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MicroCommentary

When the swimming gets tough, the tough form a biofilm

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Summary

Bacteria live either as independent planktonic cells or as members of surface-attached communities called biofilms. Motility and biofilm development are mutually exclusive events, and control of the phase of this 'swim-or-stick' switch involves the ability of the bacterium to sense and respond appropriately to a surface. Cairns *et al*. (2013) report that the *Bacillus subtilis* flagellum functions in surface-sensing. Using mutants of *B. subtilis* that prevent flagellum rotation, they measured the expression and activity of DegU, the response regulator of the two-component DegS–DegU circuit. DegU activity and *degU* transcription increased when flagellum rotation was prevented, and were dependent on the DegS kinase. Inhibiting flagellar rotation by overexpressing the EpsE flagellar 'clutch' or addition of anti-flagellin antiserum also increased *degU* transcription and activity. These results suggest *B. subtilis* senses restriction of flagellum rotation as the cell nears a surface. Inhibition of the flagellum activates the DegS–DegU circuit to turn on biofilm formation, i.e. the flagellum is acting as a mechanosensor of surfaces. *B. subtilis* joins an ever-expanding group of bacteria, including species of *Vibrio*, *Proteus* and *Caulobacter* that use the flagellum as a surface sensor.

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 (Monds and O'Toole, 2009). Formation of a biofilm is a developmental process that begins when a bacterium attaches to a surface and progresses through a series of morphological and physiological changes in the cells as the biofilm matures. Biofilms have major clinical relevance as their formation provides a protective environment against environmental stresses, immune responses, antibacterial agents and antibiotics (Karatan and Watnick, 2009). Moreover, the formation of biofilms on man-made surfaces, such as those associated with heat exchangers or the hulls of ships, often leads to decreased efficiency and performance, and increased cost to remove the bacterial layer.

It is abundantly clear that biofilm formation is regulated in response to environmental conditions and cues that vary among different species. Moreover and in contrast to the wealth of knowledge on the events and mechanisms involved in biofilm maturation, i.e. those events occurring *after* a cell's initial contact with a surface, we know relatively little about the mechanisms used by bacteria to sense a surface. How does a bacterial cell know it has contacted a surface? What mechanisms are used to detect a surface, and how does a bacterium transduce this exterior 'surface signal' into its cytoplasm to ultimately control gene expression and biofilm formation?

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 (Monds and O'Toole, 2009). Therefore, as a bacterium nears a surface, a decision occurs between remaining in a pelagic lifestyle and becoming sessile. Recent evidence suggests that what determines the phase of the 'swim-or-stick' bimodal switch in biofilm formation is not simply stochastic, rather control of this decision involves the ability of the bacterium to sense and respond appropriately to a surface, a sensory transduction mechanism referred to as surface sensing (Otto and Silhavy, 2002). In several bacterial species, flagella are not only required as a mechanical device for propulsion, but also play a critical role in the initial stages of surface adhesion that leads to the formation of a biofilm.

Bacteria swim by rotating one or more rigid helical flagella that may be subdivided into three substructures (Chevance and Hughes, 2008). The first to be assembled is the basal body that anchors the flagellum to the cell membrane, provides the power for rotation, and the apparatus for secretion of the more distal components.

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The hook 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 composed 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 (Chevance and Hughes, 2008). Synthesis of the flagellum is an ordered process controlled by a set of regulatory checkpoints that ensure proper synthesis and assembly of the flagellar components.

The rotor in the hook-basal body (HBB) consists of an axial rod and the FliF ring (Fig. 1), embedded in the cytoplasmic membrane, and the C ring, composed of FliG, FliM and FliN (Chevance and Hughes, 2008). Rotation of the HBB structure is achieved via the motor force generators, MotA and MotB, anchored around the basal body that act as stators against the C-ring rotor. MotB is a membrane protein with a peptidoglycan-binding domain, while MotA interacts with FliG. MotA, MotB and FliG are specifically involved in torque generation. The MotAB 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 (Zhou *et al*., 1998). The torque generated by the filament powers swimming through liquid and flagellum-dependent swarming motility over solid surfaces (Kearns, 2010).

Bacillus subtilis, a Gram-positive, non-pathogenic, soil-dwelling bacterium, serves as an exceptional experimental model organism to study biofilm formation. Biofilms are produced when cells in contact with a surface initiate expression of genes required for an extracellular matrix composed of multiple proteins and exopolysaccharides (EPS), such as those synthesized by the products of the EPS operon (Marvasi *et al*., 2010) and γ-poly-DL-glutamic acid (γ-PGA) synthesized by the *pgs* operon enzymes (Stanley and Lazazzera, 2005).

Control of *B. subtilis* biofilm matrix gene expression is under the control of a complex regulatory network that includes three major transcriptional proteins: Spo0A, ComA and DegU (Vlamakis *et al*., 2013). Signals relayed through this network ultimately trigger activation of a multicomponent phosphorelay that results in the phosphorylation of Spo0A (Burbulys *et al*., 1991). Low levels of Spo0A~P promote the transcription of the *tapA-sipW-tasA* and *epsA-O* operons (Fujita *et al*., 2005; Chai *et al*., 2011), which encode the extracellular biofilm matrix amyloid protein, TasA and proteins required for the synthesis of the biofilm matrix EPS respectively (Vlamakis *et al*., 2013). In addition to possessing glycosyltransferase activity required for EPS synthesis, EpsE functions as a molecular clutch that inhibits flagellar rotation by interacting with the flagellar motor switch protein, FliG (Blair *et al*., 2008; Guttenplan *et al*., 2010). This action ensures that cells shut off motility when matrix production occurs to initiate biofilm formation. A third matrix protein, BslA (formerly YuaB), is also required for biofilm formation (Kobayashi and Iwano, 2012). The expression of *bslA* gene is activated by phosphorylated DegU (DegU~P) (Kobayashi, 2007; Ostrowski *et al*., 2011).

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 (Murray *et al*., 2009), as well as synthesis of γ-PGA (Stanley and Lazazzera, 2005). 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 (Fig. 1) (Murray *et al*., 2009).

The signal(s) sensed by DegS leading to phosphorylation of DegU remain nebulous, but changes in osmolarity, carbon levels or amino acid starvation may be involved. 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 (Hsueh *et al*., 2011).

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 (Verhamme *et al*., 2009). Recently, the Kearns laboratory reported that DegU~P also indirectly inhibits σ^D (encoding the flagellar gene-specific sigma factor) by activating the expression of the anti-sigma factor FlgM (Hsueh *et al*., 2011). 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 (Hsueh *et al*., 2011). This suggests that the DegS–DegU two-component system may either directly or indirectly sense cell motility, flagellar assembly or the status of flagellar motor's operation.

Cairns *et al*. (2013) provide results showing that the *B. subtilis* flagellum serves a mechanosensory role in biofilm formation. First, deletion of *motB* resulted in cells harbouring flagella that did not rotate. $MotB^-$ colonies are mucoid due to increases in *pgsB* expression and γ-PGA synthesis, which coincide with increases in transcription of *aprE* and protease activity, all DegU~P-dependent processes. In Δ*motB* cells, transcription of *degU* is fourfold

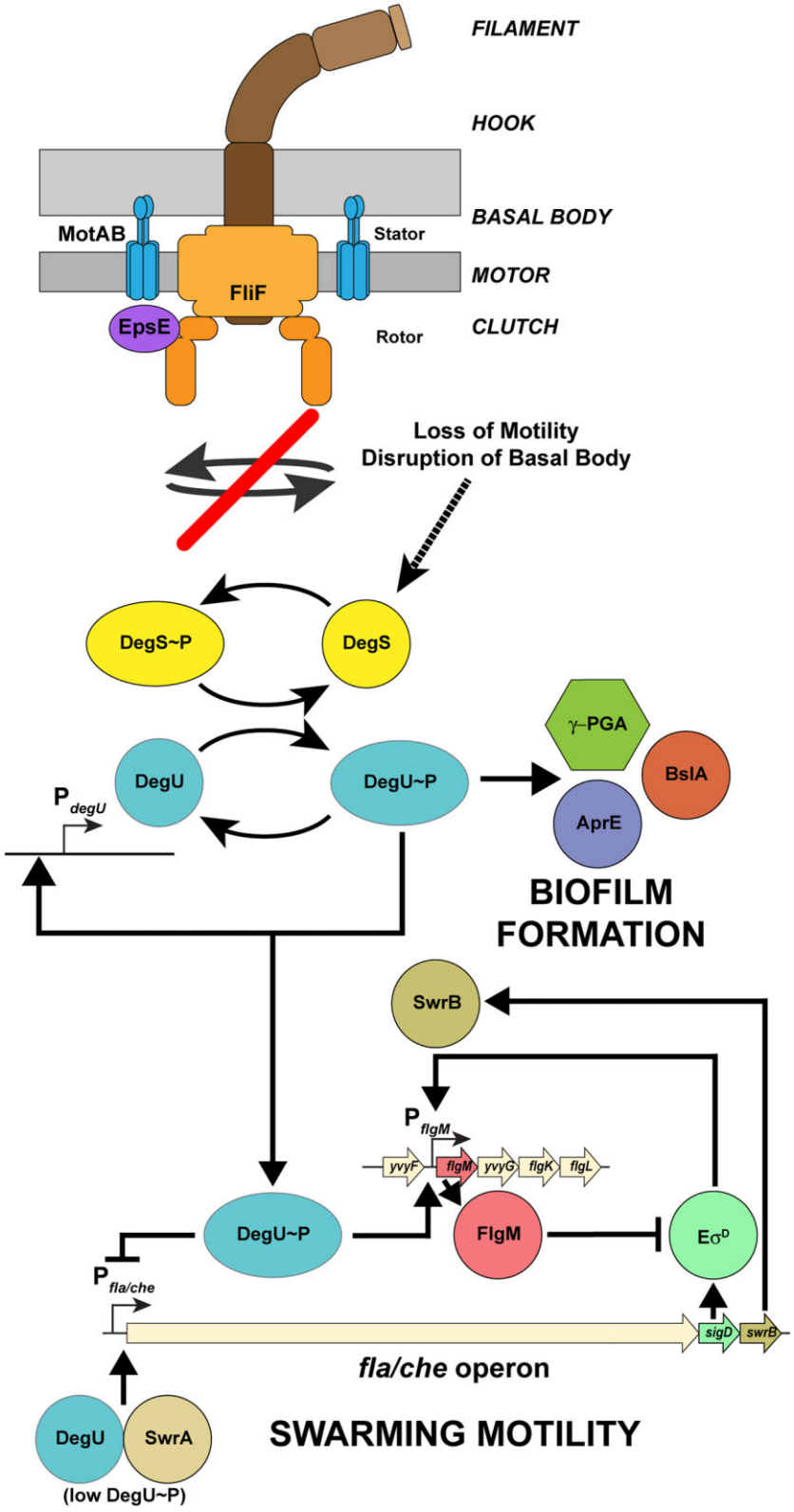

Fig. 1. Inhibition of flagellum rotation increases biofilm formation and decreases swarming motility. From top to bottom: Cartoon of a Gram-positive flagellum with the MotAB stator in blue, the rotor in orange, and EpsE (clutch) in purple. Loss of motility or disruption of assembly of the flagellum results 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, while T-end lines indicate inhibitory effects. Promoters are shown as small square-angled arrows.

higher, with a corresponding increase in DegU~P levels, which could be complemented by ectopic expression of *motB*$^+$. Similar results were obtained with a *motAB* mutant. A *degU32* hy mutation, known to increase DegU~P levels, resulted in highly mucoid colonies with elevated γ-PGA biosynthesis, while disruption of DegS in a Δ*motB* background reverted colonies to a wild-type phenotype. Therefore, DegS kinase is required for the upregulation of the DegU~P-dependent processes in Δ*motB* cells.

Is flagellar motor torque involved in DegU~P levels? A *motB* D24A mutation was constructed that, by homology to *Escherichia coli* MotB, disrupts proton flow and thereby eliminates torque. Expression of *motB* D24A (a dominant-negative allele of *motB*) at an ectopic site was unable to complement the Δ*motB* defect, and *motB* D24A cells produced more protease and γ-PGA than wild-type, suggesting that disrupting proton flow through the MotAB stator, and therefore flagellar rotation, increases DegU~P activity.

Cairns *et al*. (2013) used two other means to inhibit flagellar rotation. First, they overexpressed an *epsE* allele (*epsE* D94A), which retains the flagellar clutch but lacks glycosyltransferase activity (Blair *et al*., 2008). Cells with EpsE D94A are non-motile and have a DegU~P-dependent phenotype, i.e. increased transcription of *bslA* (the main target of DegU~P that is needed for biofilm formation), and *aprE*. Synthesis of γ-PGA also increased in EpsE D94A cells resulting in mucoid colonies, while expression of EpsE K106E, which retains clutch but lacks the glycosyltransferase activity, phenocopied wild-type. Second, an anti-Hag (flagellin) antibody added to cells containing a *degU::lacZ* transcriptional fusion disrupted swimming motility and resulted in upregulation of *degU* and an increase in exoprotease production. These results strongly implicate the *B. subtilis* flagellum as a mechanosensor of surfaces.

How does DegS sense the lack of flagellar rotation or incomplete flagellum assembly? Cairns *et al*. (2013) offer several possibilities. First, similar to *E. coli* YcgR (Boehm *et al*., 2010; Fang and Gomelsky, 2010; Paul *et al*., 2010), 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 that is part of the *Proteus mirabilis* surface-sensing mechanism (Belas and Suvanasuthi, 2005; Lee *et al*., 2013). Third, inhibited flagellar rotation is likely to cause changes in membrane potential or cellular energy status due to restricted proton (ion) flux through the MotAB stators (Kawagishi *et al*., 1996; Van Dellen *et al*., 2008), 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* Cpx surface-sensing circuit (Otto and Silhavy, 2002).

With this report, *B. subtilis* joins an ever-expanding group of bacterial species that use the flagellum to sense and respond to the environment. For example, *Vibrio parahaemolyticus* and *P. mirabilis*, both of which swarm over surfaces, sense a surface-induced inhibition of flagellar rotation and in response initiate production of differentiated swarmer cells (Belas and Suvanasuthi, 2005; Gode-Potratz *et al*., 2011). It was recently shown that upon encountering a surface with an ensuing inhibition of flagellar rotation, *Caulobacter crescentus* immediately produces holdfasts and attaches (Li *et al*., 2012). *Pseudomonas aeruginosa* biofilm formation is regulated at the level of flagellar reversal frequency, apparent in high viscosity media (Caiazza *et al*., 2007), suggesting that the flagella of this species may too be used to sense some characteristic of a surface; a point that is underscored by the notable surface-dependent expression of cyclic-di-GMP by the *P. aeruginosa* Wsp regulatory system (Guvener and Harwood, 2007). Similarly, the flagella of *Salmonella enterica* Typhimurium are considered 'drought sensors' that measure surface wetness (Mariconda *et al*., 2006).

Do other bacteria use their flagella as surface sensors? While experimental evidence is lacking, it seems nearly certain some do, especially in the genera that produce swarmer cells and swarming motility, e.g. *Aeromonas*, *Azospirillum, Rhodospirillum, Rhizobium, Serratia* and *Yersinia*. Furthermore, given the survival advantage of forming a biofilm, it would be surprising if other non-swarming bacterial species did not use their flagella to sense a surface.

There are other mechanisms by which bacteria detect surfaces, yet, there is something poetically delightful in the realization that the rotary flagellum, originally considered as functioning solely to propel bacteria, is also a sensor of surfaces. This knowledge comes with a host of new questions with potentially exciting answers, not the least of which are the nature of the 'true' surface signal sensed by DegS–DegU and molecules involved in this signal transduction pathway. Hopefully, we will not have to wait long for those answers.

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References

Belas, R., and Suvanasuthi, R. (2005) The ability of *Proteus mirabilis* to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. *J Bacteriol* **187:** 6789–6803.

Blair, K.M., Turner, L., Winkelman, J.T., Berg, H.C., and Kearns, D.B. (2008) A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* **320:** 1636–1638.

Boehm, A., Kaiser, M., Li, H., Spangler, C., Kasper, C.A., Ackermann, M., *et al*. (2010) Second messenger-mediated adjustment of bacterial swimming velocity. *Cell* **141:** 107–116.

Burbulys, D., Trach, K.A., and Hoch, J.A. (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell* **64:** 545–552.

Caiazza, N.C., Merritt, J.H., Brothers, K.M., and O'Toole, G.A. (2007) Inverse regulation of biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. *J Bacteriol* **189:** 3603–3612.

Cairns, L.S., Marlow, V.L., Bissett, E., Ostrowski, A., and Stanley-Wall, N.R. (2013) A mechanical signal transmitted by the flagellum controls signalling in *Bacillus subtilis. Mol Microbiol* **90**(1)**:** 6–21..

Chai, Y., Norman, T., Kolter, R., and Losick, R. (2011) Evidence that metabolism and chromosome copy number control mutually exclusive cell fates in *Bacillus subtilis*. *EMBO J* **30:** 1402–1413.

Chevance, F.F., and Hughes, K.T. (2008) Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol* **6:** 455–465.

Fang, X., and Gomelsky, M. (2010) A post-translational, c-di-GMP-dependent mechanism regulating flagellar motility. *Mol Microbiol* **76:** 1295–1305.

Fujita, M., Gonzalez-Pastor, J.E., and Losick, R. (2005) High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. *J Bacteriol* **187:** 1357–1368.

Gode-Potratz, C.J., Kustusch, R.J., Breheny, P.J., Weiss, D.S., and McCarter, L.L. (2011) Surface sensing in *Vibrio parahaemolyticus* triggers a programme of gene expression that promotes colonization and virulence. *Mol Microbiol* **79:** 240–263.

Guttenplan, S.B., Blair, K.M., and Kearns, D.B. (2010) The EpsE flagellar clutch is bifunctional and synergizes with EPS biosynthesis to promote *Bacillus subtilis* biofilm formation. *PLoS Genet* **6:** e1001243.

Guvener, Z.T., and Harwood, C.S. (2007) Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. *Mol Microbiol* **66:** 1459–1473.

Hsueh, Y.H., Cozy, L.M., Sham, L.T., Calvo, R.A., Gutu, A.D., Winkler, M.E., and Kearns, D.B. (2011) DegU-phosphate activates expression of the anti-sigma factor FlgM in *Bacillus subtilis*. *Mol Microbiol* **81:** 1092–1108.

Karatan, E., and Watnick, P. (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol Mol Biol Rev* **73:** 310–347.

Kawagishi, I., Imagawa, M., Imae, Y., McCarter, L., and Homma, M. (1996) The sodium-driven polar flagellar motor of marine *Vibrio* as the mechanosensor that regulates lateral flagellar expression. *Mol Microbiol* **20:** 693–699.

Kearns, D.B. (2010) A field guide to bacterial swarming motility. *Nat Rev Microbiol* **8:** 634–644.

Kobayashi, K. (2007) Gradual activation of the response regulator DegU controls serial expression of genes for flagellum formation and biofilm formation in *Bacillus subtilis*. *Mol Microbiol* **66:** 395–409.

Kobayashi, K., and Iwano, M. (2012) BslA (YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms. *Mol Microbiol* **85:** 51–66.

Lee, Y.Y., Patellis, J., and Belas, R. (2013) The activity of *Proteus mirabilis* FliL is viscosity-dependent and requires extragenic DNA. *J Bacteriol* **195:** 823–832.

Li, G., Brown, P.J., Tang, J.X., Xu, J., Quardokus, E.M., Fuqua, C., and Brun, Y.V. (2012) Surface contact stimulates the just-in-time deployment of bacterial adhesins. *Mol Microbiol* **83:** 41–51.

Mariconda, S., Wang, Q., and Harshey, R.M. (2006) A mechanical role for the chemotaxis system in swarming motility. *Mol Microbiol* **60:** 1590–1602.

Marvasi, M., Visscher, P.T., and Casillas Martinez, L. (2010) Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. *FEMS Microbiol Lett* **313:** 1–9.

Monds, R.D., and O'Toole, G.A. (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol* **17:** 73–87.

Murray, E.J., Kiley, T.B., and Stanley-Wall, N.R. (2009) A pivotal role for the response regulator DegU in controlling multicellular behaviour. *Microbiology* **155:** 1–8.

Ostrowski, A., Mehert, A., Prescott, A., Kiley, T.B., and Stanley-Wall, N.R. (2011) YuaB functions synergistically with the exopolysaccharide and TasA amyloid fibers to allow biofilm formation by *Bacillus subtilis*. *J Bacteriol* **193:** 4821–4831.

Otto, K., and Silhavy, T.J. (2002) Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci USA* **99:** 2287–2292.

Paul, K., Nieto, V., Carlquist, W.C., Blair, D.F., and Harshey, R.M. (2010) The c-di-GMP binding protein YcgR controls flagellar motor direction and speed to affect chemotaxis by a 'backstop brake' mechanism. *Mol Cell* **38:** 128–139.

Stanley, N.R., and Lazazzera, B.A. (2005) Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-gamma-dl-glutamic acid production and biofilm formation. *Mol Microbiol* **57:** 1143–1158.

Van Dellen, K.L., Houot, L., and Watnick, P.I. (2008) Genetic analysis of *Vibrio cholerae* monolayer formation reveals a key role for $\Delta\Psi$ in the transition to permanent attachment. *J Bacteriol* **190:** 8185–8196.

Verhamme, D.T., Murray, E.J., and Stanley-Wall, N.R. (2009) DegU and Spo0A jointly control transcription of two loci required for complex colony development by *Bacillus subtilis*. *J Bacteriol* **191:** 100–108.

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R., and Kolter, R. (2013) Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat Rev Microbiol* **11:** 157–168.

Zhou, J.S., Lloyd, A., and Blair, D.F. (1998) Electrostatic interactions between rotor and stator in the bacterial flagellar motor. *Proc Natl Acad Sci USA* **95:** 6436–6441.