensor. These mechanosensors utilize the rotating flagellum and are able to detect subtle changes in the function of their motors during surface contact. However, although prevalent in many bacterial species, flagellar mechanosensing is not the only means used to detect and respond to surfaces. Obviously, not all surface-sensing pathways directly involve flagella or their function, and nonmotile bacteria form biofilms without the need for flagella. For this reason, I included at least two examples of non-flagellum-mediated surface sensing (the *P. aeruginosa* Wsp surface-sensing circuit and the *P. mirabilis* LPS/O-antigen circuit). With that caveat out of the way, I firmly believe that a better understanding of the surface-sensing function of the flagellar nanomachine is critical to our understanding of biofilm formation.

In contemplating how flagellar mechanosensing function, two features stand out in the model systems described here. The first is that surface sensing takes place at the level of the flagellar stators, and second, surface contact seems to alter the flow of ions through the stators and perturb PMF or one of its components, $\Delta\Psi$ and ΔpH. Such changes could result in a hyperpolarized membrane and/or acidification of the cytoplasm (in the case of increased unabated proton flow into the cell). Understanding the molecular mechanisms the cell uses to detect and respond to a hyperpolarized membrane or to cytoplasmic acidification resulting from stator impairment is a high priority that offers great potential for future research on flagellar mechanosensory systems.

Many questions remain to be answered (Box 3). What cellular components (proteins, regulatory RNAs, small molecule effectors, and so on) constitute the surface-sensing pathway? What is the signal sensed by WspA or the *P. mirabilis* LPS/O-antigen surface-sensing circuit? For species that have multiple means of sensing a surface (for example, *P. aeruginosa* SadC and Wsp surface-sensing circuits), how is the hierarchy of biofilm control orchestrated? Do the pathways converge or remain separate, controlling separate and unique components needed to initiate biofilm formation?

I am of the opinion that study of the flagellum's function in sensing and response to surfaces is crucial for understanding biofilm development, for the development of strategies to prevent biofilm formation, and in the fabrication of stealth surfaces that go undetected by the flagellar mechanosensor. Using mechanosensing flagella as a model provides a tractable system to understand surface sensing that will have implications beyond flagellated bacteria, given the central role of the proton-driven motors identified in this review. Moreover, although the flagellar motor is the best-understood nanomachine, one big mystery is the mechanism by which ion flow generates rotational force. Gaining a better understanding of the flagellar mechanosensor is certainly a step in the right direction towards answering this question.

Box 3. Outstanding questions

- Do other motile bacteria with less apparent phenotypes use their flagella as surface sensors?
- How is inhibition of flagellar rotation coupled to transcriptional control of biofilm formation?
- Does FliL sense motor rotation or ion flow? If so, how?
- What component(s) of the flagellar motor and/or basal body does FliL interact with?
- What proteins are in the pathway leading from FliL to the regulatory proteins that control biofilm formation?
- Is FliL involved in surface sensing of *Caulobacter crescentus, Pseudomonas aeruginosa, Vibrio parahaemolyticus,* and other species?
- How does DegS sense the lack of flagellar rotation or incomplete flagellar assembly? Is FliL involved?
- What alternative mechanisms transduce inhibition of flagellar rotation to induce biofilm formation?
- What factors control the lateral placement of Wsp clusters in *P. aeruginosa*?
- How are other known surface-sensing systems, that is, the CpxR envelope stress system [18,95,96], CsgD, CsrA, RpoS, and so on, interconnected to flagellar rotation surface sensing?
- What mechanisms are involved in repressing flagellar mechanosensing of a surface, thereby permitting bacteria to leave a biofilm?

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